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Characterization of unknown mutations in the von Willebrand factor gene

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Für meine Familie und Freunde,
die mich immer unterstützt haben.

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ABSTRACT

The von Willebrand factor (vWF) is a large glycoprotein, which plays an important role in primary hemostasis. Defects in this protein lead to von Willebrand disease (VWD), the most common inherited bleeding disorder. It is caused by quantitative or qualitative defects of vWF and is classified in three subtypes. Type 1 VWD is characterized by a partial quantitative deficiency of vWF, type 2 VWD is subdivided in four subtypes and characterized by qualitative defects of vWF and type 3 VWD is the most severe form of VWD characterized by a complete absence of vWF.

In this master thesis 39 patients with reduced vWF:Ag and/or reduced vWF:RCo activity suspected to suffer from VWD were analyzed for mutations in the VWF gene. The mutations were detected by sequencing of the complete VWF gene.

In 19 individuals VWD causing mutations were identified: eight novel variants, and nine variations that are already described in the literature. Two of the already described mutation were detected twice.

Three out of the eight novel variants are putative splice site mutations. Two of them are located at the donor site of an intron (c.3379+2 T>C; c.3538+2 T>C) and one is located at the acceptor site of an intron (c.5312-2A>G). All three mutations were detected heterozygously. c.3538+2T>C in intron 26 leads to exon 26 skipping as detected by mRNA analysis. This region of the vWF protein is essential for the binding of vWF to damaged subendothelial matrix.
The other five identified novel mutations are located in exonic regions and occurred heterozygously. The putative impact of them on the function of vWF was predicted by *in silico* assessments such as PolyPhen-2, Project Hope and Align GVGD. All of them potentially leading to a qualitative defect of vWF and are associated with type 2 VWD.
Amino acid substitution p.Asn1231Ser is located in the D3 domain of vWF and is suspected to cause defective folding of the protein. As a consequence, vWF cannot be exported out of the cell.
The three exonic variations p.Arg1274Trp, p.Glu1305Asp and p.His1419Pro are located in the A1 domain of vWF and are potentially associated with an altered GpIb binding ability of vWF.
In the A3 domain of vWF variation p.Arg1830Ser was identified. This mutations is suspected to be associated with moderate to severe bleeding symptoms because of decreased vWF-collagen binding.

For final characterization of this novel exonic mutations VWF constructs have to be transfected into a mammalian host cell line to express recombinant vWF proteins. By comparing the function of vWF proteins derived from the VWF sequence of a healthy individual with those obtained by additionally introducing single

potentially causal mutations for VWD by site directed mutagenesis, the consequences of these novel mutations can be assessed *in vitro*.

In this study, in 19 out of 39 individuals mutations in the VWF gene were identified. Eight of those mutations are not described in the literature but are predicted by *in silico* assessments to cause VWD.

INHALT

Der von Willebrand Faktor (vWF) ist ein großes Glykoprotein, welches eine entscheidende Rolle in der primären Hämostase spielt. Defekte in diesem Protein führen zum von Willebrand Syndrom (vWS), der häufigsten angeborenen Blutgerinnungsstörung. vWS wird durch ein quantitativ oder qualitativ defektes vWF Protein verursacht und unterteilt sich in drei Typen. Bei Typ 1 vWS liegt ein quantitativer Defekt des Proteins vor, während Typ 2 vWS, das in vier Subtypen unterteilt wird, durch qualitative Defekte von vWF charakterisiert wird. Typ 3 vWS ist die klinisch am stärksten ausgeprägte Form des vWS, da das Protein komplett fehlt.

In dieser Masterarbeit wurden 39 Patienten mit reduziertem vWF:Ag und/oder vWF:RCo Aktivität, bei welchen ein Verdacht auf vWS vorliegt, durch die Sequenzierung des kompletten VWF Gens auf Mutationen im VWF Gen untersucht. In 19 Individuen wurden vWS assoziierte Mutationen gefunden: acht neue Mutationen und neun Mutationen, welche bereits in der Literatur beschrieben sind. Zwei dieser bereits bekannten Mutationen wurden in je zwei verschiedenen Individuen detektiert.

Bei drei der acht neuen Mutationen handelt es sind um heterozygote Spleißstellen an den Positionen c.3379+2T>C, c.3538+2T>C und c.5312-2A>G. Durch mRNA Analyse wurde festgestellt, dass die Mutation c.3538+2T>C im Intron 26 zum Überspringen von Exon 26 führt. Diese Region des vWF Proteins ist für die Bindung von vWF an die beschädigte Matrix des Subendothels verantwortlich.

Die anderen fünf neu identifizierten heterozygoten Mutationen befinden sich in exonischen Regionen des Gens. Deren Auswirkung auf die Funktion des vWF Proteins wurde durch *in silico* Methoden vorhergesagt. Verwendet wurden die Programme PolyPhen-2, Project HOPE und Align GVGD.

Der Aminosäureaustausch p.Asn1231Ser befindet sich in der D3 Domäne des vWF Proteins und führt vermutlich zu einer fehlerhaften Faltung des Proteins. Dadurch kann der vWF nicht aus der Zelle exportiert werden.

Die drei exonischen Mutationen p.Arg1274Trp, p.Glu1305Asp und p.His1419Pro befinden sich in der A1 Domäne des vWF und führen vermutlich zu einer veränderten Bindung an GpIb.

Die Mutation p.Arg1830Ser wurde in der A3 Domäne des vWF gefunden. Diese Mutation führt vermutlich – durch eine möglicherweise verminderte vWF-Kollagen Bindung – zu moderaten bis schwerwiegenden Blutgerinnungsstörungen.

Um die funktionellen Auswirkungen der gefundenen neuen Mutationen festzustellen, ist es notwendig, VWF Konstrukte, die die Mutationen enthalten, herzustellen und in Säugerzellen zu transfizieren. Mit Hilfe funktioneller vWF Tests können unterschiedliche Eigenschaften der produzierten mutierten vWF Proteine dann im Vergleich zum vWF eines gesunden Individuums *in vitro* untersucht werden.

In dieser Studie wurden in 19 von 39 Individuen Mutationen im VWF Gen gefunden. Acht dieser Mutationen sind nicht in der Literatur beschrieben und *in silico* Methoden sagen eine Auswirkung auf die Struktur des vWF Proteins voraus.

1 INTRODUCTION

Von Willebrand disease (VWD) is the most common bleeding disorder in humans with a prevalence of 1 %.[1] Almost 80 % of patients with symptomatic VWD are living in the developing world.[2]

VWD was first described in 1926 by the Finnish physician Dr. Erik Adolf von Willebrand as an inherited bleeding disorder with features distinct from haemophilia.[3] Haemophilia is characterized by a deficiency of clotting factors VIII or IX, whereas VWD is characterized by reduced blood levels and/or activity of von Willebrand factor (vWF).[4] Von Willebrand studied several members of a large family resident in Finland. A thirteen year old girl died during her fourth menstrual bleeding because of hemorrhage and four siblings, between ages 2 and 4, also died because of hemorrhage.[5] Also the parents and the other five siblings showed bleeding symptoms.[2] Their disease was initially called hereditary pseudohemophilia, von Willebrand was not able to identify the actual cause for the disorder, and it remained obscure until the identification of vWF as distinct protein and the development of immunological assays for vWF and factor VIII (FVIII) discrimination in 1971.[4,6,7]

The von Willebrand factor is a large, adhesive, multimeric glycoprotein that is stored in platelet α-granules, Weibel-Palade bodies, blood plasma and subendothelial connective tissue.[1,8–10] It circulates as a 500 to 20000 kDa multimer in plasma and plays an important role in primary hemostasis.[11] If a blood vessel is injured clotting factors and platelets form a plug which stops bleeding.[4] The critical factor is vWF with two essential functions: First, its role as carrier protein for blood clotting factor VIII, protecting it from premature proteolysis by activated protein C.[1,6,9,10] Defect vWF leads to a lack of FVIII in the circulation. Second, vWF promotes platelet adhesion to the subendothelium of injured vessels.[1,10] Absence of vWF results in a defective formation of platelet plugs at sites of vascular injury.[10]

Thus, a lack of vWF leads to a bleeding disorder due to quantitative or qualitative defects of vWF in plasma with a heterogeneous phenotype depending on the type of von Willebrand disease.[12]

1.1 VON WILLEBRAND FACTOR GENE

The von Willebrand factor gene (VWF) is located on the short arm of chromosome 12 and comprises 178 kb of genomic DNA.[13] Almost 9 kb mRNA are encoded by 52 exons ranging in size from 41 bp to 1,3 kb.[6,13] Beside of its large size and genomic complexity structural analysis of the VWF gene is complicated by the presence of a partial unprocessed 21 to 29 kb pseudogene with a nucleotide

sequence diverge of only 3,1 % from the VWF gene.[14] The unfunctional evolutionary remnant localized on chromosome 22 corresponds to 12 exons (exons 23-34) of the VWF gene.[13] Although several functional domains of the vWF protein are encoded by these exons, several stop codons in the coding region of the pseudogene suggest that it is not translated in a functional protein.[5,14]

1.2 VON WILLEBRAND FACTOR PROTEIN

The vWF protein is exclusively synthesized in megakaryocytes and endothelial cells as a 2813 amino acid (aa) pre-pro-VWF precursor.[12] 8,3 % of amino acids encode for cysteine which are later paired in disulfide bonds.[10] Several complex intracellular maturation steps are necessary to form mature vWF subunits which are secreted by the cell as multimers. Only the largest vWF multimers are hemostatically active (Figure 1).[10,13]

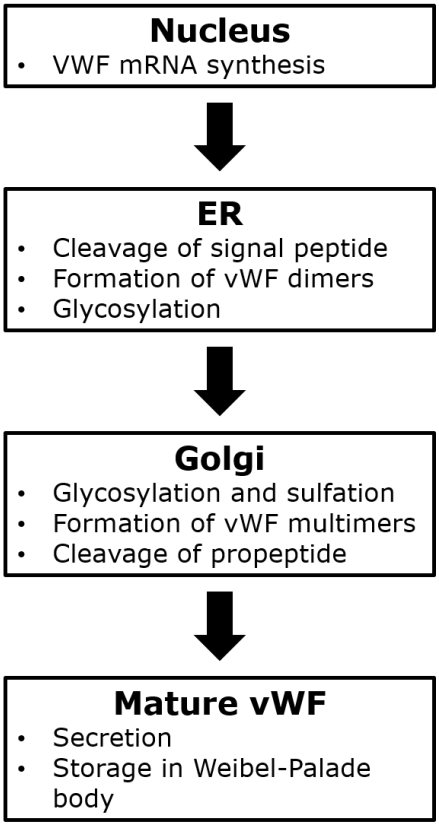

Figure 1 Intracellular maturation steps of VWF

Summary of the biosynthesis and maturation of vWF and cellular localization. Following synthesis of VWF mRNA in the nucleus the pre-pro-peptide, the signal peptide, is cleaved. In the Golgi vWF is further processed and after formation of vWF multimers the mature vWF is secreted or stored in Weibel-Palade bodies.[13]

Via the 22 amino acid pre-sequence, the pre-pro-VWF precursor enters the endoplasmic reticulum (ER). In the ER the 741 aa propeptide mediates the alignment of vWF dimers and is subsequently cleaved from the mature vWF subunit. The 2050 aa mature vWF subunits are secreted as polymerized multimers into the plasma by the cell via constitutive, continuous secretion regardless of environmental factors, and regulated pathways, secreted only in response of a specific signal, or stored in the Weibel-Palade body, a compartment within the endothelial cell.[5,6]

95 % of the primary structure of vWF displays internal repetition of the four homologous domains A, B, C and D (Figure 2).[10] Each domain is present in 2 to 5 copies in the following order: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2. The domains specifically interact with FVIII, heparin, glycoprotein Ib (GpIb), collagen or glycoprotein IIb/IIIa (GpIIb/IIIa).[10,13]

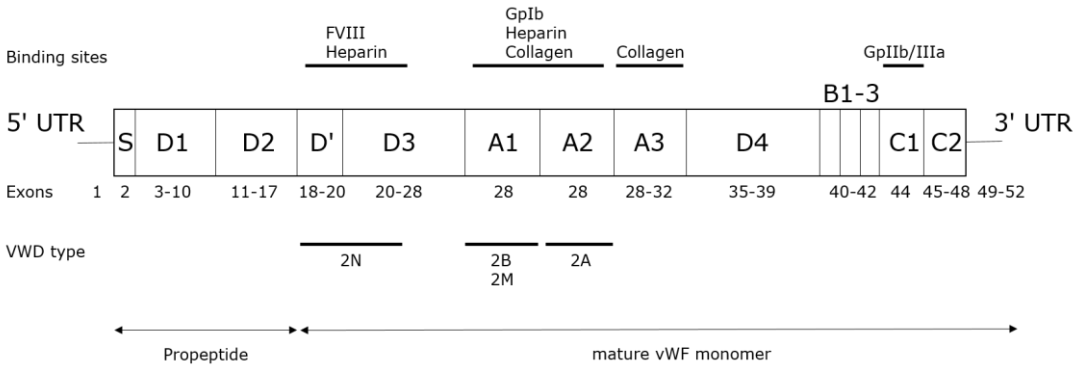

Figure 2 Domains and binding regions of VWF

The primary structure of vWF consists of four homologous domains termed A, B, C and D. Exon 1 is part of the 5' UTR and exon 2, containing the translation start codon ATG, encodes the signaling peptide, which enables the entry of the VWF mRNA into the ER. The propeptide containing the signaling peptide, domains D1 and D2 are cleaved from the protein in the Golgi and the remaining 2050 amino acids are secreted as mature vWF multimers into the plasma or stored intracellularly in Weibel-Palade bodies.[5,6,15]

The vWF protein contains binding sites for FVIII, heparin, GPIb, collagen and GpIIb/IIIa. The binding sites for FVIII, heparin, GPIb and collagen are located in exons 18 to 32. The GpIIb/IIIa binding site is located in exon 44.[10,13] Mutations associated with the type 2 VWD subtypes are primarily located in exons 18 to 28. Type 1 and type 3 VWD associated genetic variations are spread all over the gene.[10,13]

Even the mature vWF subunit starts with D', the domains D1 and D2 of the propeptide are absolutely necessary for multimer formation.[15] By interchain disulfide bonds between the domains D' and D3 identical subunits of vWF assemble to multimeric structures.[16] The D' domain and the D3 domain possess binding

domains for FVIII.[6] Binding of FVIII by vWF stabilizes the clotting factor in the plasma.[6]

The A domains contain binding sites for heparin, collagen and platelet glycoprotein Ib.[14] GPIb is a type I membrane glycoprotein of the GPIb-IX-V complex on the platelet surface.[8] Its primary platelet adhesion receptors form together with the primary platelet adhesion receptors of the leucine-rich region (LRR) and the immunoreceptor family, GPVI/FcRγ, a platelet-specific adhesion-signaling complex.[8] GPIb consists of an extracellular ligand-binding domain, a transmembrane domain, a sialomucin domain and a cytoplasmic domain.[8] The ligand-binding domain of the platelets binds to vWF and leads to the subsequent arrest of bleeding by the formation of a hemostatic platelet-plug.[8,11]

Heparin is bound by the heparin binding site located in domain A1.[17] The heparin binding site overlaps with the GpIb binding domain and is able to impair platelet binding of the vWF by covering the GpIb binding domain.[10,18]

Using the collagen binding site vWF is able to bind to damaged subendothelial matrix and this leads to recruitment, activation and aggregation of platelets.[19] By binding to damaged subendothelial matrix vWF gets activated which leads to a conformational change in the A1 domain and enables the binding to the platelet GpIb receptor.[20]

Via the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) binding site in the C1 domain and the GPIb binding site in the A1 domain vWF is able to bind to the platelets via platelet GPIb and GPIIb/IIIa receptors.[6,13].Thus, initial platelet adhesion to the injured subendothelium is mediated by GPIb receptor, which leads to activation of the platelets and the GPIIb/IIIa receptor.[17,21] This receptor can only bind to vWF if the platelets are activated.[20] The binding of GpIIb/IIIa to vWF leads to irreversible binding of the platelets to the subendothelium and to spreading and aggregation of platelets at sites of injury.[17,20]

In the A2 domain of vWF the metalloproteinase ADAMTS13 cleaves the high molecular weight multimers (HMWM) between the amino acids 1605 and 1606 into smaller and less reactive fragments. A lack of ADAMTS13 leads to microangiopathic syndrome and thrombotic thrombocytopenic purpura due to spontaneous binding of highly reactive vWF HMWMs to platelets and subsequent platelet aggregation. Thus, regulation of multimer size by ADAMTS13 is crucial for the normal physiological activity of vWF.[22,23]

The multimers contain more than 40 subunits and vary in size between 500 kDa and more than 10 million Da.[16,24] Especially defects in HMWM formation have an immense impact on bleeding disorders, since through binding to connective tissue and to platelets HMWM mediate platelet adhesion at sites of vascular injury.[24] Defects in multimer assembly can cause VWD because of a lack of HMWM (VWD type 2A, 2B and 3).[25]

1.3 VON WILLEBRAND DISEASE

Genetic defects in the VWF gene may cause VWD. This inherited bleeding disorder is subdivided in three types based on quantitative or qualitative defects of vWF (Table 1).[16]. Type 1 and 3 are characterized by a partial or almost complete quantitative absence of vWF.[9] Type 2 is caused by qualitative defects and includes the four subtypes 2N, 2M, 2B and 2A.[9] The ratio of patients who suffer from VWD is approximately 65-75 % type 1 VWD, 25-30 % type 2 VWD and 1-6 % type 3 VWD.[6] All types except type 2N and type 3 are dominant in inheritance.[26]

Table 1 6 subtypes of VWD

VWD Type	vWF level	Prevalence[6]	Symptoms
Type 1	low vWF level 20-50 % of normal level[5]	65-75 %	mild, quantitative defects of vWF
Type 2 2A 2B 2M 2N	normal vWF level, low FVIII level	25-30 %	qualitative defects of vWF
Type 3	low or absent vWF	1-6 %	severe, quantitative defects of vWF
Further information about the subtypes are listed in chapter 1.3.2.			

1.3.1 DIAGNOSIS OF VWD

Early clinical diagnosis especially of patients with type 1 or type 2 VWD is difficult because they do not have major bleeding problems in contrast to individuals carrying a type 3 VWD mutation who suffer from severe bleeding episodes.[4] Additionally, VWD phenotypes vary depending on the location of the disease causing mutation and thus on the affected domain. Accordingly, a variety of diagnostic laboratory assays is required to analyze vWF properties including vWF antigen level (vWF:Ag), vWF-platelet GPIb interaction (vWF:RCo), vWF-subendothelium-collagen interaction (vWF:CB), vWF multimer formation and factor VIII coagulant activity (FVIII:C).[4]

1.3.1.1 vWF ANTIGEN LEVEL (vWF:Ag)

vWF antigen levels are usually determined by an enzyme-linked immunosorbent assay (ELISA).[4]

Thereby, the vWF antigen is attached to the surface of a microtiter plate and a specific enzyme-linked antibody is added, which binds to the sample. Subsequently, the enzyme's substrate is added to the antigen-antibody complex and the enzyme-substrate reaction leads to a detectable signal.

1.3.1.2 vWF-PLATELET GpIb INTERACTION (vWF:RCo)

The activity of vWF is assessed by testing the interaction of vWF with the GpIb receptor of platelets. For the ristocetin cofactor assay the antibiotic ristocetin sulfate is utilized.[27] Ristocetin sulfate promotes the interaction of vWF with GpIb under static conditions *in vitro* and this leads to platelet aggregation. It is a non-physiological test but by using patient plasma platelet aggregation correlates with vWF activity.[19,27] Nevertheless, assay results are not very reliable because of a high number of coefficient variation and poor reproducibility and standardization.[19]

1.3.1.3 vWF-SUBENDOTHELIUM-COLLAGEN INTERACTION (vWF:CB)

The vWF:CB is used to test the ability of vWF to bind to collagen. The primary collagen binding site is localized in domain A3 of vWF. The results of this ELISA depend on the size of the multimers, hence, large multimers bind more eagerly than smaller multimers.[4]

1.3.1.4 vWF MULTIMER FORMATION

The multimer patterns are analyzed by gel electrophoresis using a non-reducing agarose gel in the presence of sodium dodecyl sulfate (SDS).[2,4] Following the electrophoresis, the gel is blotted on a nitrocellulose membrane and subsequently, a specific antibody is added, which binds to the multimers. Via chemiluminescence, the bands on the gel are visualized and the size of the multimers is determined by comparison with a reference sample.

1.3.1.5 FVIII COAGULANT ACTIVITY (FVIII:C)

FVIII coagulant activity is analyzed because it reflects the ability of vWF to protect factor VIII against proteolysis. Plasma FVIII:C levels normally correlate with vWF:Ag levels.[2]

1.3.2 TYPES OF VWD

For VWD type determination a combination of laboratory assay results is necessary. Table 2 shows VWD type associated assay results.

Table 2 Correlation of laboratory assay result and VWD type [19]

VWD type	vWF:Ag	vWF:RCo	vWF:CB	FVIII:C	Multimers Formation
1	low	low	low	normal to low	normal
2A	low	low to very low	low to very low	low	no HMWM vWF
2B	normal to low	low to very low	low to very low	normal to low	no HMWM vWF
2M	low	low to very low	low to very low	low	normal
2N	normal to low	normal to low	normal to low	low to very low	normal
3	very low to absent	very low to absent	very low to absent	very low	no vWF

1.3.2.1 VWD TYPE 1

With 65-75 % prevalence, type 1 is the most common form of VWD with dominant inheritance and generally milder clinical symptoms.[6,28] Symptoms are mild mucocutaneous bleeding, bruising, epistaxis and women experience heavy menstrual bleeding.[4] vWF antigen level, ristocetin cofactor activity and collagen binding is low.[19] Multimer formation is normal but multimers show reduced activity.[19] The phenotype is variable and vWF level is influenced by environmental factors such as hormones, aging and physical stress.[6] Patients with blood type 0 are overrepresented in individuals suffering from VWD type 1.[29] Studies showed that blood type 0 decreases vWF plasma levels.[29] The associated mutations are spread throughout the VWF sequence and show an immense heterogeneity like stop codons, deletions and point mutations.[30]

1.3.2.2 VWD TYPE 2

Type 2 VWD is subdivided in the four subtypes 2A, 2B, 2M and 2N. It is characterized by qualitative defects of vWF in particular in multimerization, proteolysis and interaction with vWF ligands.[30] Mutations associated with type 2 are localized in functional domains of the VWF, especially in the D'-D3 domains (type 2N, exons 18 to 25), in the A1-A2 domains (types 2A, 2B and 2M, exon 28).[30] Types 2A, 2B and 2M cause mild to moderate mucocutaneous bleeding.[4] The multimer formation of HMWM is normal in types 2M and 2N but absent in types 2A and 2B.[19]

1.3.2.2.1 VWD TYPE 2A

Type 2A is characterized by loss of HMWM and decreased ristocetin-induced platelet aggregation.[19] Additionally, multimer formation, ADAMTS13-mediated proteolysis, secretion and intracellular storage are affected.[6] Almost 80 % of the disease causing mutations leading to VWD type 2A are localized in exon 28 in domain A2.[6] The vWF A2 domain is necessary for the cleavage of the vWF HMWM by ADAMTS13 to digest the highly reactive HMWM into less reactive smaller multimers.[6] Thus, alterations within this domain may lead to a lack of HMWM and/or enhanced ADAMTS13-mediated proteolysis.[6] Type 2A can be further divided into the three subtypes IID, IIC and IIE. IID is caused by mutations at the carboxy-terminal of VWF which affects the dimerization of vWF monomers.[16,31] Mutations leading to IIC VWD influence multimerization and subtype IIE mutations impair intracellular transport and secretion of intermediate and HMWM.[16,31]

1.3.2.2.2 VWD TYPE 2B

Type 2B mutations cause a lack of vWF HMWMs, low vWF:Ag and vWF:RCo levels and a low vWF:RCo/vWF:Ag ratio.[4,6] All of the known mutations causing VWD type 2B are located in exon 28 encoding the A1 domain of vWF.[6] Some of them increase the affinity to platelet glycoprotein Ib which may cause variable thrombocytopenia.[24]

1.3.2.2.3 VWD TYPE 2M

Type 2M is caused by missense mutations, most of them localized in VWF A1 domain, and is characterized by reduced vWF:RCo/vWF:Ag ratio and reduced binding to the platelet GpIb receptor.[6] Furthermore, it leads to the loss of platelet dependent function and collagen binding defects, despite normal vWF multimer patterns.[6,24,32] Individuals, carrying a type 2M VWD mutation have a low vWF:RCo and episodes of bleeding can be severe.[4]

1.3.2.2.4 VWD TYPE 2N

In contrast to the other type 2 VWD subtypes, type 2N is autosomal recessive in inheritance, this means it requires either two mutant alleles or one mutant and one null allele.[6] Due to decreased FVIII binding capacity of vWF, type 2N leads to reduced FVIII levels in the plasma because unbound FVIII is not protected against proteolysis.[6,9,24] The symptoms typical for type 2N VWD are very similar to haemophilia A.[4] More than 25 mutations causing type 2N are localized between exons 18 to 25.[6] This region of the gene encodes the D' and D3 domain of vWF and is responsible for FVIII binding.[6]

1.3.2.3 VWD TYPE 3

Type 3 is the rarest form of VWD with a prevalence of 0,5-1 case per million.[33] This autosomal recessive inherited VWD type is the most severe bleeding disorder which leads to spontaneous bleeding.[33] It is characterized by very low levels or a complete absence of vWF in plasma.[33] Additionally, vWF:Ag, vWF:RCo and FVIII:C levels are very low.[6,24] Mutations causing VWD type 3 are spread throughout the gene and show an immense heterogeneity like type 1 VWD causing mutations.[34] One-third of the known VWD type 3 mutations are missense mutations and patients are typically homozygous or compound heterozygous for null alleles.[33] The mutations localized in domains D1, D2 and D3 cause severe defects of dimerization or multimerization.[28]

1.4 TREATMENT OF VWD

For the treatment of patients with VWD especially during surgical procedures two main therapeutic approaches are used: Treatment with Desmopressin (DDAVP) and transfusion with blood products containing the FVIII-vWF complex from plasma.[2,35,36]

Desmopressin (1-desamino-8-d-arginine vasopressin) was originally designed to treat patients with diabetes insipidus and is a synthetic analog of vasopressin, an antidiuretic hormone. In patients with mild haemophilia A or VWD DDAVP increases the plasma concentration of FVIII and vWF by releasing stored vWF and FVIII in the plasma. The mode of action is not fully understood and while it is known that endothelial cell Weibel-Palade bodies are the source of vWF, the source of FVIII is not known. The first successful clinical trial was performed in 1977 and Desmopressin is usually administered subcutaneously or intravenously at a dose of 0,3 µg/kg. It increases FVIII and vWF levels in the plasma 3-6 fold above the basal levels within 30-60 minutes.[2,35,36]

Possible occurring side effects include mild tachycardia, headache and facial flushing and very rarely hyponatremia.[2,35]

It is most effective in type 1 VWD patients and there are no therapeutic effects in patients with type 3 VWD. In patients with type 2 VWD the responsiveness varies depending on the subtype.[2,35,36]

If patients are unresponsive to DDAVP transfusion with blood products containing factor VIII-vWF complex is possible. Therefore, cryoprecipitate, the precipitate of centrifuged thawed fresh frozen plasma, is used. It carries a smaller risk of transmitting blood-borne infections than fresh frozen plasma. Furthermore, the concentration of FVIII-vWF is 5-10 fold higher than in fresh frozen plasma.[2,35,36]

2 MATERIALS AND METHODS

2.1 PATIENTS

39 patients with reduced vWF antigen and/or reduced Ristocetin Cofactor activity suspected to suffer from VWD were analyzed for mutations in the VWF gene. For these patients sequencing of exon 28 or exons 18-24 was performed previously for diagnostic purposes and no known disease causing mutation was identified.

The patients DNA samples were anonymized using a numerical code.

Archived and anonymized DNA obtained from healthy control individuals was used for establishing the methods used in this study.

The study was approved by the local ethics committee.

2.2 DNA AND RNA EXTRACTION

Genomic DNA and RNA were extracted from whole blood by using silica-membrane spin columns.

For DNA isolation the Qiasymphony^{SP} and the Qiasymphony DSP DNA Midi Kit both by Qiagen (Hilden, Germany) were used.

RNA isolation was performed by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

2.3 PRIMER DESIGN

The used primers for the amplification of all the exons and part of the 5' and the 3' genomic region of the VWF gene were designed with the aid of NCBI Primer BLAST, a tool for finding specific primers (http://www.ncbi.nlm.nih.gov/tools/primer-blast). As target sequence the VWF genomic reference sequence (GenBank no. NG_009072.1) was used. Because of the von Willebrand factor pseudogene 1 (VWFP1) in chromosome 22 with a nucleotide sequence diverge of only 3 %, the primers used for the amplification of exons 23 to 34 were designed by taking into account the precise differences between the VWF genomic sequence and the sequence of the pseudogene (GenBank no. NG_001212.3) by comparing both sequences and additionally performing a BLAST search for the primers. Primers with the highest number of mismatches were selected. The most important mismatch is the most 3' base of the primer.

Additional sequencing primers were designed, to cover all amplified regions with both a forward and reverse primer especially when sequencing large amplicons.

The final primers and the conditions used for amplification and sequencing the VWF gene are listed in Table I and Table II in the Appendix.

The primers listed in Table III in the Appendix were used to amplify and sequence VWF complementary DNA (cDNA). The forward and reverse primer for the complete cDNA amplification were designed by the recommendations mentioned in the manufacturer's instructions of the pcDNA5/FRT/V5-His TOPO® TA Expression Kit (InvitrogenTM, Carlsbad, USA). The forward primer contains three bases of the Kozak translation ignition sequence (AAG) in front of the ATG start codon. The used vector is a 5,1 kb expression vector which carries an ampicillin resistance gene for selection of transformed Escherichia coli cells and a C-terminal polyhistidine (6xHis) tag for antibody detection of the recombinant protein. The reverse primer was placed in front of the stop codon to maintain the frame through the DNA encoding the 6xHis tag.

Additional primers for polymerase chain reaction (PCR) and sequencing were designed to enable amplification and sequencing of the whole cDNA.

Oligonucleotide primers were synthesized by VBC Biotech (Vienna, Austria) and were purified by polyacrylamide gel electrophoresis (PAGE).

2.4 GENOMIC VWF DNA AMPLIFICATION

All the 52 exons and the 5' and 3' UTR of the VWF gene were amplified using a Long PCR Enzyme Mix kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions and the Mastercycler gradient by Eppendorf (Hamburg, Germany). The Long PCR Enzyme Mix kit contains nuclease free water, 10x Long PCR Buffer, 25 mM $MgCl_2$ solution, dimethylsulfoxid (DMSO) and a 5 U/µl Long PCR Enzyme Mix. The enzyme mix contains a Taq DNA polymerase and a thermostable DNA polymerase with proofreading activity. Additionally, 160 mM dNTP Mix (A C G T, 4x 10 µmol, GE Healthcare, Chalfont St Giles, Great Britain) was used for the PCR. Approximately 80 ng DNA and 10 pmol of each primer were added to the PCR solution in a total volume of 26 µl.

The final conditions for amplifying all the exons, the exon-intron boundaries and the 5' and 3'UTR of VWF are shown in Table 3 and Table 4.

Because of multiple tandem repeats in intron 37 close to the exon-intron boundary of exon 37 a variety of PCR products with different length caused by synthesis stops of the polymerase were amplified. Because of this tandem repeats exons 36 and 37 were amplified by performing a nested PCR. In the first run of the nested PCR a primer pair was chosen producing longer, more unspecific amplicons. The PCR product was a mixture of correct and shorter products caused by

amplification stops. In the second run another primer pair was used which binds within the first correct PCR product. It is very unlikely that the incorrect products from the first run contain both binding sites of the new primer pair. For the second run 1 µl of a 1:1000 dilution of the first PCR product was used.

Table II in the Appendix shows the respective PCR reaction mix and cycler conditions for each exon.

Table 3 PCR reaction mix for VWF DNA amplification

Reagents	**A**	**B**	**C**
H_2O	14,48 µl	13,48 µl	13,25 µl
10x Buffer	2,5 µl	2,5 µl	2,5 µl
25 mM $MgCl_2$	1,8 µl (1,73 mM)	2,8 µl (2,7 mM)	2,8 µl (2,7 mM)
160 mM dNTP Mix (DNA Polymerization Mix (20 mM each A, C, G, T), GE Healthcare)	0,25 µl (1,54 mM)	0,25 µl (1,54 mM)	0,35 µl (2,15 mM)
5x Rapid Load Buffer (ORIGENE, Rockville, USA)	1,6 µl	1,6 µl	1,6 µl
DMSO	1,25 µl (4,8 %)	1,25 µl (4,8 %)	1,25 µl (4,8 %)
5 U/µl Long PCR Enzyme Mix	0,12 µl (0,6 U)	0,12 µl (0,6 U)	0,25 µl (1,25 U)
		22 µl	
Primer forward (10 pmol/µl)		1 µl (10 pmol)	
Primer reverse (10 pmol/µl)		1 µl (10 pmol)	
DNA (~ 40 ng/µl)		2 µl (80 ng)	
		26 µl	

Table 4 Cycler conditions for VWF DNA amplification

	Program 1	Program 2	Program 3	Program 4
1	94 °C 5 min	94 °C 5 min	94 °C 5 min	94 °C 5 min
2	94 °C 1 min	94 °C 1 min	94 °C 1 min	94 °C 1 min
3	59 °C 1 min	62 °C 1 min	57 °C 1 min	59 °C 1 min
4	72 °C 1 min	72 °C 1 min	72 °C 3 min	72 °C 3 min
Repeats	31x	33x	33x	34x
5	72 °C 15 min	72 °C 15 min	72 °C 15 min	72 °C 15 min
6	4 °C ∞	4 °C ∞	4 °C ∞	4 °C ∞

To check the correct size of the amplicons the PCR products were separated by PAGE using Criterion™ Precast Gel 5% TBE (BIO-RAD, Hercules, USA) and 1x TBE Buffer (BIO-RAD). The size of the amplicons was determined by comparing them with the O'Range Ruler 200 bp DNA Ladder mixed 1:6 with the 6x Orange DNA Loading Dye (Thermo Scientific). Gel electrophoresis was performed at 175 V for 35 minutes. Subsequently, the double stranded PCR products were stained using a 1:10000 SYBR green nucleic acid gel strain (Life technologies, Carlsbad, USA) solution. SYBR green is a cyanine dye which binds double stranded DNA. The DNA-SYBR green complex absorbs blue light. The emitted green fluorescent signal was recorded and documented using a digital image visor (FluorChem™ SP, Biozym, Hessisch-Oldendorf, Germany).

2.5 VWF DNA SANGER SEQUENCING

Sanger sequencing is also known as terminator sequencing. DNA fragments of different length are produced by adding both dNTPs (deoxynucleotide triphosphates) and ddNTPs (dideoxynucleotide triphosphates) to the sequencing reaction mix. The randomly incorporated ddNTPs lack a 3' OH group, which is required for the formation of phosphodiester bonds between two nucleotides. Therefore, adding ddNTPs leads to chain termination. Since each ddNTP (ddATP, ddTTP, ddCTP, ddGTP) carries a unique fluorescent molecule, the synthesized and terminated DNA strands are differentially fluorescently labelled. Via capillary electrophoresis the fragments are separated by size and their fluorescence signal is recorded.[37]

Prior to the sequencing reaction, unincorporated primers and dNTPs had to be removed from the PCR product. An aliquot of 10 µl was enzymatically digested by adding 2 µl ExoSAP-IT (Affymetrix, Santa Clara, USA), which contains two hydrolytic enzymes, Exonuclease I and Shrimp Alline Phosphatase (SAP) and a specially formulated buffer. 2 µl of the purified PCR product were subjected to sequencing using the BigDye Terminator v3.1 Kit (Applied Biosystems, Carlsbad, USA) and 1 µl of the primer (listed in Table I in the Appendix) according the manufacturer's suggestions. The cycler conditions are indicated in Table 5. Subsequently, excess fluorescent dye was removed using a vacuum clean up device (Millipore, Billerica, USA). The purified sequencing reaction was subjected to capillary electrophoresis with the 3130xl Genetic Analyzer Gene Amp® PRR System 9700 (Applied Biosystems).

Table 5 Cycler conditions for sequencing reaction

	Cycler conditions	
1	96 °C	1 min
2	96 °C	10 sec
3	55 °C	5 sec
4	60 °C	4 min
Repeats	24x	
5	4 °C	∞

2.5.1 SEQUENCE ANALYSIS

The obtained signals were analyzed using the SeqScape® v2.6 software (Life technologies) and a template derived from the reference VWF sequence (GenBank no. NG_009072.1).

The nomenclature recommended by the Human Genome Variation Society (HGVS) was used for describing deviations from the reference sequence. Each variant detected by sequencing the patients' DNAs was matched to entries of the following databases: The database for single nucleotide polymorphisms (SNP, http://www.ncbi.nlm.nih.gov/snp) a website of the National Center of Biotechnology Information (NCBI) and the professional version of the Human Gene Mutation Database (HGMD, http://www.biobaseinternational.com/product/hgmd). The latter database includes disease-associated mutations, functional polymorphisms and disease associated polymorphisms and is continuously updated based on published data.

Every putative mutation was confirmed by repeating the PCR and sequencing procedures.

For variants not described in the databases mentioned above, the impact of exonic mutations on protein function was predicted by PolyPhen-2 (http://genetics.bwa.harvard.edu/pph2), Project HOPE (http://www.cmbi.ru.nl/hope/home) and Align GVGD (http://agvgd.iarc.fr/agvgd_input.php) based on the vWF protein reference sequence (GenBank no NP_000543.2).

2.6 VWF DNA 454-PYROSEQUENCING (NGS)

The GS Junior (Roche, Rotkreuz, Switzerland) utilizes pyrosequencing, a sequence-by-synthesis technique, which allows monitoring DNA synthesis in real time. It is a next-generation sequencing technique which allows parallel sequencing of different samples at one time. The region of interest is amplified by using a biotinylated reverse primer. The PCR product is attached to beads coated with streptavidin allowing a firm immobilization of the PCR product on the bead surface via avidin-biotin bonding. The antisense strand attached to the bead serves as template for the synthesized sense strand. The four dNTPs are added successively and by injection of the correct complementary dNTP, inorganic pyrophosphate (PP_i) is released during addition of the dNTP to the nascent strand by the polymerase. PP_i is converted into adenosine triphosphate (ATP), a cofactor for the oxidation of luciferin to oxyluciferin and detectable light. The light emission is proportional to the number of dNTPs added to the nascent strand.[37]

On the GS Junior (Roche) sequencing of the VWF gene was performed by using a custom-made exon amplification kit by Multiplicom (Niel, Belgium) and Roche reagents according to the manufacturer's instructions.

The specified target regions (all exons and exon/intron boundaries) of the VWF gene were amplified in three separate multiplex PCR amplification reactions per patient using the Multiplex Amplification of Specific Targets for Resequencing (MASTR) kit (Multiplicom).

Subsequently, a Universal PCR was performed using a MID kit (Multiplicom) to tag the amplicons of each patient individually with molecular identifiers (MID) and 454 specific sequencing adaptors. The three multiplex PCR products of each patient were individually tagged with the same specific barcode (MID).

The tagged amplicons were mixed per patient (Amplicon Library) and purified from small residual DNA fragments. For this purpose the Agencourt® AMPure® XP (Beckman Coulter, Brea, USA) and the DynaMag™-2 Magnetic Particle Concentrator (Invitrogen™) were used.

Afterwards, the DNA concentration was determined by using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™) and the LightCycler 480 (Roche). The Amplicon Libraries were pooled equimolarly and the resulting amplicon pool was further processed with Titanium® emPCR kits of the Lib-A type (Roche). Subsequently, the resulting bead library was sequenced on the GS Junior (Roche).

The sequencing results were analyzed by the GS Amplicon Variant Analyzer Version 2.9 (454 Life Science, Branford, USA) software.

2.7 VWF cDNA Synthesis

Total RNA from whole blood was isolated using the RNeasy Mini Kit by Qiagen following the manufacturer's instructions. cDNA synthesis was established for two different reverse transcriptase kits.

2.7.1 ProtoScript II Reverse Transcriptase Kit (New England Biolabs)

The ProtoScript II Reverse Transcriptase kit (New England Biolabs, Massachusetts, USA) contains 5x ProtoScript II Reverse Transcriptase Reaction Buffer, 0,1 M 10x Dithiothreitol (DTT), and ProtoScript II Reverse Transcriptase (200000 U/ml). Approximately 50 ng/µl RNA and 3,85 µM Oligo d(T)18 mRNA Primer (New England Biolabs) were added to the synthesis reaction displayed in Table 6.

After initial 10 minutes at 22 °C to allow the primers to bind to the poly(A) tail of the template, the cDNA synthesis reaction was performed at 42 °C for 50 minutes followed by 20 minutes at 65 °C for enzyme inactivation.

Table 6 PCR reaction mix for VWF cDNA synthesis using ProtoScript II Reverse Transcriptase Kit

Reagents	1x
5x ProtoScript II Reverse Transcriptase Reaction Buffer	5 µl
0,1 M 10x DTT	2 µl
160 mM dNTP (DNA Polymerization Mix (20 mM each A, C, G, T), GE Healthcare)	2 µl (12,3 mM)
50 µM Oligo d(T)18 mRNA Primer	2 µl (3,85 µM)

20 U/µl RNase Inhibitor (Applied Biosystems)	3 µl (60 U)
200000 U/ml ProtoScript II Reverse Transcriptase	2 µl (400 U)
	16 µl
total RNA (~ 50 ng/µl)	10 µl (~ 500 ng)
	26 µl

2.7.2 TRANSCRIPTOR HIGH FIDELITY CDNA SYNTHESIS KIT (ROCHE)

The Transcriptor High Fidelity cDNA Synthesis Kit (Roche) contains 5x Transcriptor High Fidelity Reaction Buffer, Protector RNase Inhibitor (40 U/µl), Deoxynucleotide Mix (10 mM each dATP, dCTP, dGTP, dTTP), Anchored-oligo(dT)$_{18}$ Primer (50 µM), 0,1 M DTT and Transcriptor High Fidelity Reverse Transcriptase.

Table 7 PCR reaction mix for VWF cDNA synthesis using Transcriptor High Fidelity cDNA Synthesis Kit

Reagents	**1x**
Template-Primer Mix	11,4 µl
5x Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer	4 µl
40 U/µl Protector RNase Inhibitor	0,5 µl (20 U)
10 mM each Deoxynucleotide Mix	2 µl (1 mM each)
0,1 M DTT	1 µl (5 mM)
Transcriptor High Fidelity Reverse Transcriptase	1,1 µl (10 U)
	20 µl

First step was the denaturation of a template-primer mix at 65 °C for 10 minutes to ensure denaturation of mRNA secondary structures. The mixture contained ~500 ng mRNA and 2,5 µM of anchored-oligo$(dT)_{18}$ primers in a total volume of 11,4 µl. Following the denaturation the remaining components listed in Table 7 were added and the reaction was incubated for 1 hour at 45 °C and another 5 minutes at 85 °C for enzyme inactivation.

Better results were obtained by using ProtoScript II Reverse Transcriptase Kit (New England Biolabs).

2.8 VWF cDNA AMPLIFICATION

Nucleotides c.-3 to c.8439 of the VWF cDNA (GenBank no. NM_000552.3) were amplified using Long PCR Enzyme Mix kit (Thermo Scientific) and primers cDNAfw and cDNArvHis as indicated in Table 8 below and in Table III in the Appendix.

Table 8 PCR reaction mix for VWF cDNA amplification

Reagents	**1x**
H_2O	13,05 µl
10x Buffer	2,5 µl
25 mM $MgCl_2$	2,8 µl (2,7 mM)
160 mM dNTP (DNA Polymerization Mix (20 mM each A, C, G, T), GE Healthcare)	0,5 µl (3,08 mM)
5x Rapid Load Buffer (ORIGENE)	1,6 µl
DMSO	1,25 µl (4,8 %)
5 U/µl Long PCR Enzyme Mix	0,3 µl (1,5 U)
	22 µl
Primer cDNAfw (10 pmol/µl)	1 µl (10 pmol)

Primer cDNArv (10 pmol/µl)	1 µl (10 pmol)
cDNA	2 µl
	26 µl

Cycling was performed in two steps (Table 9). After initial denaturation at 95 °C for 5 minutes the first 10 cycles comprised a 30 seconds denaturation period at 95 °C, followed by 30 seconds annealing at 60 °C and elongation at 68 °C for 14 minutes. In the subsequent 17 cycles the annealing temperature was increased to 65 °C and elongation was extended for additional 20 seconds each cycle. Because of the high number of base pairs to be amplified (8442 bp) long elongation times were necessary. The low annealing temperature during the first 10 cycles facilitates the binding of the primers to the cDNA. The higher temperature in the following 17 cycles increases specificity.

Table 9 Cycler conditions for VWF cDNA amplification

	Cycler conditions	
1	95 °C	5 min
2	95 °C	30 sec
3	60 °C	30 sec
4	68 °C	14 min
Repeats	9x	
5	95 °C	30 sec
6	65 °C	30 sec
7	68 °C	14 min
Repeats	17x + 20 sec each repeat	
8	68°C	30 min
9	4 °C	∞

The resulting PCR product was separated on a 0,8 % low melting agarose gel. Therefore, 0,33 g low melting agarose (BIO-RAD) were solved in 40 ml 1x TBE. For size determination the DNA Molecular Weight Marker X (0,07-12,2 kbp) by Roche was used. Gel electrophoresis was performed at 90 V for 2 hours, the gel was subsequently stained for one hour with a 1:750 SYBR green (Life technologies) solution and the PCR product was visualized with UV-light and a digital image visor (FluorChem™ SP, Biozym).

The band of correct size was cut out of the gel and stored in an Eppendorf tube until further use.

2.8.1 SINGLE EXON AMPLIFICATION OF VWF CDNA

For amplification of single exons of the cDNA to detect exon skipping caused by splice site mutations and for cDNA sequencing Long PCR Enzyme Mix kit (Thermo Scientific) and primers shown in Table III in the Appendix were used. In Table 10 the PCR mix for one reaction and in Table 11 the cycler conditions are displayed. The cDNA was diluted 1:100 and 1 µl of this dilution was used for PCR.

Table 10 PCR mix for amplification of single exons of the VWF cDNA

Reagents	**1x**
H_2O	13,48 µl
10x Buffer	2,5 µl
25 mM $MgCl_2$	2,8 µl (2,7 mM)
160 mM dNTP Mix (DNA Polymerization Mix (20 mM each A, C, G, T), GE Healthcare)	0,25 µl (1,54 mM)
5x Rapid Load Buffer (ORIGENE)	1,6 µl
DMSO	1,25 µl (5 %)
5 U/µl Long PCR Enzyme Mix	0,12 µl (0,6 U)
	22 µl
Primer forward (10 pmol/µl)	1 µl (10 pmol)

Primer reverse (10 pmol/µl)	1 µl (10 pmol)
cDNA (1:100)	1 µl
	25 µl

Table 11 Cycler conditions for amplification of single exons of the VWF cDNA

	Cycler conditions	
1	94 °C	5 min
2	94 °C	1 min
3	60 °C	1 min
4	72 °C	1 min
Repeats	33x	
5	72 °C	15 min
6	4 °C	∞

The PCR products were separated by PAGE as described above (Chapter 2.4).

2.9 VWF cDNA Sanger Sequencing

For cDNA sequencing PCR products were digested with ExoSAP-IT (Affymetrix) and subjected to Sanger sequencing as described above (Chapter 2.5).

2.9.1 VWF cDNA Sequencing analysis

The obtained sequences were aligned with the reference VWF mRNA sequence (GenBank no. NM_00552.3) and analyzed using SeqScape® v2.6 software (Life technologies).

2.10 Cloning

The amplified VWF cDNA (c.-3 to c.8689) was inserted into a pcDNA5/FRT/V5-His-TOPO® vector (Invitrogen™) and amplified in One Shot® TOP10 Chemically Competent Escherichia coli cells (Invitrogen™) as described below.

2.10.1 GENERATING VWF CONSTRUCT

The pcDNA5/FRT/V5-His-TOPO® vector (Invitrogen™) is an already linearized, double stranded DNA molecule used for cloning and expression of VWF cDNA. It combines the Flp-In™ System with the TOPO® Cloning technology. The Flp-In™ System allows the integration of the vector into the Flp-In™ host cell line. The TOPO® Cloning technology allows the ligation of Taq-amplified PCR-products with the vector without ligase or post-PCR procedures. This vector carries features necessary for amplification in bacteria and subsequent expression of recombinant proteins in mammalian cells:

- Human cytomegalovirus (CMV) immediate-early promoter/enhancer for high-level expression of the gene of interest in mammalian cells.[38]
- TOPO® Cloning site for insertion of Taq-amplified PCR products in frame without ligase.
- V5 epitope and polyhistidine (6xHis) tag for detection and purification of the recombinant protein.
- Bovine growth hormone (BGH) polyadenylation signal and SV 40 early polyadenylation signal for transcription termination and polyadenylation of mRNA.[39]
- Flp Recombination Target (FRT) site serves as the binding and cleavage site for Flp recombinase.
- Hygromycin resistance gene for selection of stable transfectants in mammalian cells.
- pUC origin for high-copy number replication and growth in Escherichia coli.
- Ampicillin (bla) resistance gene (β-lactamase) and bla promoter for expression of the ampicillin resistance gene and selection of transformants in E. coli.

For successful ligation the insert has to have a 3' A-overhang. The used Long PCR Enzyme Mix for VWF cDNA amplification consists of a Taq DNA Polymerase, which adds A-overhangs on the 3' end of the amplicon and a thermostable DNA polymerase with proofreading activity. To ensure that all cDNA transcripts carrying an A-overhang the cut out gel slice from a 0,8 % low melting agarose gel was melted at 65 °C. 9 µl of the melted gel slice were incubated with 10x Taq Polymerase Buffer, 5 U/µl AmpliTaq Gold®, 25 mM $MgCl_2$ (AmpliTaq Gold® DNA Polymerase with Buffer II & $MgCl_2$, Applied Biosystems) and 100 mM 2'-Deoxyadenosine 5'-Triphosphate for 15 minutes at 72 °C. The complete reaction mix for one reaction is shown in Table 12.

The TOPO® Cloning reaction was performed by following the instructions of the manual of the pcDNA5/FRT/V5-His TOPO® TA Expression Kit (Invitrogen™). 4 µl of the 3' A-overhangs post-amplification reaction were incubated with 1 µl salt

solution provided in the kit and 1 µl already linearized pcDNA5/FRT/V5-His-TOPO® vector for 15 minutes at 37 °C.

Table 12 Reaction mix for 3' A-overhangs post-amplification

Reagents	1x
10x Taq Polymerase Buffer	1 µl
25 mM $MgCl_2$	0,125 µl (0,31 mM)
100 mM dATP	0,25 µl (2,5 mM)
5 U/µl AmpliTaq Gold®	0,25 µl (1,25 mM)
Melted cDNA gel slice	9 µl

2.10.2 TRANSFORMATION OF BACTERIA

One Shot® TOP10 Chemically Competent Escherichia coli (Invitrogen™) cells were transformed with this VWF-vector construct by adding 2 µl of TOPO® Cloning reaction into a vial of E. coli cells and incubating them 30 minutes on ice. After heat shocking the cells for 30 seconds at 42 °C, 250 µl SOC medium were added and horizontally shaked at 37 °C for 1 hour at 300 rpm. 200 µl of the transformed One Shot® TOP10 Chemically Competent E. coli cells were spread on a prewarmed LB agar plate and incubated at 37 °C over night. Subsequently, colonies were picked and cultivated in 2 ml LB medium for 12 hours in a horizontal shaker at 225 rpm at 37 °C.

For preparation of 1 l LB medium 1 % peptone (GIBCOBRL, Carlsbad, USA), 0,5 % yeast extract (GIBCOBRL) and 1 % NaCl (MERCK, Darmstadt, Germany) were mixed in 950 ml deionized water (B.Braun, Melsungen, Germany) and pH was adjusted to 7,0 with NaOH (MERCK). The volume was filled up to 1 l with deionized water and autoclaved on liquid cycle for 20 minutes at 140 °C.

For preparation of LB agar plates 15 g/l agar (GIBCOBRL) were added before autoclaving. After cooling medium to 55 °C after autoclaving 100 µg/ml ampicillin (Roche) were added. The medium was stored at 4 °C and the medium for LB agar plates was poured into 10 cm plates and stored at 4 °C as well.

2.10.3 Analysis of Positive Colonies

To analyze successfully transformed colonies the plasmid was cut by restriction enzyme BamHI-HF® (New England Biolabs). High fidelity (HF) restriction enzymes are fully active in a broad range of conditions and minimizing off-target products. A restriction enzyme in general recognizes specific nucleotide sequences and cleaves dsDNA at the target site. Both the vector and the insert contain a single BamHI recognition sequence shown in Figure 3. The size of the obtained fragments provides information on the 5' to 3' orientation of the insert.

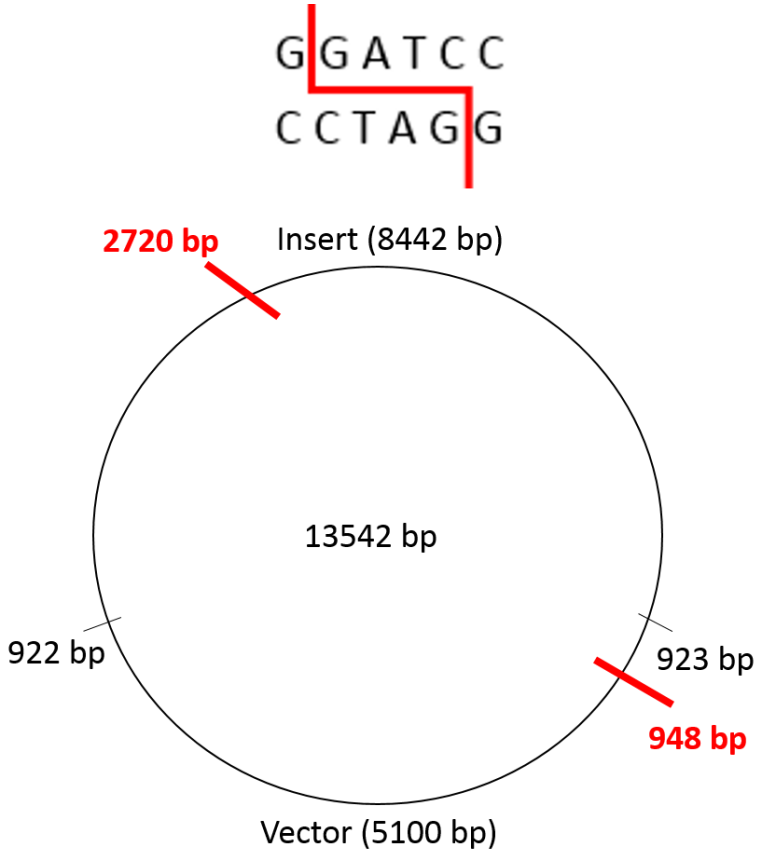

Figure 3 BamHI recognition sequence and plasmid cutting side

In the top of the figure the recognition sequence of BamHI is shown.
The insert was ligated in the 5100 bp comprising vector between basepair 922 and 923. The recognition sequence of BamHI in the vector starts at basepair 948 and the BamHI recognition sequence in the insert starts at basepair 2720.
After incubating the 13542 bp comprising plasmid with BamHI two fragments of different size are present. In case the insert is ligated in correct orientation from 5' to 3' the reaction product contains fragments of 7795 bp and 5747 bp. If the insert was not inserted correctly fragments containing 10797 bp and 2745 bp are produced.

Before restriction digest plasmid preparation was performed by using the Qiaprep® Spin Miniprep Kit (Qiagen). 1 ml of earlier cultivated bacteria were used for preparation following the manufacturer's instructions.

The isolated plasmid was diluted 1:10 (~30-50 ng plasmid DNA) and mixed with 1 µl BamHI-HF (20000 U/ml), 2 µl 10x CutSmart™ Buffer and 16 µl water.

The restriction enzyme digest was performed at 37 °C for 30 minutes and the reaction was stopped by adding 3 µl of 6x gel loading dye, included in the kit, to 20 µl of the reaction.

The digested vector was separated on a 0,8 % agarose gel: 0,33 g agarose (BIO-RAD) were solved in 40 ml 1x TBE. For size determination the DNA Molecular Weight Marker X (0,07-12,2 kbp) by Roche was used. Gel electrophoresis was performed at 90 V for 2 hours and the gel was subsequently stained for one hour with a 1:750 SYBR green (Life technologies) solution. The digested plasmid was visualized with UV-light and a digital image visor (FluorChem™ SP, Biozym).

To verify that one single colony is amplified 20 µl of colonies containing an insert in the right orientation were spread on a LB agar plate containing 100 µg/ml ampicillin (Roche) with 180 µl LB medium and were incubated over night at 37 °C. Subsequently, colonies were picked and cultivated over night in 2-5 ml LB medium containing 100 µg/ml ampicillin (Roche). Following plasmid preparation, the plasmid was sequenced by using the primers listed in Table III in the Appendix, the PCR reaction mix displayed in Table 10 and the cycler conditions shown in Table 11.

2.10.4 SITE DIRECTED MUTAGENESIS

To generate a plasmid carrying the VWF sequence of a healthy individual the cDNA based on the mRNA of a healthy control was ligated in the vector. In Table IV in the Appendix the sequence of the VWF insert is shown. Some allele variations are more common in the European population (frequencies from HAP MAP CEU) than those indicated in the reference VWF mRNA sequence (GenBank no. NM_000552.3), these positions are marked in the sequence indicated in the Appendix.

Synthesis errors in the plasmid in the VWF cDNA sequence which are leading to an amino acid substitution have to be eliminated. For this purpose QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, USA) was used. A complementary primer pair for each synthesis error was designed following the manufacturer's instructions (Table 17 in the Results). Each primer contains of ca. 30 bp and a GC-content of ca. 40 %. The base which has to be corrected has to be located in the middle of the sequence. Instead of the incorrect base the correct base has to be introduced in the primers sequence.

In separate reactions for each synthesis error the correct base was introduced in the VWF sequence by using the designed primer pair and the plasmid as template. By performing a PCR plasmids carrying the correct base were synthesized. Subsequently, the incorrect, parental plasmids were digested by Dpn I, an endonuclease specific for methylated and hemimethylated DNA.

For mutant strand synthesis reaction the reagents provided in the kit were mixed with the primers and the plasmid DNA as indicated in Table 13.

Table 13 Reaction mix for mutant strand synthesis reaction

Reagents	**1x**
10x QuikChange Lightning Buffer	5 µl
dsDNA template (80 ng/µl)	1 µl (80 ng)
fw primer (10 pmol/µl)	1 µl (10 pmol)
rv primer (10 pmol/µl)	1 µl (10 pmol)
dNTP mix	1 µl
QuikSolution reagent	1,5 µl
ddH_2O	39,5 µl
	50 µl
QuikChange Lightning Enzyme	1 µl
	51 µl

The PCR conditions for the mutant strand synthesis reaction are displayed in Table 14. The elongation time was calculated specifically for this plasmid by following equation: 30 seconds/kb of plasmid length → 30 sec x 13,548 kb = 6,42 minutes.

Table 14 Cycler conditions for mutant strand synthesis reaction

	Cycler conditions	
1	95 °C	2 min
2	95 °C	20 sec
3	60 °C	10 sec
4	68 °C	6,42 min
Repeats	17x	
5	68 °C	5 min

Subsequently, 2 µl of Dpn I were added to the mutant strand synthesis reaction product and incubated for 5 minutes at 37 °C to digest the unaffected parental strands.

2 µl of the Dpn I-treated DNA were transferred to a vial of One Shot® TOP10 Chemically Competent Escherichia coli cells (Invitrogen™) and the transformation reaction was incubated on ice for 30 minutes. Following a 30 second heat pulse at 42 °C, 250 ml SOC medium were added and the tubes shaked for one hour at 37 °C. Afterwards, 200 µl of transformed bacteria were spread on a prewarmed LB-agar plate and incubated over night at 37 °C.

After picking and cultivating colonies the plasmid was isolated (Chapter 2.10.3) and sequenced (Chapter 2.8.1 and Chapter 2.9). Colonies in which plasmid the specific base was successfully substituted were used as template for the next mutant strand synthesis reaction.

2.10.5 TRANSFECTION OF HEK293T

HEK293 are originally human embryonic kidney cells transformed with sheared fragments of adenovirus type 5 DNA.[40] HEK293T cells carry an additional neomycin resistance compared to normal HEK293 cells.

In future experiments the generated plasmid has to be transfected into HEK293T cells. Therefore, the HEK2093T cells have to be thawed and transferred to a 75 cm^2 tissue culture flask with 15 ml medium containing 1x DMEM (Gibco®, Carlsbad, USA), 10 % fetal bovine serum (FBS, Biochrom AG, Berlin, Germany), 1 % Penicillin/Streptomycin solution (Pen Strep, Gibco®) and 1 % L-Glutamine (PAA Laboratories,Pasching, Austria).

For transfection the cells have to be cultured in a 6-well plate until they are 80-90 % confluent. In two separate reactions 4 ng of the vector have to be mixed with 300 µl DMEM and 15 µl Lipofectamine® 2000 Transfection Reagent (Invitrogen™) have to be mixed with 285 µl DMEM. After 5 minutes of incubation the vector-DMEM mixture has to be added to the Lipofectamine-DMEM mixture and incubated for another 20 minutes at room temperature. In the meantime, the medium of the cells has to be discarded and the cells be washed with prewarmed 1x DPBS (BioWhittaker®, Walkersville, USA). The Lipofectamine-vector mixture has to be added to the cells and incubated for 5 hours at 37 °C in a CO_2 incubator (Heraeus Cytoperm 2, Thermo Scientific). Subsequently, 1 ml full growing medium has to be added and the cells have to be incubated for another 72 hours.

To check transfection efficiency the cells have to be co-transfected with a GFP-plasmid. With the aid of an UV microscope the success of the transfection can be determined. HEK293T cells when exposed to UV light exhibits green fluorescence if they express the GFP gene.

3 RESULTS

3.1 SEQUENCING AND ANALYSIS OF GENOMIC VWF DNA

For genomic VWF DNA sequencing PCRs were performed to amplify each of the 52 exons encoding for the VWF gene. Subsequently, the samples were sequenced and analyzed by using the SeqScape® software.

In Table V in the Appendix all identified polymorphisms, detected in all the 39 patients and 3 controls, are indicated. Their frequency in the European population (Frequencies from HAP MAP CEU and pilot_1_CEU_low_coverage_panel (of 1000 genome project)) is shown as well as their frequency in the analyzed population in this master thesis. Some detected polymorphisms are described as functional polymorphisms influencing the activity of vWF and are listed in Table 15.

Table 15 Functional polymorphisms in VWF

Position	Frequency in % (SNP) AA:Aa:aa	Frequency in % (42 samples) AA:Aa:aa	rs number
5'UTR c.-250-670GT[20]	n/a	>20 GT 45 %	rs72106596
Exon 18 c.2365A>G; p.Thr789Ala	43:46:11 [A]	64:29:7	rs1063856
Exon 20 c.2555G>A; p.Arg852Gln	80:19:1 [A]	74:26:0	rs216321
Exon 28 c.4141A>G; p.Thr1381Ala	20:37:43 [A]	10:48:42	rs216311
Exon 28 c.4414G>C; p.Asp1472His	n/a	86:14:0	rs1800383
Exon 28 c.4693G>T; p.Val1565Leu	87:10:3 [A]	81:17:2	rs1800385
[A] Frequencies from HAP MAP CEU			

In the promoter region starting at position c.-250-670GT[20] the GT-tandem repeat is located. Longer GT repeats (>20 repeats) increase VWF promoter activity.[41]

Variant c.2365A>G in exon 18 is associated with higher vWF:Ag levels and FVIII:C activity.[26,30]

Polymorphism c.2555G>A is located in exon 20 and is associated with increased FVIII levels and decreased collagen-induced platelet aggregation.[42]
The functional polymorphism c.4141A>G in exon 28 leads to the amino acid substitution of threonine by alanine on amino acid position p.1381. A substitution in both alleles is associated with an increased affinity for the platelet receptor GpIbα.[11,43]
Variant c.4414G>C in exon 28 affects binding to ristocetin in an *in vitro* performed vWF:RCo assay and increases vWF:Ag levels.[30,44]
A small increase in the proteolysis of vWF multimers by ADAMTS13 is associated with variant c.4693G>T.[45]

The mutations shown in Table 16 are identified putative causal mutations for VWD, not described in the literature, found in different patients suspected to suffer from VWD. These patients do not carry any other mutation associated with VWD. Additionally, the results of vWF antigen level assay, vWF-platelet GPIb binding assay and FVIII coagulant activity assay are listed in the table.

Table 16 Putative causal mutations for VWD not described in literature

Putative causal mutation	vWF:Ag (in %)	vWF:RCo (in %)	FVIII:C (in %)
Intron 25 c.3379+2T>C	13	12	48
Intron 26 c.3538+2T>C	18	12	40
Exon 28 c.3692A>G p.Asn1231Ser	64	63	69
Exon 28 c.3820A>T p.Arg1274Trp	91	14	65
Exon 28 c.3915G>C p.Glu1305Asp	103	88	100
Exon 28 c.4256A>C p.His1419Pro	28	17	65
Intron 30 c.5312-2A>G	25	19	53
Exon 32 c.5488C>T p.Arg1830Ser	56	44	101

The indicated eight novel mutations in Table 16 were further analyzed to proof their causality for VWD. They were found in eight different patients and all of them occurred heterozygously. Three of them are potential splice site mutations

located in the intron close to the exon-intron boundary. Two of them are located at the donor site of the intron and the other one at the acceptor site. All of them probably leading to the expression of a shortened and not functional protein. The other five mutations are exonic mutations leading to amino acid substitutions, which may influence the amount and/or activity of the expressed vWF protein.

The intronic mutation in intron 26 (c. 3538+2T>C) was analyzed by sequencing the patient's cDNA to determine if the mutation influences splicing.

The exonic mutations have to be analyzed by generating a recombinant protein in mammalian cell lines and the amount and activity of the expressed vWF have to be compared to a recombinant protein based on the sequence of a healthy control.

In Table VI in the Appendix all detected disease causing mutations for VWD, which are already described in the literature, are listed.

3.2 Characterization of novel Splice Site Mutations

Splice site mutations are suspected to influence splicing of the pre-mRNA into mRNA. For the export of the mRNA out of the nucleus the removal of the noncoding introns is necessary otherwise the mRNA stays in the nucleus to avoid translation.[46] For splicing three positions on the RNA are necessary: The splice site on the 5' end, called splice donor, and is marked by a conserved and invariant GU dinucleotide, the splice site on the 3' end of an intron, called splice acceptor, which is hallmarked by an AG dinucleotide and the branch point in the intronic sequence.[47,48] The splicing is performed by the spliceosome, an assembly of protein and RNA molecules, which recognizes the mentioned three positions on the RNA.[48]

Incorrect spliced VWF may lead to a shortened, non- or less active protein in the circulation.

3.2.1 INTRON 26: C.3538+2T>C

The intronic mutation c.3538+2T>C was detected by sequencing genomic VWF DNA and the obtained electropherogram is shown in Figure 4.

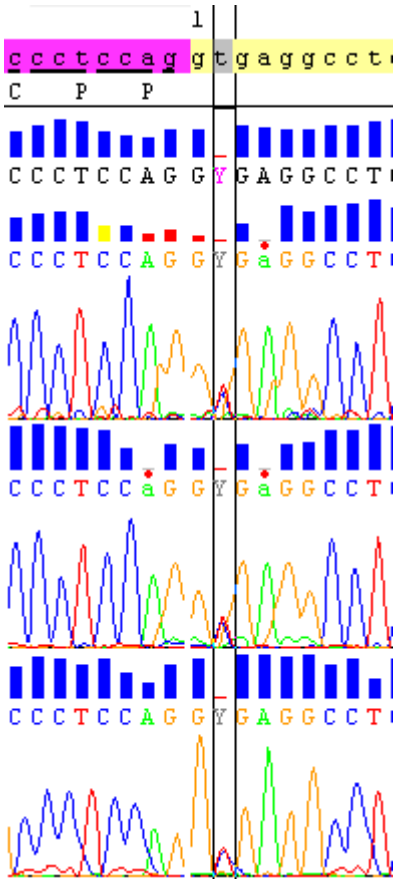

Figure 4 Sequencing analysis by SeqScape® of mutation c.3538+2T>C in intron 26

In this figure three different reads of the boundary of exon and intron 26 are shown. Exon 26 is marked in pink and intron 26 in yellow. The heterozygous mutation on position +2 is highlighted.

To verify exon skipping caused by a thymidine to cytosine substitution on position +2 in intron 26 the mRNA was isolated from the patient's peripheral blood. mRNAs are already spliced RNAs and by sequencing them the effect of mutations in splice site sequences can be detected.

A cDNA from the patient's mRNA was generated and primers were chosen which cover exons 25, 26 and 27 and parts of exon 28 to verify the presence of exon 26. The amplified region comprises 1330 bp if exon 26 is present. If the 159 bp containing exon 26 is missing a PCR product consisting of 1171 bp was expected. For size determination of the amplicons the PCR product was separated by PAGE (Figure 5).

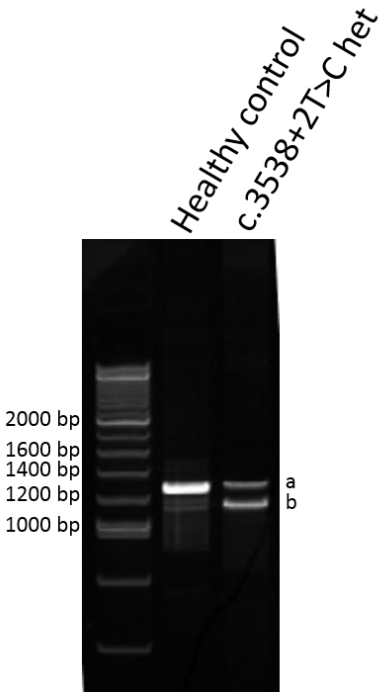

Figure 5 Gel electrophoresis of cDNA PCR product of intronic mutation c.3538+2T>C in intron 26

The gel shows the separated PCR products of cDNA amplification. The first lane shows the marker for size determination. The second lane displays a healthy control, who has no mutation in the amplified region. The third lane shows the PCR product of the cDNA of the patient. Because the mutation was detected heterozygously there are two bands visible. The alleles of the healthy control are spliced correctly. Band a in the third lane correlates with the correctly spliced alleles of the healthy control. Band b is smaller and is caused by a missing exon 26 in the amplicons.

To verify the skipping of exon 26 the two bands of the patient's PCR product were cut out of the gel and reamplified separately. Subsequently, the PCR products were sequenced and the obtained electropherograms are shown in Figure 6.

In Figure 6 a the sequencing results of band a are shown. The splicing is correct and exon 26 is linked to exon 25 and exon 27. In Figure 6 b the sequencing results of band b are shown. In this sample the 3' end of exon 25 is linked immediately to exon 27 indicating that exon 26 is missing in the mRNA of one allele.

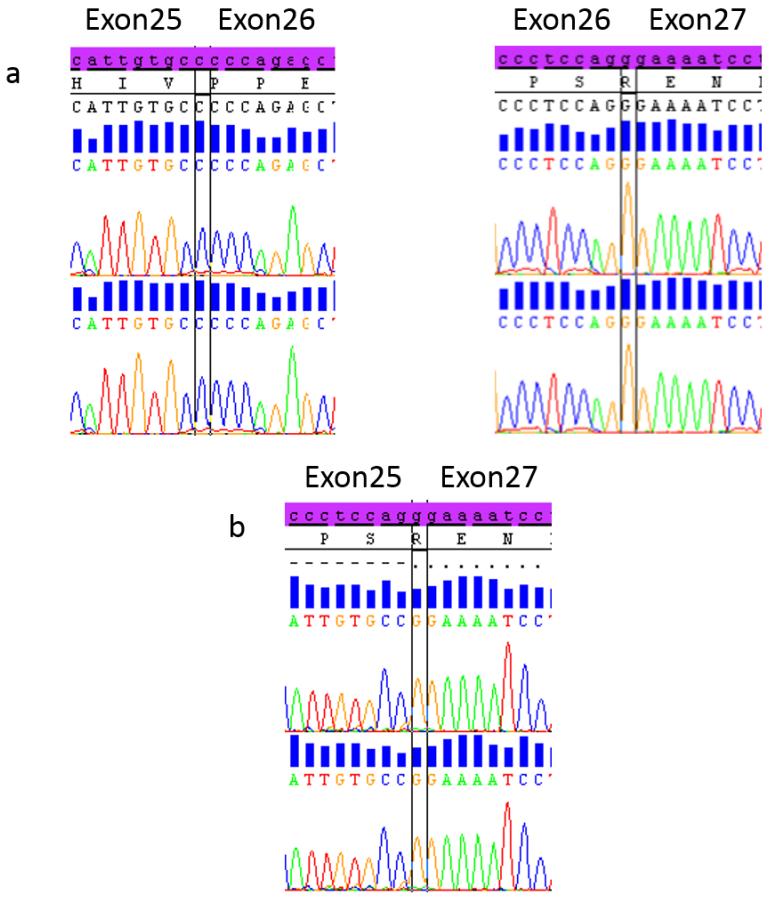

Figure 6 Sequencing results of the two separately sequenced gel bands of patient's cDNA

Figure a shows the sequencing results of band a, which correlates with the PCR product of a healthy control (Figure 5). The left figure pictures the exon boundary of exon 25 and 26, the right figure shows the boundary of exon 26 and 27.
In figure b the sequencing analysis of band b is shown. In the upper, violet part the correct sequence is shown but the sequencing curves show that exon 27 follows immediately on exon 25 and exon 26 is missing.

3.2.2 ADDITIONAL NOVEL SPLICE SITE MUTATIONS

Next to the intronic mutation c.3538+2T>C in intron 26 two other potential splice site mutations were identified by sequencing genomic VWF DNA. On the one hand a mutation at the donor site of intron 25 at position c.3379+2T>C and on the other hand a sequence variation at the acceptor site of intron 30 at position c.5312-2A>G. Both mutations are located in the conserved splice sites of the introns like the characterized mutation in intron 26. Consequently, it is very likely that these two mutations cause a modified protein. In case of the intron 25 mutation a skipped exon 25 is likely and in case of the mutation located at the acceptor site of intron 30 it is possible that exon 31 is skipped. It is very likely that both mutations lead to a shortened protein which function is altered.

3.3 CHARACTERIZATION OF NOVEL EXONIC MUTATIONS

The five novel exonic mutations indicated in Table 16 are not described in the literature and all of them lead to an amino acid change. The causality for VWD of these mutations was predicted by PolyPhen-2, Align GVGD and Project HOPE. Effects on the vWF protein are dependent on the position of the mutation in the VWF sequence and the respective domain of the vWF protein.

3.3.1 EXON 28: C.3692A>G, P.ASN1231SER

The adenine to guanine transition on position c.3692 causes an amino acid substitution from asparagine to serine on position p.1231. The obtained electropherogram of the sequencing results is shown in Figure 7.

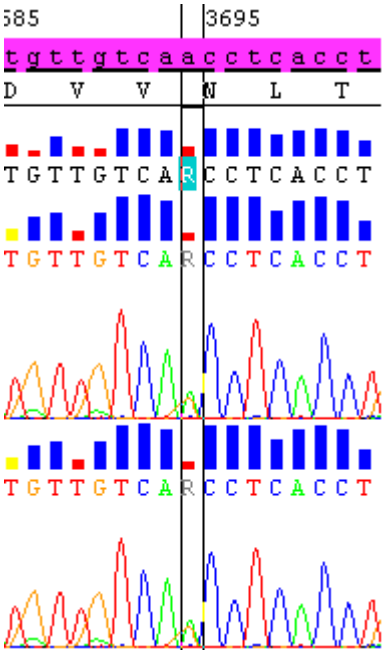

Figure 7 Sequencing results of mutation c.3692A>G, p.Asn1231Ser in exon 28

The heterozygous mutation on position c.3692 is highlighted. The transition leads to an asparagine to serine substitution.

All three prediction tools predicted the mutation as less risky for a functional interference of vWF. PolyPhen-2 predicted only a probability for the mutation of being damaging of 0,7 % (Figure 8).

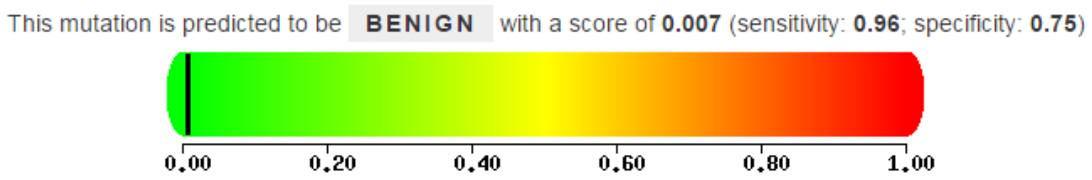

Figure 8 PolyPhen-2 prediction of mutation p.Asn1231Ser

PolyPhen-2 prediction of the putative causality of the exon 28 c.3692A>G mutation. The program predicts a possible causality for VWD of 0,7 %.

3.3.2 Exon 28: c.3820A>T, p.Arg1274Trp

This mutation leads to an amino acid switch from arginine to tryptophan on position p.1274. The analysis of the sequencing results is displayed in Figure 9.

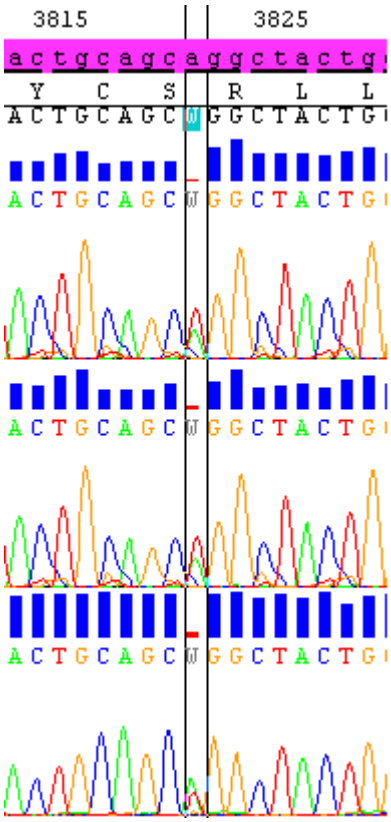

Figure 9 Sequencing results of mutation c.3820A>T, p.Arg1274Trp in exon 28

The heterozygous point mutation on position c.3820 is highlighted. The transversion leads to an arginine to tryptophan substitution.

The prediction tools Align GVGD and PolyPhen-2 predicted that this mutation is possibly damaging. PolyPhen-2 calculated the possibility for the mutation to be

damaging with a score of 93,5 % (Figure 10) and Project HOPE identified this mutation as possibly damaging.

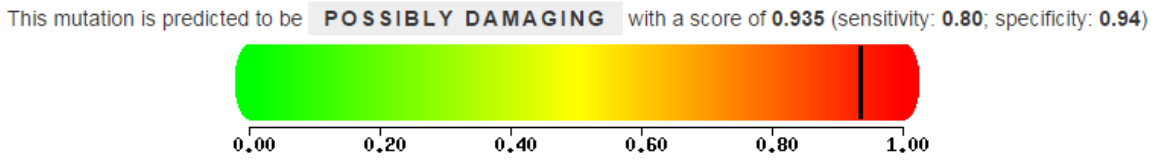

Figure 10 PolyPhen-2 prediction of mutation p.Arg1274Trp

PolyPhen-2 prediction of the putative causality of the exon 28 c.3820A>T mutation. The program predicts a causality for a defective protein caused by the tested mutation of 93,5 %.

3.3.3 Exon 28: c.3915G>C, p.Glu1305Asp

Sequencing analysis of heterozygous mutation c.3915G>C in exon 28 is shown in Figure 11.

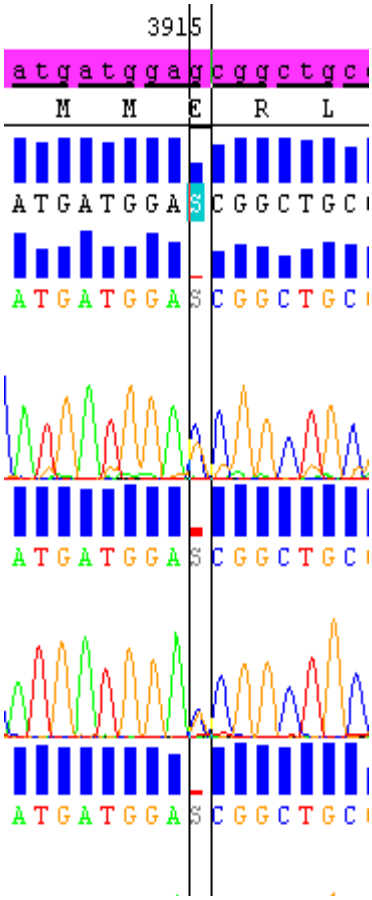

Figure 11 Sequencing results of mutation c.3915G>C, p.Glu1305Asp in exon 28

The figure shows the electropherogram of the sequencing results of mutation c.3915G>C.

This transversion from guanine to cytosine leads to an amino acid change from glutamic acid to aspartic acid on position p.1305.

This mutation was predicted as possibly damaging by Project HOPE and PolyPhen-2 with a score of more than 95 % (Figure 12). Align GVGD predicted a moderate impact of this glutamic acid to aspartic acid switch on the functionality of the protein.

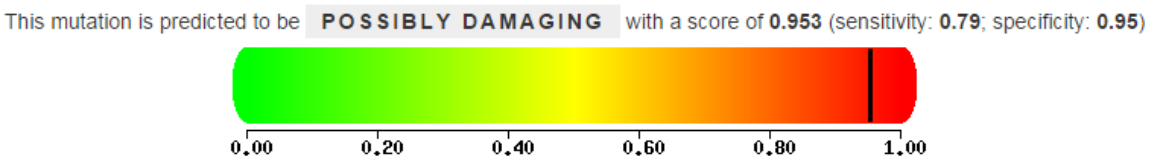

Figure 12 Polyphen2 prediction of mutation p.Glu1305Asp

PolyPhen-2 prediction of the putative causality of the exon 28 c.3915G>C mutation. The program predicts a possible causality for a defective vWF of 95 %.

3.3.4 Exon 28: c.4256A>C, p.His1419Pro

By sequencing the genomic VWF DNA of a patient suspected to suffer from VWD a heterozygous mutation on position c.4256 was detected. The presence of adenine on the WT allele and cytosine on the mutated allele is visible in the electropherogram shown in Figure 14.

The negative influence of this mutation on vWF is very likely because prediction tool PolyPhen-2 predicted a score of 99 % for this mutation to be probably damaging (Figure 13). Also Project HOPE and Align GVGD predicted a probably damaging impact of this mutation on the expressed protein.

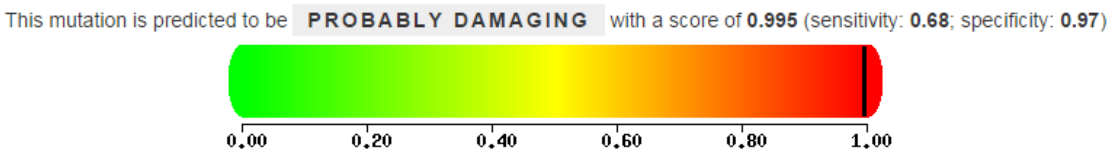

Figure 13 PolyPhen-2 prediction of mutation p.His1419Pro

PolyPhen-2 prediction of the putative causality of the exon 32 c.4256A>C mutation on the protein. The program predicts the mutation to be possible damaging with a score of 99,5 %.

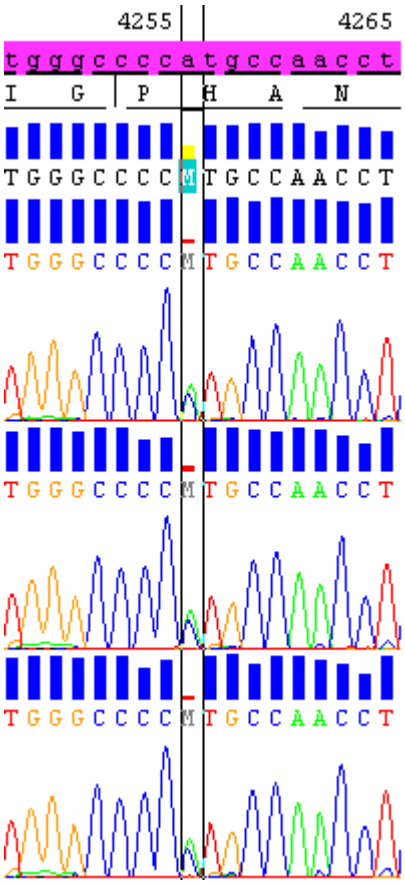

Figure 14 Sequencing results of mutation c.4256A>C, p.His1419Pro in exon 28

The heterozygous transversion from adenine to cytosine on position c.4256 is highlighted in the picture.

3.3.5 Exon 32: c.5488C>T, p.Arg1830Ser

The detected transition from cytosine to thymidine on position c.5488 leads to an amino acid change from arginine to serine on position p.1830. The sequencing results are analyzed and shown in Figure 15.

The possible impact of the mutation on the protein was predicted by PolyPhen-2, Align GVGD and Project HOPE. PolyPhen-2 (Figure 16) predicted the mutation to be possible damaging with a score of 91 %. Also Project HOPE and Align GVGD calculated a possibly damaging impact of this mutation on the vWF protein.

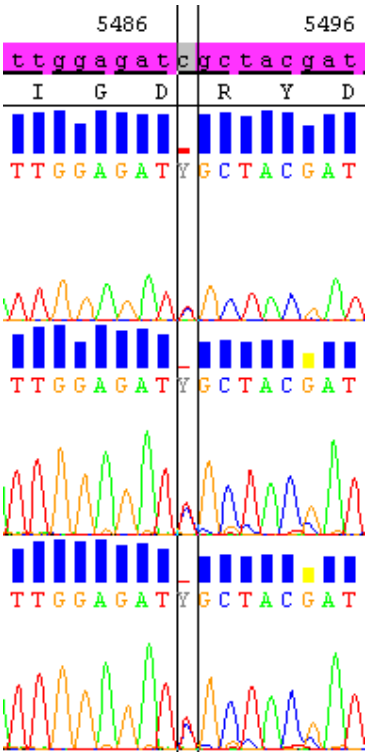

Figure 15 Sequencing results of mutation c.5488C>T, p.Arg1830Ser in exon 32

The highlighted base in the sequencing results show the heterozygous variant on position c.5488 in exon 32.

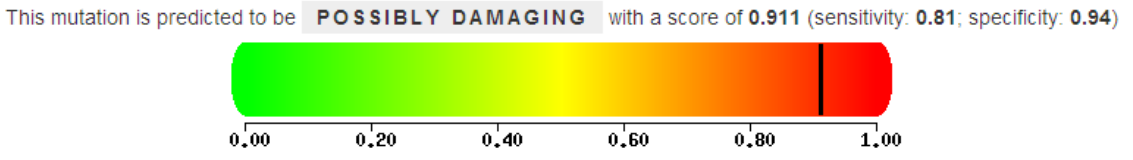

Figure 16 PolyPhen-2 prediction of mutation p.Arg1830Ser

PolyPhen-2 prediction of the putative damaging impact of the exon 32 c.5488C>T mutation on the protein. The program predicts a causality of 91 %.

3.4 GENERATING VWF CONSTRUCTS

The generated reference plasmid is based on the mRNA of a healthy control individual and is referred to as WT. To characterize the identified novel exonic mutations these mutations should be individually introduced into the WT plasmid by site directed mutagenesis. Figure 17 shows the PCR product of the VWF cDNA of a healthy control.

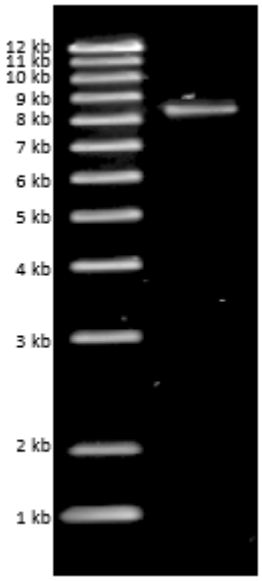

Figure 17 VWF cDNA agarose gel electrophoresis

VWF cDNA size determination by agarose gel electrophoresis. The cDNA consists of 8442 bp.

Subsequent sequencing verified the full length and correct sequence from c.-3 to c.8439 of the cDNA corresponding to the reference mRNA sequence of VWF (GenBank no. NM_00552.3).

After checking the sequence and cutting out the band of the gel the amplicon of the cDNA was ligated into a linearized pcDNA5/FRT/V5-His TOPO® TA expression vector and subsequently transformed into E. coli cells.

After cultivating the transformed E. colis, the plasmid was isolated and digested with the aid of BamHI-HF® to determine the presence and the correct orientation of the insert from 5' to 3' in the vector. Both, the vector and the insert carry an unique cutting site for BamHI. The size of the bands gives information on the direction of the insert (Figure 18).

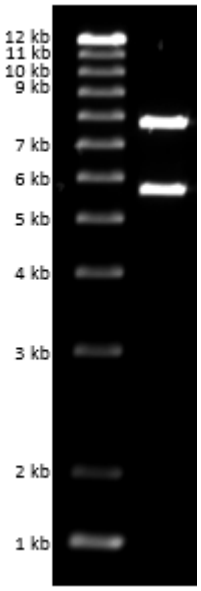

Figure 18 Plasmid digested with BamHI-HF®

The plasmid was digested with BamHI and the samples are separated on a 0,8 % agarose gel. There are two distinct bands visible: The DNA in the upper band consists of almost the whole vector and 2720 bp of the VWF insert (7795 bp). The DNA in the lower band consists of 5722 bp of the VWF insert and 25 bp of the vector (5747 bp).

Plasmids containing the insert in the correct orientation were sequenced and the plasmid with the insert carrying the least synthesis errors was used as target for site directed mutagenesis to generate the VWF WT construct. The designed primers used for site directed mutagenesis are listed in Table 17, the mutated base is highlighted in the table.

Table 17 Primes for site-directed mutagenesis to generate a WT plasmid

Position of the Mutation	Primer sequence
c.502T>C p.Phe168Cys	fw: 5'TGGCAACTTTAACATC**T**TTGCTGAAGATGAC 3' rv: 5'GTCATCTTCAGCAA**A**GATGTTAAAGTTGCCA 3'
c.853T>A p.Trp285Arg	fw: 5'ATGGTGCTGTACGGC**T**GGACCGACCAC 3' rv: 5'GTGGTCGGTCC**A**GCCGTACAGCACCAT 3'
c.2555G>A p.Arg852Gln	fw: 5'ACTTGTGTCTGTC**G**GGACCGGAAGTG 3' rv: 5'CACTTCCGGTCC**C**GACAGACACAAGT 3'

c.2979T>A p.Cys993stop	fw: 5'AGAAAGTGTG**T**GGCCTGTGTGGG 3' rv: 5'CCCACACAGGCC**A**CACACTTTCT 3'
c.4309G>A p.Ala1437Thr	fw: 5'TGAGAACAAG**G**CCTTCGTGCTGAG 3' rv: 5'CTCAGCACGAAGG**C**CTTGTTCTCA 3'
c.4693G>A p.Val1565Met	fw: 5'ACATCCTGCAGCGG**G**TGCGAGAGATC 3' rv: 5'GATCTCTCGCA**C**CCGCTGCAGGATGT 3'
c.5788A>G p.Ser1930Gly	fw: 5'TTCGTGCCCTAAC**A**GCCAGTCCCCTG 3' rv: 5'CAGGGGACTGGC**T**GTTAGGGCACGAA 3'
c.6085G>A p.Val2029Ile	fw: 5'TGAATGGGAGACTG**G**TCTCTGTTCCTTACG 3' rv: 5'CGTAAGGAACAGAGA**C**CAGTCTCCCATTCA 3'
c.7586T>A p.Leu2529His	fw: 5'GAGAACCCCTGCC**T**CATCAATGAGTGTG 3' rv: 5'CACACTCATTGATG**A**GGCAGGGGTTCTC 3'

The incorrect base is corrected in the primer sequence and highlighted.

After correction of all synthesis errors by using a site directed mutagenesis kit a plasmid is generated encoding for a vWF protein of a healthy individual. This plasmid is used as reference for the novel exonic mutations, which are introduced into separate plasmids. For this purpose a pair of forward and reverse primer for each exonic mutation of interest was designed. The primers are listed in Table 18. The missense mutation is highlighted in the table.

Table 18 Primes for site-directed mutagenesis to generate a mutant plasmid

Position of the mutation of interest	**Primer sequence**
c.3692A>G p.Asn1231Ser	fw: 5'CCACTGTGATGTTGTCA**A**CCTCACCTGTGAAGCC 3' rv: 5'GGCTTCACAGGTGAGG**T**TGACAACATCACAGTGG 3'
c.3820A>T p.Arg1274Trp	fw: 5'ATTTCTACTGCAGC**T**GGCTACTGGACCTGG 3' rv: 5'CCAGGTCCAGTAGCC**A**GCTGCAGTAGAAAT 3'
c.3915G>C p.Glu1305Asp	fw: 5'GGTGGACATGATGGA**C**CGGCTGCGCATCT 3' rv: 5'AGATGCGCAGCCG**G**TCCATCATGTCCACC 3'
c.4256A>C p.His1419Pro	fw: 5'GCATTGGGCCCC**C**TGCCAACCTCAAGCA 3' rv: 5'TGCTTGAGGTTGGCA**G**GGGGCCCAATGC 3'

c.5488C>T p.Arg1830Ser	fw: 5'ATTGGAATTGGAGAT**T**GCTACGATGCAGCCC 3' rv: 5'GGGCTGCATCGTAGC**A**ATCTCCAATTCCAAT 3'

The novel exonic mutation of interest is highlighted.

For testing the impact of the novel exonic mutation on the function and the expression of the vWF protein, mammalian cells e.g. HEK-cells have to be transfected with these constructs.

With the obtained recombinant proteins functional assays vWF:Ag, vWF:CB and vWF:RCo have to be performed and multimer formation has to be determined.

4 DISCUSSION

VWD is the most common autosomal inherited bleeding disorder caused by quantitative or qualitative defects of the vWF protein. It leads to minor to severe bleeding symptoms and is classified in three types. VWD types 1 and 3 are characterized by reduced or absent vWF levels. VWD type 2 is characterized by quantitative defects and is further subdivided into the four subtypes 2A, 2B, 2M and 2N.[49]

In this study 39 patients suspected to suffer from VWD were analyzed by sequencing the complete VWF gene. The analyzed patients presented with reduced vWF:Ag levels and/or vWF:RCo activity.

17 mutations in 19 individuals were identified, nine known disease causing mutations and eight mutations not described in the literature so far. Out of the eight novel mutations detected in eight distinct individuals, three are located at splice sites whereas the other five variations are exonic point mutations and are suspected to cause qualitative vWF defects. The eight mutations occurred heterozygously and no other putative disease causing mutation in the VWF gene was identified in these patients.

4.1 NOVEL SPLICE SITE MUTATIONS

4.1.1 INTRON 26: C.3538+2T>C

The intronic mutation c.3538+2T>C is located at the 5' end of intron 26 and disrupts a splice site consisting of a highly conserved GU dinucleotide, which marks the splice site for the spliceosome.[47,48] Thus, this mutation leads to skipping of exon 26 (Figure 6) and probably to a shortened and functionally deficient vWF protein.

Exon 26 is located in the D3 domain of VWF. This domain is responsible for FVIII binding and mutations in this region may lead to defective multimerization or dimerization of the protein.[50] Although the FVIII binding site of vWF is located between exon 18 and 23, mutations beyond this region i.e. form exon 23 to 27 are also associated with dramatically decreased binding of vWF to FVIII.[51] The defective multimerization and dimerization of VWF caused by mutations in the D3 domain explain the very low level of vWF antigen in the plasma of the affected patient, which is only 18 % of normal. The decreased binding to FVIII leads to a reduced FVIII:C activity of only 40 % because FVIII is not stabilized by vWF. Interestingly, this individual carries a heterozygous functional polymorphism in exon 20 (c.2555G>A) which can increase FVIII levels. However, the FVIII:C activity is low.

In other studies mutation c.3538+1G>A, as well located in the D3 domain of VWF, was identified as type 2A VWD causing variant with very similar vWF:Ag levels, vWF:RCo activity and FVIII:C activity.[52,53] Thus, the loss of an exon in this region seems to be associated with type 2A VWD, which suggests that the mutation identified in this study is also a type 2A VWD causing variant.

Since this patient carries one unaffected VWF allele, in case of elevated risk for bleeding (for example surgery) desmopressin might help to increase vWF:Ag levels and to ameliorate the bleeding tendency. In previous studies DDAVP prompted the secretion of all correctly folded vWF proteins out of endothelial cells.[54]

4.1.2 ADDITIONAL NOVEL SPLICE SITE MUTATIONS

The intronic mutation c.3379+2T>C is located in intron 25 disrupting a highly conserved splice site. As well as the intronic mutation in intron 26 it is located in the D3 domain of the protein, which is responsible for FVIII binding.[6,50] The vWF:Ag level and the vWF:RCo activity of this patient are very low (13 % and 12 % respectively) and thus FVIII:C activity is only 48 %. This mutation most likely causes exon 25 skipping and mutations in this region of VWF are associated with dimerization and multimerization defects of vWF and reduced FVIII binding of vWF.[51]

Mutation c.5312-2A>G is located in intron 30 and is most likely leading to exon 31 skipping. Since this region of VWF encodes for the A3 domain, which is responsible for collagen binding, mutations in this region will influence the binding of vWF to the subendothelial matrix.[55] However, due to the skipping of exon 31 protein levels will be low as reflected by the patient's low vWF:Ag levels of 25 %, vWF:RCo activity is only 19 % and FVIII:C activity 53 %.

In the patients VWF sequence also functional polymorphisms c.-250-670GT[18];[21] and c.4693G>T were detected. The GT tandem repeat in the VWF promoter region is associated with an increased promoter activity.[41] Since only in one allele more than 20 GT repeats are present, which causes increased promoter activity, it is possible that it is on the same allele as the intronic mutation. Also the VWD associated polymorphism c.4693G>T was detected heterozygously without knowledge on the affected allele. Thus, we cannot exclude that more than one variation is located on the same allele. However, if the point mutation c.4693G>T, which leads to increased proteolysis of vWF HMWM by ADAMTS13, is located on the allele not carrying the intronic mutation vWF level would be further reduced.[45]

4.2 NOVEL EXONIC MUTATIONS

All of the five newly identified exonic mutations lead to heterozygous amino acid substitutions that might affect the function or the structure of the vWF protein.

Based on their localization in the VWF gene and thus on their potential impact on the function of the mutated protein, these mutations can be assigned to three different groups: multimerization defects, GpIb platelet binding defects, and collagen binding defects.

Four novel mutations were identified in exon 28. Three of them are located in the A1 domain of the vWF protein and one is located in the D3 domain. With the A1 domain vWF binds to collagen types I, III and VI and mutations in this domain are associated with deficient platelet binding to sites of vascular injury.[55,56] Collagen type VI effects vWF-dependent platelet binding under conditions of low shear which enables platelets to be recruited to sites of superficial injuries without stimulating vessel occlusion.[56] The D3 domain of vWF is involved in the multimerization of the protein via disulfide bridge formation between the domains D3 and D' and the FVIII binding domain.[57]

The fifth novel mutation detected in this study is located in exon 32 in the A3 domain. Mutations in this domain of the vWF are associated with defective collagen binding and protein assembly is affected.[55]

4.2.1 EXON 28: C.3692A>G, P.ASN1231SER

The missense mutation c.3692A>G, p.Asn1231Ser is located in exon 28 in the D3 domain of the vWF protein.

The three *in silico* prediction tools PolyPhen-2, Project HOPE and Align GVGD predicted a not very likely influence of this mutation on protein function (Figure 8). Thus, functional studies with the mutated protein are required to rule out or to confirm an impact of this mutation on vWF function. In the affected patient vWF:Ag level (56 %) and vWF:RCo (44 %) are slightly reduced suggesting the presence of a genetic alteration with a minor impact on vWF function. Since the blood type of the patient is not known vWF:Ag levels could additionally be influenced by blood type – individuals with blood type 0 usually have reduced vWF levels.[29]

4.2.2 EXON 28: C.3820A>T, P.ARG1274TRP

Although c.3820A>T, p.Arg1274Trp is not described in the literature, in several studies mutations in the close proximity of this genetic alteration are characterized. A mutation of cysteine to serine, arginine, glycine or phenylalanine on amino acid position p.1272 leads to the decrease or absence of HMWM.[58–60] The con-

sequence of a cysteine to glycine or arginine substitution is an increased GpIb binding because of a conformational change of vWF.[58–60] In case of a arginine to serine or phenylalanine substitution the GpIb binding of vWF is decreased.[59,61] The mutations were identified in type 2A VWD patients.[62]

The patient analyzed in this study shows antigen levels within the normal range (91 %) but a dramatically reduced vWF:RCo activity of only 14 % suggesting a major impact of this mutation on platelet GpIb binding. These data are in line with the effects of the p.Cys1272Ser or Phe substitution which is located only two codons upstream of this novel mutation. Furthermore, the three *in silico* prediction tools PolyPhen-2, Project HOPE and Align GVGD predicted a very likely influence of this mutation on protein function (Figure 10). Conformational changes of the protein are possible because the arginine is smaller and positively charged whereas tryptophan is bigger than arginine, neutral and more hydrophobic.

4.2.3 Exon 28: c.3915G>C, p.Glu1305Asp

c.3915G>C, p.Glu1305Asp is located in the A1 domain of vWF. This domain represents the contact area of GpIbα which was shown in a study where the gain-of-function mutation p.Arg1306Gln was characterized.[63] This mutation was found in patients suffering from type 2B VWD.[63,64] The amino acid substitution p.Arg1306Trp is described as one of the most common type 2B VWD mutations and leads to an increased binding to platelet GpIbα.[65] Accordingly, c.3915G>C, p.Glu1305Asp was predicted to be possibly damaging by Project HOPE and PolyPhen-2 (Figure 12). Mutations in this region of VWF responsible for binding GpIbα will thus affect the number of platelets in the circulation causing mild to moderate thrombocytopenia.[66]

4.2.4 Exon 28: c.4256A>C, p.His1419Pro

c.4256A>C, p.His1419Pro is located in the A1 domain of vWF. In other studies an isoleucine to threonine or asparagine substitution at amino acid position p.1416 was described as responsible for type 2 VWD.[67] Mutations at p.Ile1416 are associated with reduced expression of vWF and decreased GpIbα binding but they do not affect collagen binding or multimer pattern formation.[67]

The analyzed patient has a vWF:Ag level of 28 % and a vWF:RCo activity of 17 %. This correlates with the described consequences for mutations at p.Ile1416 and indicates a major impact of c.4256A>C, p.His1419Pro on vWF structure and function. Accordingly, PolyPhen-2, Project HOPE and Align GVGD predicted this mutation to be probably damaging (Figure 13).

4.2.5 EXON 32: C.5488C>T, P.ARG1830SER

This exonic mutation c.5488C>T, p.Arg1830Ser is located in exon 32 in the A3 domain of vWF. Mutations in this region are associated with low vWF:Ag levels and defective collagen binding as described previously.[55]

The VWD causing mutation p.Pro1824His leads to low vWF:Ag levels due to intracellular retention of the defective vWF protein.[55] Additionally, reduced GpIIb/IIIa and GpIbα binding was detected.[55] The mutation is located in the main collagen types I and III binding domain and leads to reduced collagen binding and moderate to severe bleeding symptoms.[68]

The patient analyzed in this study carries a mutation in close proximity to p.Pro1824His at position p.Arg1830 and shows reduced vWF:Ag levels of 56 % and vWF:RCo levels of 44 %, which may reflect the compromised GpIbα binding caused by p.Arg1830Ser. Accordingly, PolyPhen-2, Align GVGD and Project HOPE predicted the mutation to be possible damaging (Figure 16).

4.2.6 LIMITATIONS OF THIS STUDY AND OUTLOOK

The results shown in this study are only predictions of the causality of the distinct mutation for VWD based on *in silico* assessments and comparisons to published studies. The fact that they are all located in functionally import domains of the protein makes it very likely that they have a negative influence on vWF function. To evaluate the causality of a detected mutation for VWD a large cohort of healthy individuals has to be analyzed for this mutation to determine its frequency in the general population without bleeding symptoms. Additionally, by genetic analysis of affected and unaffected family members of the respective patient the impact of the identified mutation on vWF could be further estimated. To finally prove the functional consequences of newly identified mutations recombinant vWF of a healthy control should be compared with vWF containing the respective mutation.

So far a plasmid carrying the VWF sequence of a healthy control individual was created and the mutation of interest was intended to be introduced into its VWF sequence by site directed mutagenesis. Since the sequence of the expressed recombinant proteins would differ at one particular position only, a direct comparison of vWF assays (vWF:Ag, vWF:RCo, vWF:CB, multimere pattern) performed with these proteins could demonstrate a functional consequence of the novel mutation on vWF structure and/or function.

However, upon sequencing of the primary VWF constructs several deviations from the original VWF sequence were detected – most likely due to synthesis errors of the reverse transcriptases used in this study (n=2 and one of them had supposedly proof reading qualities). However, considering the enormous size of

the template (~8,4 kb) the probability for synthesis errors is not low. Thus every plasmid we sequenced contained a number of unique mutations. The most promising construct contained only nine additional variations including missense mutations and stop codons that might be corrected by individual site directed mutagenesis in future studies to create the reference VWF construct. Subsequently the mutation of interest will be introduced into this plasmid – again by site directed mutagenesis. Following transfection of the reference and the mutated VWF construct into mammalian cells such as HEK293 the obtained recombinant vWF proteins will be analyzed as mentioned above. Differences in vWF:Ag, vWF:RCo and vWF:CB should reflect the impact of the respective mutation on vWF function.

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ABBREVIATIONS

µg ..... microgram
µl ..... microliter
µM ..... micromolar
µmol..... micromole
A..... Adenine
aa ..... amino acid
AF ..... allele frequency
Ag ..... antigen
Ala ..... alanine
Arg..... arginine
Asn..... asparagine
Asp..... aspartic acid
ATP ..... adenosine triphosphate
BGH ..... Bovine Growth Hormone
bla ..... Ampicillin resistance gene
bp ..... base pair
C..... Celsius, Cytosine
CB..... collagen binding
cDNA ..... complementary Deoxyribonucleic acid
cm ..... centimeter
cm^2 ..... square centimetre
CMV ..... Cytomegalovirus
CO_2 ..... carbon dioxide
CTP ..... Cytidine triphosphate
Cys..... cysteine
Da..... Dalton
DDAVP..... 1-Desamino-8-D-Arginin-Vasopressin
ddH_2O..... double-distilled water
ddNTP..... dideoxynucleotide triphosphate
del ..... deletion
DMEM ..... Dulbecco's Modified Eagle Medium
DMSO ..... dimethylsulfoxid
DNA ..... deoxyribonucleic acid
dNTP ..... deoxynucleotide triphosphate
DPBS ..... Dulbecco's Phosphate-buffered Saline
dsDNA ..... double stranded deoxyribonucleic acid
DTT ..... dithiothreitol
dup ..... duplication
E. coli ..... Escherichia coli
ELISA ..... enzyme-linked immunosorbent assay
ER..... Endoplasmatic reticulum
FBS ..... Fetal bovine serum
FRT ..... Flippase recognition target
fs ..... frameshift
FVIII..... factor VIII
fw ..... forward
g..... gram

G ..... Guanine
GFP ..... Green fluorescent protein
Gln ..... glutamine
Glu ..... glutamic acid
Gly ..... glycine
GPIb ..... glycoprotein Ib
GpIIb/IIIa ..... Glycoprotein IIb/IIIa
GpVI ..... Glycoprotein VI
GTP ..... Guanosine triphosphate
h ..... hour
H_2O ..... water
HEK ..... Human Embryonic Kidney
het ..... heterozygous
HF ..... high fidelity
HGMD ..... Human Gene Mutation Database
HGVS ..... Human Genome Variation Society
His ..... histidine
HMWM ..... high molecular weight multimer
Ile ..... isoleucine
kb ..... kilobases
kbp ..... kilobase pairs
kDa ..... kilo Dalton
kg ..... kilogram
l ..... liter
LB ..... Lysogeny broth
Leu ..... leucine
LRR ..... Leucin-rich region
Lys ..... lysine
MASTR ..... Multiplex Amplification of Specific Targets for Resequencing
Met ..... methionine
$MgCl_2$ ..... magnesiumchlorid
MID ..... molecular identifier
min ..... minute
ml ..... milliliter
mM ..... millimolar
mRNA ..... messenger ribonucleic acid
n/a ..... not available
NaCl ..... Sodium chloride
NaOH ..... Sodium hydroxide
NCBI ..... National Center for Biotechnology Information
ng ..... nanogram
NGS ..... next generation sequencing
PAGE ..... polyacrylamide gel electrophoresis
PCR ..... polymerase chain reaction
Pen/Strep ..... penicillin/streptomycin
Phe ..... phenylalanine
pmol ..... picomole
PP_i ..... inorganic pyrophosphate
Pro ..... proline
RCo ..... Ristocetin cofactor

RNA..... ribonucleic acid
rpm..... revolutions per minute
rv..... reverse
SAP..... Shrimp Alline Phosphatase
SDS..... sodium dodecyl sulfate
sec..... second
Ser..... serine
SNP..... Single Nucleotide Polymorphism
SOC..... Super Optimal broth with Catabolite repression
T..... Thymine
TBE..... Tris Borate Ethylenediaminetetraacetic acid
TE..... Tris Ethylenediaminetetraacetic acid
Thr..... threonine
Trp..... tryptophan
TTP..... Thymidine triphosphate
Tyr..... tyrosine
U..... unit, uracil
UTR..... untranslated region
UV..... ultra violet
V..... volt
Val..... valine
VWD..... von Willebrand disease
VWF..... von Willebrand factor gene
vWF..... von Willebrand factor protein
VWFP1..... von Willebrand pseudogene 1
vWS..... von Willebrand Syndrom
WT..... wild type

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APPENDIX

Table I PCR & sequencing primers for DNA amplification

Amplified region	**PCR primers & sequencing primers (S) primer position** (primer position referred to GenBank no. NG_009072.1)	**Product size**
5' region	**vWFP1fw:** 5'ATCTGTTGACCTTGTGATCC 3' 2501 … 2520 **vWFP1rv:** 5'ACATTACAAGAATGGGCAGT 3' 3373 … 3392 **vWFP1Sfw:** 5'GAATTCATCGTCAAGAGAGCT 3' 2819 … 2839 **vWFP1Srv:** 5'CCCAGCTAATTTTTTGTATT 3' 2994 … 3013	852 bp
	vWFP2-3fw: 5'GAGACAGGCAAACTTCCTTA 3' 3230 … 3249 **vWFP2-3rv:** 5'AGCTTTGTAGAGTTTGGAGG 3' 4896 … 4915 **vWFP2Srv:** 5'ATTTCCACTTCCAGCTCTTA 3' 3621 … 36940 **vWFP3Sfw:** 5'ACCACGCCTG GCTAATTTTT 3' 3811 … 3830 **vWFP2-3Sfw:** 5'CTGTCTCATGCTGATTCTGCCTC 3' 3976 … 3998 **vWFP2-3Srv:** 5'CTGCTTCTCCATGATACCTC 3' 4127 … 4146 **vWFP3Srv:** 5'CAGGGCAAGCTGATACAGGC 3' 4741 … 4760	1646 bp
Exon 1	**vWFE1fw:** 5'TTTTCTCAACTTCCCTGGAG 3' 4667 … 4685	698 bp

	vWFE1rv: 5'GTATTAGGATCATCCCTGGC 3' 5384 … 5403	
Exon 2	**vWFE2fw:** 5'GAGCATTCTCTATGTCTGGG 3' 6038 … 6057 **vWFE2rv:** 5'TGGAATACAAGTCAAGGGTG 3' 6684 … 6703	626 bp
Exon 3	**vWFE3fw:** 5'AGTTATTGCTGTGAGTCTGG 3' 8137 … 8156 **vWFE3rv:** 5'GTTCCAAAGCACCTTCTCTA 3' 8797 … 8816	640 bp
Exons 4 & 5	**vWFE4-5fw:** 5'CTACCTGAGGGCAATGTATC 3' 18478 … 18497 **vWFE4-5rv:** 5'AGGAGGATCGACTCAAACTA 3' 19566 … 19595 **vWFE4-5Sfw:** 5'AAGGTGGGAGAGACATCCAG 3' 19023 … 19042 **vWFE4-5Srv:** 5'AACATTTGCTTCCATTCTCT 3' 18891 … 18910	1068 bp
Exon 6	**vWFE6fw:** 5'GTGTTAGGTTGAGCACTGTA 3' 33625 … 33644 **vWFE6rv:** 5'AGACTCTAGCCACAGTTAGT 3' 34340 … 34359 **vWFE6Sfw:** 5'ACCACCAGCAGACCTAGAAT 3' 34002 … 34021	695 bp
Exon 7	**vWFE7fw:** 5'CCTTAGCCTTGGTCTACTTC 3' 53910 … 53929 **vWFE7rv:** 5'CGGTGAAGACAAAAACCAAA 3' 54664 … 54683	734 bp
Exon 8	**vWFE8fw:** 5'AAAGGGTTTTAGGTGAAGGG 3' 55617 … 55636	594 bp

	vWFE8rv: 5'CAGTACAACAGGACAACGTA 3' 56231 … 56250	
Exon 9	**vWFE9fw:** 5'ACAAGTTCTTTGAGCTTCCT 3' 57111 … 57130 **vWFE9rv:** 5'CCCCATCTTCAAGGCTTATT 3' 57538 … 57557	407 bp
Exon 10	**vWFE10fw:** 5'GTTTGGGGACTGTGATAACT 3' 58231 … 58250 **vWFE10rv:** 5'TTAGAACTGTCCCTTCAAGC 3' 58757 … 58776	506 bp
Exon 11	**vWFE11fw:** 5'ATCTTGCAGTTTTAGGAGCA 3' 54214 … 54233 **vWFE11rv:** 5'TGGGTTTCTGGATGAATGAG 3' 64676 … 64695	442 bp
Exon 12	**vWFE12fw:** 5'CTGGGAGAATGGAGTTGTAG 3' 65149 … 65168 **vWFE12rv:** 5'AGGCTTCCTTCTCTAGTCTT 3' 65621 … 65640	452 bp
Exon 13	**vWFE13fw:** 5'CTTCAGTGCTACCATCCTTT 3' 66506 … 66525 **vWFE13rv:** 5'ACCAGCCTCATAAACAAGAG 3' 66861 … 66880	335 bp
Exon 14	**vWFE14fw:** 5'TCTGATGATTTGGGAACAGG 3' 71404 … 71423 **vWFE14rv:** 5'GTGCTGACGGTAAAAACAAA 3' 71951 … 71970 **vWFE14Sfw:** 5'AAAACCATCTCATTAGCTAA 3' 71549 … 71568 **vWFE14Srv:** 5'GGTGGGAGCACTGCCTCCGGA 3' 71891 … 71901	527 bp

Exon 15	**vWFE15fw:** 5'AGCTTACAAACTCAGCGTTA 3' 72405 … 72424 **vWFE15rv:** 5'GAAAAGTGGGTGCAAATGAA 3' 72954 … 72973	529 bp
Exon 16	**vWFE16fw:** 5'AGTGGGAAAGGAAGTTGATG 3' 76645 … 76664 **vWFE16rv:** 5'AGACCTAAAGCCCAGAAATG 3' 77389 … 77408	724 bp
Exon 17	**vWFE17fw:** 5'ATATTGAAAGGCCAGGCAG 3' 82502 … 82520 **vWFE17rv:** 5'ACTCACACAAACCCAGAAAT 3' 83092 … 83111	571 bp
Exon 18	**vWFE18fw:** 5'AAGCCCAGGTGAGAAGATGC 3' 85030 … 85049 **vWFE18rv:** 5'CAGCACCACCTCCATTGCTA 3' 85491 … 85510	441 bp
Exon 19	**vWFE19fw:** 5'CCCGTAGGCTCAAGTCTCA 3' 93077 … 93096 **vWFE19rv:** 5'GTAACCAGGTTCCCACAGGG 3' 93379 … 93398	272 bp
Exon 20	**vWFE20fw:** 5'CATACTGGGCCTATGAAGTC 3' 94670 … 94689 **vWFE20rv:** 5'CTGGGAAGTACTCCAGTAGA 3' 95098 … 95117	408 bp
Exon 21	**vWFE21fw:** 5'GAGGTTGACACACCTGAGGG 3' 97893 … 97912 **vWFE21rv:** 5'CACACTCTGCACTGTCGCTA 3' 98438 … 98457	525 bp
Exon 22	**vWFE22fw:** 5'TTTTCTTCAGCTCTGTCTCC 3' 99691 … 99710	1769 bp

	vWFE22rv: 5'TCATGCAAGTTCCAACAAAC 3' 101480 … 101499 **vWFE22Srv:** 5'TAGTATCTGATAAAACAGTC 3' 100536 … 100555	
Exons 23 & 24	**vWFE23-24fw:** 5'TCCATTGCCGCCAGGAAT 3' 103534 … 103551 **vWFE23-24rv:** 5'GCAGATCATGTCAAGACACG 3' 104187 … 104206	635 bp
Exon 25	**vWFE25fw:** 5'CCACATGCTCATGAGGCT 3' 105535 … 105552 **vWFE25rv:** 5'ATCCAGTCCCTACTAACACT 3' 106150 … 106169	597 bp
Exon 26	**vWFE26fw:** 5'ATAGCAAGACCCCATCTG 3' 106548 … 106565 **vWFE26rv:** 5'AACTGCGTGTGGCTATTTAC 3' 107281 … 107300	715 bp
Exon 27	**vWFE27fw:** 5'GAGATGCTCCTTGAGTGATG 3' 107475 … 107494 **vWFE27rv:** 5'TTACCCAAAACCTAGTCTCTA 3' 107833 … 107853	338 bp
Exon 28	**vWFE28fw:** 5'CTTGGATGTGGAATGGTCCA 3' 109780 … 109799 **vWFE28rv:** 5'GGATTAGAACCCGAGTCG 3' 111388 … 111405 **vWFE28S1fw:** 5'AAGGCCTTCGTGCTGAGCA 3' 110559 … 110577 **vWFE28S2fw:** 5'GGTTCTGGATGTGGCGTTC 3' 110738 … 110756 **vWFE28S1rv:** 5'GGAACAGTGTGTATTTCAAGA 3' 110332 … 110352	1588 bp

	vWFE28S2rv: 5'CCTTGCTCCTGTTGAAGTC 3' 110790 … 110808	
Exons 29 & 30 & 31	**vWFE29-31fw:** 5'TAAATCTGAGGTAGTCCGGA 3' 112586 … 112605 **vWFE29-31rv:** 5'GGAAAATGTGGAGAAATTAAAGA 3' 113812 … 113834 **vWFE30Sfw:** 5'GACGAAGAGGCTCTTTTTGT 3' 112949 … 112968 **vWFE30Srv:** 5'CGGCTCGACACCCTGTCTTA 3' 113378 … 113397	1206 bp
Exon 32	**vWFE32fw:** 5'CATCTTCCTCATAGGGCTGA 3' 115888 … 115907 **vWFE32rv:** 5'TCCCATGAACAGAAACTTAAAG 3' 116337 … 116358	429 bp
Exons 33 & 34	**vWFE33-34fw:** 5'CTCAGCCTCATGTCCCTAT 3' 117377 … 117395 **vWFE33-34rv:** 5'AGAAAAGCAATTCTTCCTTCCA 3' 118301 … 118322 **vWFE34Sfw:** 5'TCCTTGCTGTGTAGGCCT 3' 117764 … 117781 **vWFE33Srv:** 5'GATAATAGTAAAAGGAAGCA 3' 117682 … 117701	905 bp
Exon 35	**vWFE35fw:** 5'TTATCTGTGTGCAACTTGGT 3' 133352 … 133371 **vWFE35rv:** 5'ACCCCTCTAAGAAAGTGGTA 3' 133802 … 133821	430 bp
Exons 36 & 37	**vWFE36-37fw1:** 5'CTAGATGCTCTTGGAGACAC 3' 134688 … 134707 **vWFE36-37rv1:** 5'AACATTTGCCCCTTGATTTG 3' 136761 … 136780	2053 bp

	vWFE36-37fw2: 5'GCAGTTGAGTGTGATGAATG 3' 134961 … 134980 **vWFE36-37rv2:** 5'GTTACACAGCCCTTTACAGA 3' 136169 … 136188 **vWFE37Sfw:** 5'CTTGGATGCTTATATTTCAG 3' 135331 … 135350 **vWFE37Srv:** 5'CAGGTCATACGAAATACAGGG 3' 135913 … 135933 **vWFE36Srv:** 5'TTGATGCCAGAGACAGGCCA 3' 135391 … 135410	1188 bp
Exon 38	**vWFE38fw:** 5'TGGCAAGTTTGTCTGATGAT 3' 137554 … 137573 **vWFE38rv:** 5'CACAATACTGCCTCCCTATC 3' 137980 … 137999	406 bp
Exons 39 & 40	**vWFE39-40fw:** 5'CTTTCAGCCTGCTTTTGTTT 3' 143853 … 143872 **vWFE39-40rv:** 5'TTTTTGGAGTATCCAGTCGG 3' 144751 … 144770 **vWFE40Sfw:** 5'CGCCTCTGGTCCCCAGCAAC 3' 144321 … 144340 **vWFE39Srv:** 5'GTCCCCATGTTCCTGTTGAC 3' 144381 … 144400	878 bp
Exon 41	**vWFE41fw:** 5'TGCATGAATAGGAATGTAGCA 3' 146326 … 146346 **vWFE41rv:** 5'CTCTGTTCCAGATGTACTCC 3' 146650 … 146669	303 bp
Exon 42	**vWFE42fw:** 5'CAGATGGACTGCTTTTGGTA 3' 147507 … 147527 **vWFE42rv:** 5'CTGGGTAGGATGCAAATCTT 3' 148027 … 148046	499 bp

Exon 43	**vWFE43fw:** 5'TAGCTTTTCTAAGGCTGGTG 3' 153208 … 153227 **vWFE43rv:** 5'CCAGAGGTCACTAAAGAACC 3' 153755 … 153774 **vWFE43Srv:** 5'AAAAAGAACCTTTCTTACCC 3' 153631 … 153650	527 bp
Exon 44	**vWFE44fw:** 5'GCTGGTTGACAACAAAGATG 3' 157820 … 157839 **vWFE44rv:** 5'GGCTTCTCCCAAAATCTACA 3' 158271 … 158290	431 bp
Exon 45	**vWFE45fw:** 5'CAGGTGATTTTGACCCAAAC 3' 160071 … 160090 **vWFE45rv:** 5'GTCTTGCCATGTAGTGATCT 3' 160649 … 160668	558 bp
Exons 46 & 47	**vWFE46-47fw:** 5'CTCCAGCAGTAGGACCC 3' 161335 … 161351 **vWFE46-47rv:** 5'TATTTCTGAGGCATGCAGTT 3' 162347 … 162366 **vWFE46Srv:** 5'TCCCGTCCTCCCACCTCCAG 3' 161901 … 161920	995 bp
Exon 48	**vWFE48fw:** 5'TTCCTTTTGGATGCAAGTCT 3' 175753 … 175772 **vWFE48rv:** 5'CACCAAGTACACTCTTGTCA 3' 176379 … 176398	606 bp
Exons 49 & 50	**vWFE49-50fw:** 5'TCCCTTCGTTCATCTCTAGT 3' 176848 … 176867 **vWFE49-50rv:** 5'GAGGGAAAAGGCACATAAGA 3' 178114 … 178133 **vWFE50Sfw:** 5'AGTGTGTGATTCTTGAATAA 3' 177501 … 177520	1246 bp

	vWFE49Srv: 5'CAGTTGAGTCATCAGGATCT 3' 177561 … 177580	
Exon 51	**vWFE51fw:** 5'TTGCCCATTAGCTTCTGAAT 3' 179417 … 179436 **vWFE51rv:** 5'GCGAATTTTCCAGATCCTTC 3' 179966 … 179985	529 bp
Exon 52	**vWFE52fw:** 5'TACAAATTGCCTAGCTGTCC 3' 180315 … 180334 **vWFE52rv:** 5'GAAGTTGGCAGTATCTCACA 3' 180909 … 180928	574 bp

Table II Used PCR reaction mix, cycler conditions and sequencing primers for DNA amplification and sequencing

Region	PCR reaction mix	Cycler conditions	Sequencing primers
5' region	B	2	P1fw P1rv P1Sfw P1Srv
	B	2	P2-3fw P2-3rv P 2Srv P 3Sfw P 2-3Sfw P 2-3Srv P 3Srv

Exon 1	B	2	E1fw 2x E1rv
Exon 2	A	1	E2fw E2rv
Exon 3	A	1	E3fw E3rv
Exons 4 & 5	B	2	E4-5fw E4-5rv E4-5Sfw E4-5Srv
Exon 6	B	2	E6fw 2x E6rv E6Sfw
Exon 7	B	2	E7fw E7rv
Exon 8	A	1	E8fw E8rv
Exon 9	A	1	E9fw E9rv
Exon 10	A	1	E10fw E10rv
Exon 11	A	1	E11fw E11rv

Exon 12	A	1	E12fw E12rv
Exon 13	B	2	E13fw E13rv
Exon 14	B	2	E14fw E14rv E14Sfw E14Srv
Exon 15	A	1	E15fw E15rv
Exon 16	B	2	2x E16fw 2x E16rv
Exon 17	B	2	E17fw E17rv
Exon 18	B	2	E18fw E18rv
Exon 19	B	2	E19fw E19rv
Exon 20	A	1	E20fw E20rv
Exon 21	A	1	E21fw E21rv

Exon 22	B	1	2x E22fw E22Srv
Exons 23 & 24	B	2	E23-24fw E23-24rv
Exon 25	A	1	E25fw E25rv
Exon 26	A	1	E26fw E26rv
Exon 27	A	1	E27fw E27rv
Exon 28	B	2	E28fw E28rv E28S1fw E28S2fw E28S1rv E28S2rv
Exons 29, 30 & 31	B	3	E29-31fw E29-31rv E30Sfw E30Srv
Exon 32	A	1	E32fw E32rv

Exon 33 & 34	A	1	E33-34fw E33-34rv E34Sfw E33Srv
Exon 35	A	1	E35fw E35rv
Exon 36 & 37	C	4	E36-37fw2 E36-37rv2 E37Sfw E37Srv E36Srv
Exon 38	A	1	E38fw E38rv
Exons 39 & 40	A	1	E39-40fw E39-40rv 2x E40Sfw E39Srv
Exon 41	B	1	E41fw E41rv
Exon 42	B	2	E42fw E42rv

Exon 43	B	2	E43fw E43rv E43Srv
Exon 44	B	2	E44fw E44rv
Exon 45	B	2	E45fw E45rv
Exons 46 & 47	A	1	E46-47fw 2x E46-47rv E46Srv
Exon 48	A	1	E48fw E48r
Exons 49 & 50	A	1	E49-50fw E49-50rv E50Sfw E49Srv
Exon 51	B	2	E51fw E51rv
Exon 52	B	2	E52fw E52rv

Table III Primers for cDNA amplification and sequencing

Primers	Primer sequence and position (primer position referred to GenBank no. NM_000552.3)
cDNAfw	5'AAGATGATTCCTGCCAGATTTGCCGGGG 3' 248 … 275
rs1rv	5'ATGCCCGTTCACACCACTGT 3' 841 … 860
rs0fw	5'TGTCAATGGTACCGTGACAC 3' 541 … 560
rs0rv	5'CAAAGCTGTACATGCTCCCA 3' 391 … 410
rs1fw	5'AGTCCTGCTGTCAGACAGAT 3' 691 … 710
rs2rv	5'GGAATGCACGCAGGGACACT 3' 1290 … 1309
rs1.1fw	5'GAGAAGACTTTGTGTGAGTG 3' 1001 … 1020
rs2fw	5'TGGTATGGAGTATAGGCAGT 3' 1141 … 1160
rs3rv	5'CAGTCCCCGTGCAGCTTCCA 3' 1907 … 1926
rs2.1fw	5'CAGTGTGCTGATGACCGCGA 3' 1541 … 1560
rs2.1rv	5'CTGAAGGTGAAGTATCTGTT 3' 1451 … 1570
rs3fw	5'ACTGAAGCATGGGGCAGGAG 3' 1621 … 1640
rs4rv	5'TCCTTTTGCTGCGATGAGAC 3' 2521 … 2540

rs3.1rv	5'TCGGGCAGTTCAGCTCACAG 3' 2191 … 2210
rs4fw	5'GACCTGCCGCTCTCTCTCTT 3' 2251 … 2270
rs5rv	5'CCCAGCAGCAGAATGATGTA 3' 3131 … 3150
rs4.1fw	5'ACCATCACACCATGTGCTAC 3' 2421 … 2440
rs5fw	5'CTGCACAGACCATGTGTGTG 3' 2821 … 2840
rs6rv	5'CCCTCCACACACTGCACA 3' 3748 … 3765
rs5.1fw	5'TGCAAGAAACGGGTCACCAT 3' 3011 … 3030
rs5.1rv	5'CACTTTTCTGGTGTCAGCAC 3' 3342 … 3361
rs6fw	5'CTTACCAGTGACGTCTTCCA 3' 3437 … 3456
vWFE28S1rv	5'GGAACAGTGTGTATTTCAAGA 3' 4329 … 4349
rs7rv	5'AGTAGAAATCGTGCAACGGCG 3' 4044 … 4064
rs7fw	5'GTGGTGGACATGATGGAGCG 3' 4148 … 4167
rs8rv	5'CAGCACTGACACCTGAGTGA 3' 5430 … 5449
vWFE28S1fw	5'AAGGCCTTCGTGCTGAGCA 3' 4556 … 4574
vWFE28S2fw	5'GGTTCTGGATGTGGCGTTC 3' 4735 …4753

vWFE28S2rv	5'CCTTGCTCCTGTTGAAGTC 3' 4787 … 4805
rs8fw	5'CCCACCCTCTCCCCTGCA 3' 5282 … 5299
rs9rv	5'AGCCCCCGGTCACAGTTG 3' 6001 … 6018
rs8.1rv	5'CCAGGATGACCACCGCCTTT 3' 5629 … 5648
rs9fw	5'CCTCCACAAACTGTGCTCTG 3' 5851 … 5870
rs10rv	5'GAGCTGTCGGGGACAAGACA 3' 6641 … 6660
rs9.1fw	5'TGGAGGTGATTCTCCATAAT 3' 6201 … 6220
rs9.1rv	5'GATTGAATCTGACCTCATGCATG 3' 6388 … 6410
rs10fw	5'CAAAGACGTATGGTCTGTGTG 3' 6486 … 6506
rs11rv	5'GCAGCACTGGTCTGCATTCT 3' 7191 … 7210
rs11fw	5'CAACTGCACAACGCAGCCCT 3' 7117 … 7136
rs12rv	5'CATTGATGAGGCAGGGGTTC 3' 7825 … 7844
rs11.1fw	5'AGGAGGAGTGCAAAAGAGTG 3' 7341 … 7360
rs11.1rv	5'CAAGTACCCAAGGGGACAGC 3' 7461 … 7480
rs12fw	5'TCGGTCGGGCTTCACTTACG 3' 7681 … 7700

rs13rv	5'CCTGTGACCCTCTTCTCCCA 3' 8306 … 8325
rs13fw	5'AGGTGAATGTTGTGGGAGAT 3' 8161 … 8180
cDNArvHis	5'CTTGCTGCACTTCCTGGGGGAGCATTT 3' 8663 … 8689
rs14fw	5'TCAACGATGTGCAGGACCAG 3' 8541 … 8560

Table IV Reference sequence of VWF mRNA (GenBank no. NM_000552.3)

The reference sequence of the VWF mRNA shows bases c.-3 to c.8439. The starting codon ATG is highlighted and the more common allele variations in the European population (Frequencies from HAP MAP CEU) are marked in green.

```
  -3        aag atgattcctg ccagatttgc cggggtgctg cttgctctgg ccctcatttt
  51 gccagggacc ctttgtgcag aaggaactcg cggcaggtca tccacggccc gatgcagcct
 111 tttcggaagt gacttcgtca acacctttga tgggagcatg tacagctttg cgggatactg
 171 cagttacctc ctggcagggg gctgccagaa acgctccttc tcgattattg gggacttcca
 231 gaatggcaag agagtgagcc tctccgtgta tcttggggaa ttttttgaca tccatttgtt
 291 tgtcaatggt accgtgacac agggggacca aagagtctcc atgccctatg cctccaaagg
 351 gctgtatcta gaaactgagg ctgggtacta caagctgtcc ggtgaggcct atggctttgt
 411 ggccaggatc gatggcagcg gcaactttca agtcctgctg tcagacagat acttcaacaa
 471 gacctgcggg ctgtgtggca actttaacat ctttgctgaa gatgacttta tgacccaaga
 531 agggaccttg acctcggacc cttatgactt tgccaactca tgggctctga gcagtggaga
 591 acagtggtgt gaacgggcat ctcctcccag cagctcatgc aacatctcct ctggggaaat
 651 gcagaagggc ctgtgggagc agtgccagct tctgaagagc acctcggtgt ttgcccgctg
 711 ccaccctctg gtggaccccg agccttttgt ggccctgtgt gagaagactt tgtgtgagtg
 771 tgctgggggg ctggagtgcg cctgccctgc cctcctggag tacgcccgga cctgtgccca
 831 ggagggaatg gtgctgtacg gctggaccga ccacagcgcg tgcagcccag tgtgccctgc
 891 tggtatggag tataggcagt gtgtgtcccc ttgcgccagg acctgccaga gcctgcacat
 951 caatgaaatg tgtcaggagc gatgcgtgga tggctgcagc tgccctgagg gacagctcct
1011 ggatgaaggc ctctgcgtgg agagcaccga gtgtccctgc gtgcattccg gaaagcgcta
1071 ccctcccggc acctccctct ctcgagactg caacacctgc atttgccgaa acagccagtg
1131 gatctgcagc aatgaagaat gtccagggga gtgccttgtc acaggtcaat cacacttcaa
1191 gagctttgac aacagatact tcaccttcag tgggatctgc cagtacctgc tggcccggga
1251 ttgccaggac cactccttct ccattgtcat tgagactgtc cagtgtgctg atgaccgcga
1311 cgctgtgtgc acccgctccg tcaccgtccg gctgcctggc ctgcacaaca gccttgtgaa
1371 actgaagcat ggggcaggag ttgccatgga tggccaggac gtccagctcc ccctcctgaa
1431 aggtgacctc cgcatccagc Gtacagtgac ggcctccgtg cgcctcagct acggggagga
1491 cctgcagatg gactgggatg gccgcgggag gctgctggtg aagctgtccc ccgtctatgc
1551 cgggaagacc tgcggcctgt gtgggaatta caatggcaac cagggcgacg acttccttac
1611 cccctctggg ctggcggagc cccgggtgga ggacttcggg aacgcctgga agctgcacgg
1671 ggactgccag gacctgcaga agcagcacag cgatccctgc gccctcaacc cgcgcatgac
1731 caggttctcc gaggaggcgt gcgcggtcct gacgtccccc acattcgagg cctgccatcg
1791 tgccgtcagc ccgctgccct acctgcggaa ctgccgctac gacgtgtgct cctgctcgga
```

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1851 cggccgcgag tgcctgtgcg gcgccctggc cagctatgcc gcggcctgcg cggggagagg
1911 cgtgcgcgtc gcgtggcgcg agccaggccg ctgtgagctg aactgcccga aaggccaggt
1971 gtacctgcag tgcgggaccc cctgcaacct gacctgccgc tctctctctt acccggatga
2031 ggaatgcaat gaggcctgcc tggagggctg cttctgcccc ccagggctct acatggatga
2091 gaggggggac tgcgtgccca aggcccagtg cccctgttac tatgacggtg agatcttcca
2151 gccagaagac atcttctcag accatcacac catgtgctac tgtgaggatg gcttcatgca
2211 ctgtaccatg agtggagtcc ccggaagctt gctgcctgac gctgtcctca gcagtcccct
2271 gtctcatcgc agcaaaagga gcctatcctg tcggcccccc atggtcaagc tggtgtgtcc
2331 cgctgacaac ctgcgggctg aagggctcga gtgtaccaaa acgtgccaga actatgacct
2391 ggagtgcatg agcatgggct gtgtctctgg ctgcctctgc cccccgggca tggtccggca
2451 tgagaacaga tgtgtggccc tggaaaggtg tccctgcttc catcagggca aggagtatgc
2511 ccctggagaa acagtgaaga ttggctgcaa cacttgtgtc tgtcgggacc ggaagtggaa
2571 ctgcacagac catgtgtgtg atgccacgtg ctccacgatc ggcatggccc actacctcac
2631 cttcgacggg ctcaaatacc tgttccccgg ggagtgccag tacgttctgg tgcaggatta
2691 ctgcggcagt aaccctggga cctttcggat cctagtgggg aataagggat gcagccaccc
2751 ctcagtgaaa tgcaagaaac gggtcaccat cctggtggag ggaggagaga ttgagctgtt
2811 tgacggggag gtgaatgtga agaggcccat gaaggatgag actcactttg aggtggtgga
2871 gtctggccgg tacatcattc tgctgctggg caaagccctc tccgtggtct gggaccgcca
2931 cctgagcatc tccgtggtcc tgaagcagac ataccaggag aaagtgtgtg gcctgtgtgg
2991 gaattttgat ggcatccaga acaatgacct caccagcagc aacctccaag tggaggaaga
3051 ccctgtggac tttgggaact cctggaaagt gagctcgcag tgtgctgaca ccagaaaagt
3111 gcctctggac tcatcccctg ccacctgcca taacaacatc atgaagcaga cgatggtgga
3171 ttcctcctgt agaatcctta ccagtgacgt cttccaggac tgcaacaagc tggtggaccc
3231 cgagccatat ctggatgtct gcatttacga cacctgctcc tgtgagtcca ttggggactg
3291 cgcctgcttc tgcgacacca ttgctgccta tgcccacgtg tgtgcccagc atggcaaggt
3351 ggtgacctgg aggacggcca cattgtgccc ccagagctgc gaggagagga atctccggga
3411 gaacgggtat gagtgtgagt ggcgctataa cagctgtgca cctgcctgtc aagtcacgtg
3471 tcagcaccct gagccactgg cctgccctgt gcagtgtgtg gagggctgcc atgcccactg
3531 ccctccaggg aaaatcctgg atgagctttt gcagacctgc gttgaccctg aagactgtcc
3591 agtgtgtgag gtggctggcc ggcgttttgc ctcaggaaag aaagtcacct tgaatcccag
3651 tgaccctgag cactgccaga tttgccactg tgatgttgtc aacctcacct gtgaagcctg
3711 ccaggagccg ggaggcctgg tggtgcctcc cacagatgcc ccggtgagcc ccaccactct
3771 gtatgtggag gacatctcgg aaccgccgtt gcacgatttc tactgcagca ggctactgga
3831 cctggtcttc ctgctggatg gctcctccag gctgtccgag gctgagtttg aagtgctgaa
3891 ggcctttgtg gtggacatga tggagcggct gcgcatctcc cagaagtggg tccgcgtggc
3951 cgtggtggag taccacgacg gctcccacgc ctacatcggg ctcaaggacc ggaagcgacc
4011 gtcagagctg cggcgcattg ccagccaggt gaagtatgcg ggcagccagg tggcctccac
4071 cagcgaggtc ttgaaataca cactgttcca aatcttcagc aagatcgacc gccctgaagc
4131 ctcccgcatc Gccctgctcc tgatggccag ccaggagccc caacggatgt cccggaactt
4191 tgtccgctac gtccagggcc tgaagaagaa gaaggtcatt gtgatcccgg tgggcattgg
4251 gccccatgcc aacctcaagc agatccgcct catcgagaag caggcccctg agaacaaggc
4311 cttcgtgctg agcagtgtgg atgagctgga gcagcaaagg gacgagatcg ttagctacct
4371 ctgtgacctt gcccctgaag cccctcctcc tactctgccc cccgacatgg cacaagtcac
4431 tgtgggcccg gggctcttgg gggtttcgac cctggggccc aagaggaact ccatggttct
4491 ggatgtggcg ttcgtcctgg aaggatcgga caaaattggt gaagccgact tcaacaggag
4551 caaggagttc atggaggagg tgattcagcg gatggatgtg ggccaggaca gcatccacgt
4611 cacggtgctg cagtactcct acatggtgac Cgtggagtac cccttcagcg aggcacagtc
4671 caaaggggac atcctgcagc gggtgcgaga gatccgctac cagggcggca acaggaccaa
4731 cactgggctg gccctgcggt acctctctga ccacagcttc ttggtcagcc agggtgaccg
4791 ggagcaggcg cccaacctgg tctacatggt caccggaaat cctgcctctg atgagatcaa
4851 gaggctgcct ggagacatcc aggtggtgcc cattggagtg ggccctaatg ccaacgtgca
4911 ggagctggag aggattggct ggcccaatgc ccctatcctc atccaggact ttgagacgct
4971 cccccgagag gctcctgacc tggtgctgca gaggtgctgc tccggagagg ggctgcagat
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5031 ccccaccctc tcccctgcac ctgactgcag ccagcccctg gacgtgatcc ttctcctgga
5091 tggctcctcc agtttcccag cttcttattt tgatgaaatg aagagtttcg ccaaggcttt
5151 catttcaaaa gccaatatag ggcctcgtct cactcaggtg tcagtgctgc agtatggaag
5211 catcaccacc attgacgtgc catggaacgt ggtcccggag aaagcccatt tgctgagcct
5271 tgtggacgtc atgcagcggg agggaggccc cagccaaatc ggggatgcct tgggctttgc
5331 tgtgcgatac ttgacttcag aaatgcatgg tgccaggccg ggagcctcaa aggcggtggt
5391 catcctggtc acggacgtct ctgtggattc agtggatgca gcagctgatg ccgccaggtc
5451 caacagagtg acagtgttcc ctattggaat tggagatcgc tacgatgcag cccagctacg
5511 gatcttggca ggcccagcag gcgactccaa cgtggtgaag ctccagcgaa tcgaagacct
5571 ccctaccatg gtcaccttgg gcaattcctt cctccacaaa ctgtgctctg gatttgttag
5631 gatttgcatg gatgaggatg ggaatgagaa gaggcccggg gacgtctgga ccttgccaga
5691 ccagtgccac accgtgactt gccagccaga tggccagacc ttgctgaaga gtcatcgggt
5751 caactgtgac cgggggctga ggccttcgtg ccctaacagc cagtccctg ttaaagtgga
5811 agagacctgt ggctgccgct ggacctgccc ctgcgtgtgc acaggcagct ccactcggca
5871 catcgtgacc tttgatgggc agaatttcaa gctgactggc agctgttctt atgtcctatt
5931 tcaaaacaag gagcaggacc tggaggtgat tctccataat ggtgcctgca gccctggagc
5991 aaggcagggc tgcatgaaat ccatcgaggt gaagcacagt gccctctccg tcgagctgca
6051 cagtgacatg gaggtgacgg tgaatgggag actggtctct gttccttacg tgggtgggaa
6111 catggaagtc aacgtttatg gtgccatcat gcatgaggtc agattcaatc accttggtca
6171 catcttcaca ttcactccac aaaacaatga gttccaactg cagctcagcc ccaagacttt
6231 tgcttcaaag acgtatggtc tgtgtgggat ctgtgatgag aacggagcca atgacttcat
6291 gctgagggat ggcacagtca ccacagactg gaaaacactt gttcaggaat ggactgtgca
6351 gcggccaggg cagacgtgcc agcccatcct ggaggagcag tgtcttgtcc ccgacagctc
6411 ccactgccag gtcctcctct taccactgtt tgctgaatgc cacaaggtcc tggctccagc
6471 cacattctat gccatctgcc agcaggacag ttgccaccag gagcaagtgt gtgaggtgat
6531 cgcctcttat gcccacctct gtcggaccaa cggggtctgc gttgactgga ggacacctga
6591 tttctgtgct atgtcatgcc caccatctct ggtctacaac cactgtgagc atggctgtcc
6651 ccggcactgt gatggcaacg tgagctcctg tggggaccat ccctccgaag gctgtttctg
6711 ccctccagat aaagtcatgt tggaaggcag ctgtgtccct gaagaggcct gcactcagtg
6771 cattggtgag gatggagtcc agcaccagtt cctggaagcc tgggtcccgg accaccagcc
6831 ctgtcagatc tgcacatgcc tcagcgggcg gaaggtcaac tgcacaacgc agccctgccc
6891 cacggccaaa gctcccacgt gtggcctgtg tgaagtagcc cgcctccgcc agaatgcaga
6951 ccagtgctgc cccgagtatg agtgtgtgtg tgacccagtg agctgtgacc tgcccccagt
7011 gcctcactgt gaacgtggcc tccagcccac actgaccaac cctggcgagt gcagacccaa
7071 cttcacctgc gcctgcagga aggaggagtg caaaagagtg tccccaccct cctgcccccc
7131 gcaccgtttg cccacccttc ggaagaccca gtgctgtgat gagtatgagt gtgcctgcaa
7191 ctgtgtcaac tccacagtga gctgtcccct tgggtacttg gcctcaacCg ccaccaatga
7251 ctgtggctgt accacaacca cctgccttcc cgacaaggtg tgtgtccacc gaagcaccat
7311 ctaccctgtg ggccagttct gggaggaggg ctgcgatgtg tgcacctgca ccgacatgga
7371 ggatgccgtg atgggcctcc gcgtggccca gtgctcccag aagccctgtg aggacagctg
7431 tcggtcgggc ttcacttacg ttctgcatga aggcgagtgc tgtggaaggt gcctgccatc
7491 tgcctgtgag gtggtgactg gctcaccgcg gggggactcc cagtcttcct ggaagagtgt
7551 cggctcccag tgggcctccc cggagaaccc ctgcctcatc aatgagtgtg tccgagtgaa
7611 ggaggaggtc tttatacaac aaaggaacgt ctcctgcccc cagctggagg tccctgtctg
7671 cccctcgggc tttcagctga gctgtaagac ctcagcgtgc tgcccaagct gtcgctgtga
7731 gcgcatggag gcctgcatgc tcaatggcac tgtcattggg cccgggaaga ctgtgatgat
7791 cgatgtgtgc acgacctgcc gctgcatggt gcaggtgggg gtcatctctg gattcaagct
7851 ggagtgcagg aagaccacct gcaacccctg ccccctgggt tacaaggaag aaaataacac
7911 aggtgaatgt tgtgggagat gtttgcctac ggcttgcacc attcagctaa gaggaggaca
7971 gatcatgaca ctgaagcgtg atgagacgct ccaggatggc tgtgatactc acttctgcaa
8031 ggtcaatgag agaggagagt acttctggga gaagagggtc acaggctgcc cacccttga
8091 tgaacacaag tgtctggctg agggaggtaa aattatgaaa attccaggca cctgctgtga
8151 cacatgtgag gagcctgagt gcaacgacat cactgccagg ctgcagtatg tcaaggtggg
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8211 aagctgtaag tctgaagtag aggtggatat ccactactgc cagggcaaat gtgccagcaa
8271 agccatgtac tccattgaca tcaacgatgt gcaggaccag tgctcctgct gctctccgac
8331 acggacggag cccatgcagg tggccctgca ctgcaccaat ggctctgttg tgtaccatga
8391 ggttctcaat gccatggagt gcaaatgctc ccccaggaag tgcagcaag
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Table V Detected polymorphisms and not disease causing variations

Position	Frequency in % (SNP) AA:Aa:aa	Frequency in % (42 patients) AA:Aa:aa	rs number
5' UTR:			
c.-250-2173G>A	n/a	98:2:0	n/a
c.-250-2125T>C	n/a	98:2:0	n/a
c.-250-1997A>T	n/a	98:2:0	rs12315619
c.-250-1890A>G	n/a	98:2:0	rs113248472
c.-250-1835delA (WT=13A)	n/a	0:100:0	n/a
c.-250-1794delG	n/a	98:2:0	rs66999924
c.-250-1794G>C	n/a	10:50:40	rs7966230
c.-250-1597T>C	n/a	98:2:0	rs73266901
c.-250-1235C>T	n/a	7:52:41	rs7964777
c.-250-1187A>G	n/a	18:41:41	rs7954855
c.-250-1141A>G	n/a	98:2:0	rs112531308
c.-250-1053G>A	AF 35:65 [B]	9:50:41	rs7965413
c.-250-1048C>T	n/a	91:9:0	rs151088025
Intron 1:			
c.1-64C>T	10:48:42 [A]	12:52:36	rs2286608
Intron 2:			
c.56-47A>C	n/a	98:2:0	rs369855929
c.56-46G>T	n/a	98:2:0	rs41299142
c.56-40C>T	AF 99:1 [B]	95:5:0	rs113081282
Intron 3:			
c.220+52T>C	AF 2:98 [B]	0:7:93	rs6489694
c.220+71G>A	n/a	95:5:0	n/a
Intron 5:			
c.533-153delT	n/a	9:50:41	rs5796209
Intro 6:			
c.657+11A>C	AF 70:30 [B]	93:7:0	rs7980045

Intron 7:			
c.875-274A>G	n/a	76:24:0	n/a
Exon 8:			
c.954T>A; p.Asn318Lys	95:5:0 [A]	93:7:0	rs1800387
Intron 8:			
c.998-27C>T	AF 92:8 [B]	83:17:0	rs55907031
Exon 9:			
c.1062A>G; p.Glu354=	n/a	98:2:0	n/a
c.1077C>T; p.Pro359=	n/a	98:2:0	rs71582884
Intron 9:			
c.1110-73T>A	2:12:86 [A]	83:17:0	rs2239156
Exon 11:			
c.1173A>T; p.Thr391=	AF 88:12 [B]	76:24:0	rs1800375
c.1182A>C; p.Ser394=	83:15:2 [A]	74:26:0	rs1800376
Exon 12:			
c.1411G>A; p.Val471Ile	AF 91:9 [B]	79:19:2	rs1800377
Exon 13:			
c.1451A>G; p.His484Arg	20:44:36 [A]	7:57:36	rs1800378
Intron 13:			
c.1534-82delT (WT=12T)	n/a	2:98:0	rs370498560
Exon 14:			
c.1548T>C; p.Tyr516=	43:46:11 [A]	60:38:2	rs1800379
c.1626G>A, p.Ala542=	AF 91:9 [B]	86:14:0	rs35365059
Intron 15:			
c.1945+24C>T	AF 98:2 [B]	93:7:0	rs61908661
c.1946-17_-15 dupTCT	n/a	86:7:7	rs34584762
c.1946-19_-17 dupCTT	n/a	17:24:59	rs10622288

Intron 17:			
c.2281+138G>A	AF 97:3 [B]	98:2:0	rs71582862
c.2282-133T>C	AF 10:90 [B]	2:19:79	rs216294
c.2282-122G>A	AF 66:34 [B]	64:29:7	rs1558520
c.2282-121T>C	AF 66:34 [B]	69:24:7	rs1558519
c.2282-42C>A	21:52:27 [A]	10:48:42	rs216293
Exon 18:			
c.2385T>C; p.Tyr795=	43:46:11 [A]	64:29:7	rs1063857
Intron 18:			
c.2442+38G>T	AF 99:1 [B]	95:5:0	rs77372340
Intron 19:			
c.2546+25C>T	10:43:47 [A]	5:50:45	rs216325
c.2547-146C>T	66:29:5 [A]	72:26:2	rs12810426
Intron 21:			
c.2821-48C>T	AF 90:10 [B]	86:14:0	rs60831677
Exon 22:			
c.2880G>A; p.Arg960=	62:32:6 [A]	72:26:2	rs1800380
Intron 22:			
c.2967+98A>G	AF 81:19 [B]	81:17:2	rs11063999
c.2967+123A>G	n/a	86:14:0	rs12307335
c.2968-53G>A	AF 83:17 [B]	72:26:2	rs34877178
Intron 23:			
c.3108+85G>T	AF 82:18 [B]	72:26:2	rs56068059
c.3109-90G>C	n/a	72:26:2	rs56121649
Intron 24:			
c.3222+31C>T	AF 81:19 [B]	72:26:2	rs73051263
c.3223-126A>G	85:14:1 [A]	88:12:0	rs2854871
Exon 26:			
c.3390C>T; p.Cys1130=	n/a	98:2:0	n/a
c.3414C>T; p.Asn1138=	n/a	84:14:2	n/a
Intron 26:			
c.3538+135C>T	n/a	93:7:0	rs148530033

Intron 27:			
c.3675-75A>G	23:44:33 [A]	12:50:38	rs216312
Exon 28:			
c.4443G>T; p.Gly1481=	n/a	98:2:0	rs144796763
c.4641T>C; p.Thr1547=	n/a	10:48:42	rs216310
c.4665A>C; p.Ala1555=	87:13:0 [A]	81:17:2	rs1800384
Intron 31:			
c.5455+37C>T	n/a	98:2:0	rs200311828
c.5455+199A>G	AF 92:8 [B]	90:10:0	rs2854866
Intron 33:			
c.5665-118G>A	n/a	14:50:36	rs216305
Intron 34:			
c.5843-8C>G	AF 3:97 [B]	98:2:0	rs34444862
Exon 35:			
c.5844C>T; p.Cys1948=	42:40:18 [A]	36:43:21	rs216902
Intron 35:			
c.6063+25G>A	83:17:0 [A]	79:21:0	rs3741906
c.6064-71C>T	76:24:0 [A]	86:4:10	rs17491334
c.6064-50A>G	AF 98:2 [B]	98:0:2	rs71579337
Intron 36:			
c.6257-26T>C	n/a	98:2:0	n/a
Exon 37:			
c.6532G>T; p.Ala2178Ser	AF 97:3 [B]	98:0:2	rs34230288
Intron 37:			
c.6598+121C>T	n/a	79:0:21	n/a
c.6598+126G>A	AF 94:6 [B]	96:2:2	rs216900
Intron 38:			
c.6798+14C>T	99:1:0 [A]	98:2:0	rs7315124
c.6799-106G>C	AF 7:93 [B]	81:12:7	rs11063974
c.6799-14C>T	AF 17:83 [B]	21:12:67	rs177702
Exon 39:			

c.6846A>G; p.Thr2282=	76:24:0 [A]	74:14:12	rs1053523
Intron 39:			
c.6902-210G>C	AF 93:7 [B]	76:12:12	rs2070883
c.6902-105G>A	AF 93:7 [B]	76:12:12	rs2070884
Intron 40:			
c.6976+59C>T	AF 93:7 [B]	76:12:12	rs2070885
c.6976+85_+86 delGT	n/a	74:14:12	rs144331815
c.6976+111G>A	AF 99:1 [B]	98:0:2	rs61909702
c.6977-22 C>T	AF 89:11 [B]	74:24:2	rs55867239
Intron 41:			
c.7082-103T>G	n/a	98:2:0	rs71581024
c.7082-48G>A	AF 93:7 [B]	76:24:0	rs2286938
c.7082-13G>C	n/a	98:2:0	rs71581025
c.7082-7C>T	AF 70:30 [B]	47:43:10	rs216868
Exon 42:			
c.7239T>C; p.Thr2413=	0:21:79 [A]	5:14:81	rs216867
Intron 42:			
c.7287+60C>T	88:12:0 [A]	76:24:0	rs2286937
c.7288-87T>C	4:37:59 [A]	7:38:55	rs216855
c.7288-21C>T	AF 93:7 [B]	76:24:0	rs3819539
c.7288-19C>T	AF 93:7 [B]	76:24:0	rs3819540
Intron 43:			
c.7437+96dupT	n/a	5:95:0	rs5796198
Intron 44:			
c.7548+170C>T	9:51:40 [A]	14:48:38	rs11063965
c.7548+188C>G	AF 33:67 [B]	12:50:38	rs2238110
c.7549-59A>C	10:46:44 [A]	17:43:40	rs2270239
Exon 45:			
c.7682T>A; p.Phe2561Tyr	AF 96:4 [B]	86:14:0	rs35335161

Intron 45:			
c.7730-122G>T	n/a	93:7:0	n/a
Intron 46:			
c.7770+77T>C	AF 97:3 [B]	88:12:0	rs11063963
c.7771-173T>C	AF 0:100 [B]	0:2:98	rs216851
c.7771-13C>T	AF 88:12 [B]	79:21:0	rs11063962
Intron 47:			
c.7887+12T>C	AF 97:3 [B]	98:2:0	rs55687637
c.7888-223A>T	35:47:18 [A]	10:45:45	rs7958883
c.7888-134T>C	AF 92:8 [B]	93:7:0	rs57040304
c.7888-129T>C	AF 80:20 [B]	48:40:12	rs12297442
c.7888-118C>T	36:43:21 [A]	21:55:24	rs3759321
c.7888-93G>A	18:47:35 [A]	10:48:42	rs3759320
Intron 48:			
c.7987-262A>G	AF 46:54 [B]	10:45:45	rs7133777
c.7987-124C>A	n/a	98:2:0	n/a
Exon 49:			
c.8094A>G; p.Glu2698=	n/a	98:2:0	rs144030544
c.8113G>A; p.Gly2705Arg	84:16:0 [A]	93:7:0	rs7962217
Intron 49:			
c.8115+66T>C	62:31:7 [A]	71:24:5	rs2286646
c.8115+216G>A	AF 93:7 [B]	93:7:0	rs7961998
c.8116-20A>C	62:31:7 [A]	71:24:5	rs2270152
Intron 50:			
c.8155+50C>T	72:26:2 [A]	76:19:5	rs2270151
c.8156-298A>G	n/a	98:2:0	n/a
Intron 51:			
c.8253+32T>C	AF 80:20 [B]	93:7:0	rs2362483
c.8253+39C>T	AF 92:8 [B]	93:7:0	rs2362482
3' UTR:			
c.*204C>T	60:35:5 [A]	84:14:2	rs7976955

[A] Frequencies from HAP MAP CEU
[B] Frequencies from pilot_1_CEU_low_coverage_panel (of 1000 genome project)

Table VI Detected disease causing mutations described in the literature

Exon	Position	rs number	VWD Type
Exon 17	c.2269_2270delCT; p.Leu757Valfs	rs61748465	Type 3 [53,69]
Exon 18	c.2344C>T; p.Arg782Trp	rs61748471	Type 2N [70,71]
Exon 20	c.2561G>A; p.Arg854Gln	rs41276738	Type 2N [72,73]
Exon 21	c.2771G>A; p.Arg924Gln	rs33978901	Type 2N [74,75]
Exon 25	c.3314C>A; p.Ala1105Asp	n/a	Type 2A [76]
Exon 26	c.3437A>G; p.Tyr1146Cys	rs267607326	Type 1 [52]
Exon 28	c.3863T>C; p.Leu1288Pro	n/a	Type 2A [53]
Exon 36	c.6187C>T; p.Pro2063Ser	rs61750615	Type 1 [52,76]
Exon 48	c.7940C>T; p.Thr2647Met	rs61751302	Type 1 [52]