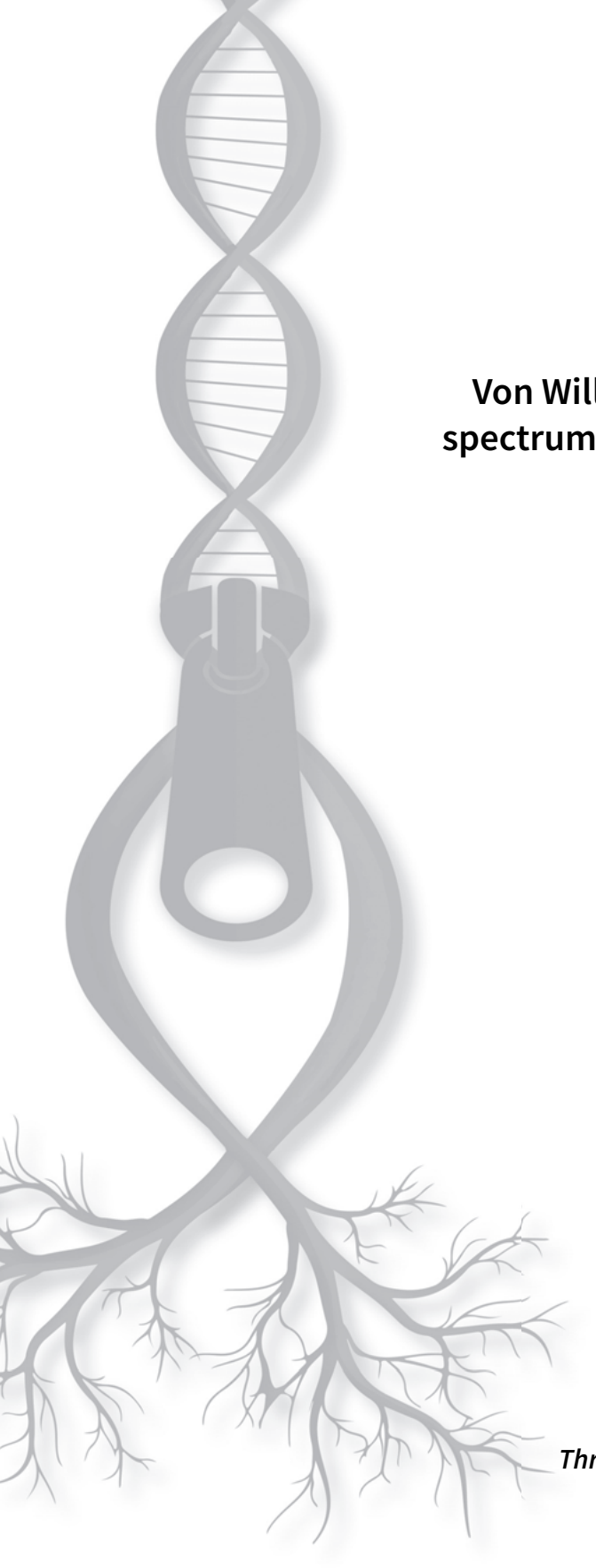
Personalized treatment for von Willebrand disease by RNA-targeted therapies

Jong, A. de



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Von Willebrand disease mutation spectrum and associated mutation mechanisms

Annika de Jong
Jeroen Eikenboom

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Abstract

Von Willebrand disease (VWD) is a bleeding disorder that is mainly caused by mutations in the multimeric protein von Willebrand factor (VWF). These mutations may lead to deficiencies in plasma VWF or dysfunctional VWF. VWF is a heterogeneous protein and over the past three decades, hundreds of VWF mutations have been identified. In this review we have organized all reported mutations, spanning a timeline from the late eighties until early 2017. This resulted in an overview of 750 unique mutations that are divided over the VWD types 1, 2A, 2B, 2M, 2N and 3. For many of these mutations the disease-causing effects have been characterized *in vitro* through expression studies, *ex vivo* by analysis of patient-derived endothelial cells, as well as in animal or (bio)physical models. Here we describe the mechanisms associated with the VWF mutations per VWD type.

Introduction

Von Willebrand factor (VWF) is a multimeric hemostatic protein produced solely by endothelial cells and megakaryocytes.[1,2] *VWF* is transcribed from the small arm of chromosome 12 (12p13.31) and translates into a 2813 amino acid protein. Newly synthesized VWF consists of 16 domains: a 22 amino acid signal peptide at the N-terminal end of the protein, a propeptide comprising the D1 and D2 domains, and mature VWF comprising the D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK domains (Fig. 1A).[3] The signal peptide is cleaved off after synthesis in the endoplasmic reticulum (ER). Then, proVWF undergoes several post-translational modifications, like glycosylation and C-terminal dimerization.[4,5] In the trans-Golgi network (TGN), the propeptide is cleaved by furin, but remains non-covalently linked to mature VWF and helps to chaperone mature VWF in the multimerization process.[6] VWF multimers can contain up to 80 subunits, which are translocated from the TGN to the alpha granules in megakaryocytes and to the cigar-shaped vesicles called Weibel-Palade bodies in endothelium.[7,8] Upon vascular damage, VWF multimers are released from the endothelial cells and through the aid of vascular flow form ultra-large VWF strings attached to the exposed collagen.[9] The unwinding of VWF into large strings exposes the binding site for platelet glycoprotein Ib (GPIb) in the A1 domain, thereby attracting platelets to sites of vascular damage and starting primary hemostasis.[10] Opening up the A2 domain exposes the cleavage site of the metalloprotease ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin motif repeats 13), which cleaves VWF between amino acid positions 1605 and 1606.[9] This leads to the release of smaller and larger VWF multimers into the circulation. In the circulation factor VIII (FVIII) is bound to VWF, which extends the half-life of FVIII.[11] VWF has a half-life of 8-12 hours and remains in the circulation until it is cleared from the system by macrophages in the liver and spleen.[12,13]

Malfunction in one of the processes described above may lead to von Willebrand disease (VWD), the most common inherited bleeding disorder that is mainly associated with mucocutaneous and surgical bleeding.[14] Based on the plasma phenotype, VWD patients are categorized into one of the VWD types: 1, 2A, 2B, 2M, 2N and 3.[15] VWD type 1 and 3 patients are typified by a partial or complete deficiency in plasma VWF respectively. These deficiencies are caused by loss of production, reduced secretion from endothelial cells and platelets, or increased clearance of VWF from the circulation. Four different qualitative defects lead to VWD type 2, and include a defect in plasma multimers in type 2A, increased binding to GPIb in type 2B, decreased binding to GPIb or collagen in type 2M and defective binding to coagulation FVIII in type 2N.

In 1985 four independent groups succeeded to clone and sequence the *VWF* gene.[16-19] From this moment on many groups sequenced *VWF* of VWD patients and this led to the identification of hundreds of variations in *VWF*. To prove that the identified variations are indeed disease-causing, VWF constructs containing many of these variants have been overexpressed in

heterologous cell systems. For VWF, studies have been performed in stable furin producing BHK, COS-7, AtT-20, HEK293, HEK293T and HEK293 EBNA cells, with only HEK293 and AtT-20 cells being able to store VWF in pseudo-Weibel-Palade bodies.[20] Expression studies led to the identification of secretion and multimerization defects and binding defects to FVIII and GPIb. Recent advances also allow for the culture of endothelial cells from VWD patients directly and have been helpful in unraveling mutation mechanisms in a patient-specific environment.[21,22] Although the *in vitro* systems have been useful, the effects of flow on VWF and clearance of the protein are difficult to determine. However, by the use of several plasma parameters and VWD mouse models, clearance defects and the *in vivo* effect of ADAMTS13 on VWF have also been investigated.[23-26]

In this review we endeavored to organize all published VWF mutations and their disease causing mechanism based on an extensive literature search (search term in supplemental information). Throughout this review the mutations and mechanisms will be explained per VWD type. Some mutations have been assigned in literature to different VWD types, which could be explained in various ways: a patient could have been misdiagnosed, the mutation may truly result in different phenotypes or a mutation may have combined phenotypic characteristics of multiple VWD types. We have depicted mutations in this review as they were assigned in literature. Furthermore, it is important to note that if more than one mutation was reported for a single patient and the disease-causing mutation was not certain, the mutations were not included in this review. Also for many candidate mutations the disease causing effect has not been proven yet, however for simplicity we call them mutations throughout this review.

VWD type 1

VWD type 1 is the most common type comprising about 60-70% of the patient population.[27] Type 1 patients show a partial deficiency in VWF with a comparable decrease of VWF antigen (VWF:Ag) and VWF activity (VWF ristocetin cofactor activity, VWF:RCo). A VWF:Ag below a cut-off of 30 IU/dL in combination with a VWF:RCo/VWF:Ag ratio > 0.6 and a normal VWF collagen binding is diagnostic for type 1 VWD, thereby accepting minor abnormalities in VWF multimers.[14,15,28] VWF:Ag levels between 30 and 50 IU/dL may be considered as VWD depending on the bleeding phenotype. Although VWD type 1 is the most frequent type of VWD, extensive investigations to the mutation mechanisms of VWD type 1 started only between 2000 and 2010 with three large studies conducted in the United Kingdom, Europe and Canada.[29-31] In these studies mutations were identified in 53-70% of the patients. Later studies in Sweden and Canada confirmed these numbers.[32,33] Importantly, some type 1 patients included in the European MCMDM-1VWD study showed minor multimer abnormalities and were later reassessed to other VWD types. Those cases with minor multimer abnormalities

showed a higher proportion of mutations, whereas in individuals with completely normal VWF multimers mutations were identified in approximately 50% of the individuals.[34] More recently, large VWD population studies increased the number of identified type 1 mutations, with the most recent studies reporting identification of mutations in about 90% of the patients.[27,35,36] These studies, however, had strict inclusion criteria increasing the chance to identify mutations. Overall, a little more than 250 unique mutations have been assigned to VWD type 1 patients (Table S1A-D; in supplemental data online). However, the disease-causing effect was only proven by *in vitro* investigations for about one quarter of these mutations.

Mutations associated with VWD type 1 are dispersed throughout the whole VWF protein (Fig. 1A-C). Most mutations identified are heterozygous missense mutations, however also (small) deletions/insertions, splice site and nonsense mutations have been reported. The mutation mechanisms can roughly be divided in three groups: decreased VWF production, decreased secretion and increased clearance.

Decreased VWF production

A lower VWF production has mostly been reported for patients heterozygous for a null allele. These heterozygous null alleles can be the result of nonsense mutations, frameshift mutations caused by (small) deletions or insertions and by splice site mutations. Heterozygosity for a null allele leads to production of protein from the non-mutated allele only, resulting in an expected production of only 50% of the normal VWF production. Since normal levels of VWF in plasma range between ~50 and 200 IU/dL, heterozygosity for a null allele will lead to VWF levels ranging between ~25 and 100 IU/dL.[37] Therefore, some people at the lower end of this distribution will be diagnosed as VWD type 1 and some people will be considered unaffected and are probably asymptomatic.

Small deletions and insertions often lead to a frameshift, which generates a premature stop codon usually within a few amino acids (indicated as for example p.Pro812Arg fs*31). Premature stop codons caused by frameshift or nonsense mutations lead to an mRNA product which is mostly degraded by nonsense-mediated decay. Splice site mutations are also found in VWD type 1 patients and may lead to exon skipping or intron retention. Depending on the reading frame of an exon, this could lead either to the production of a truncated protein or to a premature stop codon. Whether exon skipping forms a truncated or nonsense allele is partly predicted by the reading frame (Fig. 1B), however this can only be proven by investigating RNA products in platelets or patient-derived endothelial cells.[38-41] For example, in the case of c.1534-3C>A and for c.5842+1G>C, this results in three different mRNA products.[39,41] Although not frequently reported, some mutations have been identified in the promoter region of *VWF* as well.[29-31,42] These may lead to altered binding of transcription factors to the *VWF* promoter region.[42]

Decreased secretion

Some patients have normal VWF production, but a decreased secretion of VWF from the endothelial cells. Existence of secretion defects from platelets is likely but has so far not been studied. Secretion defects encompass retention of VWF in the ER or Golgi, decreased Weibel-Palade body exocytosis and possibly degradation of mutant VWF by the proteasome. Secretion defects have been studied on a cellular level by *in vitro* overexpression of mutant VWF constructs in heterologous cell systems, or by studies on patient-derived endothelial cells. Decreased secretion from mutant VWF producing cells compared to normal VWF producing cells has been identified for many of the VWD type 1 mutations tested *in vitro* and decreased secretion is often seen in combination with an *in vitro* multimerization defect (Fig. 1A, Table S1A-D; in supplemental data online). Whether decreased secretion of VWF is caused by retention of VWF in the ER or Golgi can easily be studied by co-staining of VWF with an ER or Golgi marker and this defect has been identified for among others: p.Arg782Gln, p.Cys1130Phe, p.Cys1149Arg, p.Ser1285Pro, p.Val1822Gly and p.Cys2693Tyr.[22,43-47] For many mutations decreased VWF:Ag levels were found in conditioned medium, while normal VWF:Ag levels were found in the cell lysates. A suggested mechanism for the loss of VWF within the cell is the degradation of mutant VWF by the proteasome, which has been studied by Bodó *et al*. for VWF p.Cys1149Arg.[48,49] Besides *in vitro* investigations, the response of a patient to DDAVP could also be indicative for a secretion defect. However, DDAVP unresponsiveness can also relate to production defects, and therefore only *in vitro* studies can be conclusive in the identification of secretion defects.

Increased clearance

VWF has a half-life in the circulation of about 8-12 hours. Increased clearance rates result in lower steady state plasma VWF:Ag levels and are associated with VWD type 1 and type 2B. Clearance defects of VWF are identified by an increased VWF propeptide to VWF:Ag ratio (VWFpp/VWF:Ag) and by shortened survival of plasma VWF:Ag levels after a DDAVP infusion.[26,50,51] Also, mouse experiments in which mutant VWF constructs are intravenously administered, or expressed in the hepatocytes after a hydrodynamic tail vein injection, proved clearance defects for a few mutations.[26,52-54] For VWD type 1, some mutations have been described that cause a clearance defect. Most mutations described are located in the D3 domain and include mutations at positions p.Cys1130, p.Trp1144, p.Cys1149 and p.Arg1205. The most common described variant is the VWD Vicenza mutation, p.Arg1205His, for which a macrophage-dependent increased clearance has been described.[54,55] Besides mutations in the D3 domain, also mutations in the A1, A3 and D4 domains have been associated with increased clearance in VWD type 1.[30,34-36,51,56,57]

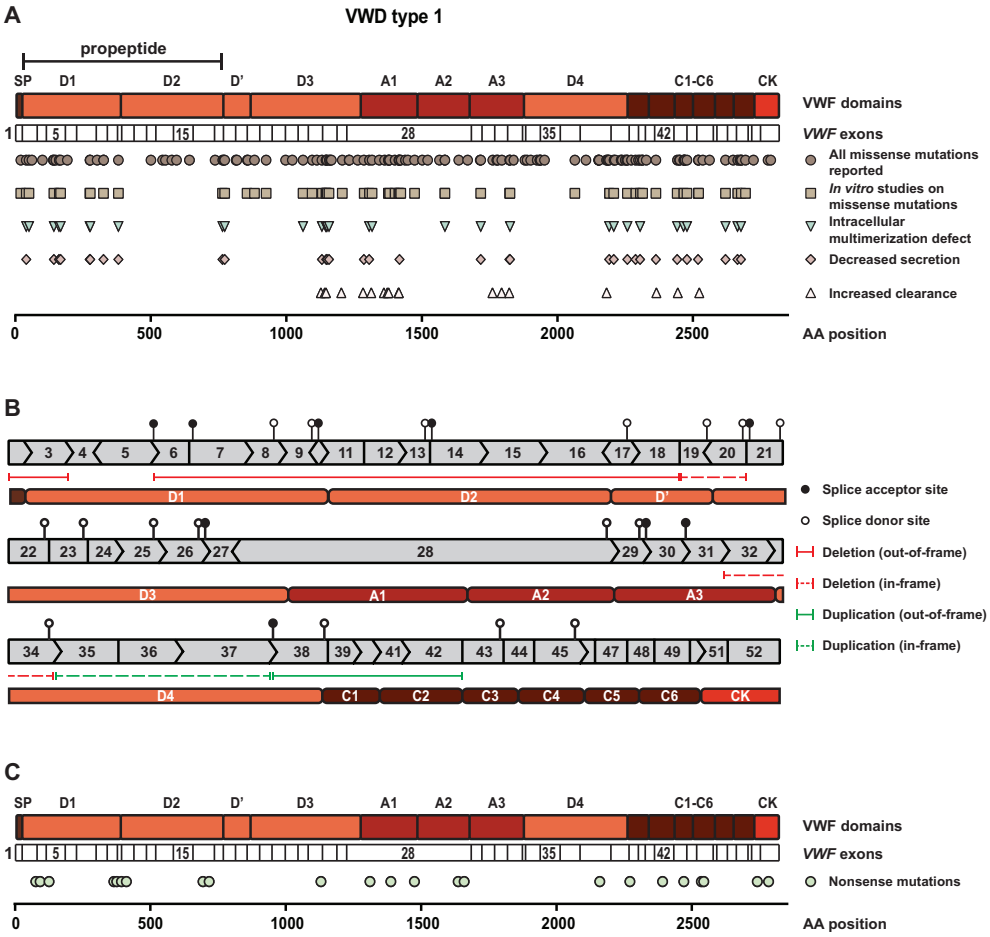

Figure 1. Overview of VWD type 1 mutations. (A) Representation of the VWD type 1 missense mutations dispersed throughout the VWF protein. The distribution of all VWD type 1 missense mutations reported so far is indicated below the VWF domain structure and the *VWF* exon numbering. In the second line all missense mutations are displayed for which the disease-causing mechanisms have been studied in *in vitro* studies in heterologous cell systems, or patient-derived endothelial cells. In the middle line all missense mutations are shown in which intracellular multimerization defects have been observed. In the fourth line all missense mutations are displayed showing decreased secretion in *in vitro* studies. And in the lower line all missense mutations are indicated that have been associated with increased clearance, which was based on increased VWFpp/VWF:Ag ratio, low survival after DDAVP infusion, or *in vivo* studies. Some mutations have multiple defects and are indicated multiple times in the figure. (B) Representation of the open reading frame of *VWF* including all VWD type 1 deletions and duplications and mutations described in splice acceptor (solid circles) and splice donor sites (open circles). All exons are depicted to scale. When the exon ends with >, the end of the exon contains one nucleotide of a new codon. When the exon ends with <, the end of the exon contains two nucleotides of a new codon. When the exon ends with |, the end of the exon contains a whole codon. Exon skipping/duplication caused by splice site mutations or deletions/duplications usually results in a truncated protein when the reading frame is maintained (>>, <<, II). Exon skipping usually results in a null allele when the reading frame gets disrupted (<>, ><, >|, etc.). Below the reading frame are the VWF domains depicted matching the color code of panel A. (C) Representation of all VWD type 1 nonsense mutations throughout the VWF protein.

VWD type 2A

VWD type 2A is associated with defective plasma multimers as a result of an intracellular multimerization defect or an increased susceptibility of VWF for cleavage by ADAMTS13. Diagnosis is based on a decreased VWF activity (VWF:RCo/VWF:Ag < 0.6), absent ristocetin induced platelet aggregation (RIPA) using low-dose ristocetin and lack of high molecular weight (HMW) multimers visualized by the VWF multimer analysis.[28] In total, a little more than 170 different type 2A mutations have been described in literature and for about half of these mutations, the disease-causing mechanism has been proven by *in vitro* studies (Fig. 2A, Table S2A-C; in supplemental data online). Type 2A mutations can be found in many VWF domains, although there is a cluster in the region D3-A2. Several different mutation mechanisms can be distinguished based on the location within the protein (Fig. 2A).

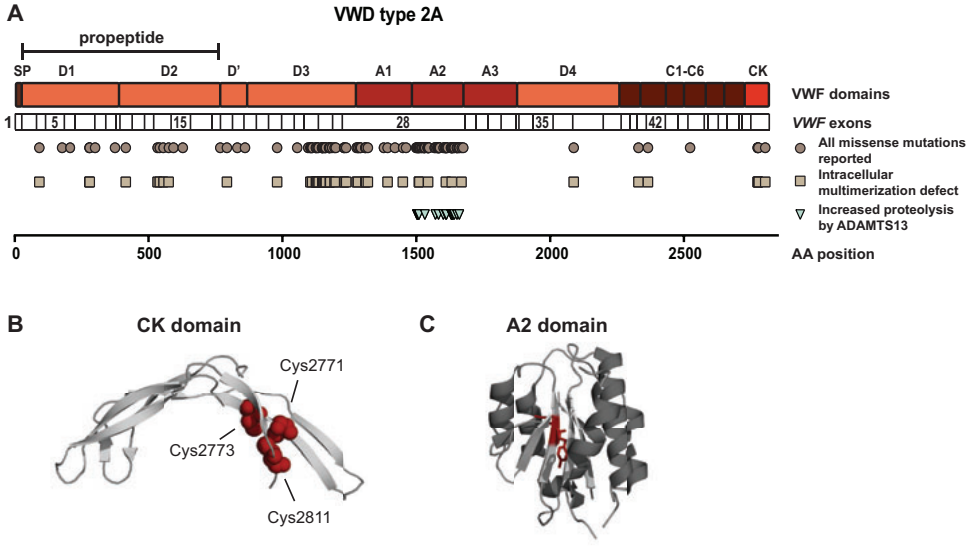

Figure 2. Overview of VWD type 2A mutations. (A) Representation of VWD type 2A mutations. The distribution of all VWD type 2A missense mutations reported so far is indicated in the upper line below the VWF domain structure and the *VWF* exon numbering. In the second line all missense mutations are displayed that have been associated with an intracellular multimerization defect proven by *in vitro* studies. In the lower line all missense mutations are displayed that result in increased proteolysis by ADAMTS13. (B) Crystal structure of the CK domain of VWF that is involved in the C-terminal dimerization process of VWF (PDB: 4NT5). The three amino acids, p.Cys2771, p.Cys2773 and p.Cys2811, involved in intermolecular disulfide bonding are indicated in red. Mutations p.Cys2771Arg/Ser/Tyr and p.Cys2773Ser/Arg are associated with VWD subtype 2A (IID). (C) Crystal structure of the VWD A2 domain (PDB: 3ZQK), which contains the cleavage site for ADAMTS13. ADAMTS13 cleaves VWF between Tyr 1605 and Met1606. Both amino acids are buried in the A2 domain and are indicated as ribbon structure in red.

Intracellular multimerization defects

VWF dimerization and multimerization takes place in the ER and TGN of endothelial cells and megakaryocytes. First, VWF dimers are formed by C-terminal dimerization

of the cysteine knot (CK) (Fig. 2B).[58] This process involves the formation of three intermolecular disulfide bonds Cys2771-2773', Cys2771'-2773 and Cys2811-2811' which was recently shown to be catalyzed by protein disulfide isomerase (PDI) (Fig. 2B).[58] Mutations described at positions Cys2771 and Cys2773 are associated with an intracellular dimerization defect, showing loss of HMW VWF and the presence of uneven bands.[59-61] These mutations have historically been subcategorized as subtype 2A (IID). Also a few other mutations in the CK domain have been assigned to subtype 2A (IID), including homozygous p.Ala2801Asp and heterozygous p.Ser2775Cys.[62,63] Interestingly, homozygous cysteine mutations in the CK domain causing disruption of intramolecular disulfide bonds are associated with VWD type 3.[64]

After dimerization of VWF, two VWF monomers will align to form a so-called dimeric bouquet.[65] The low pH and high Ca^{2+} concentration present in the TGN facilitate dimerization of two D1-D2 domains and allow intermolecular disulfide bond formation between two cysteine residues (p.Cys1099-p.Cys1099′ and p.Cys1142-p.Cys1142′) of the D3 domain.[66,67] Defects in N-terminal dimerization can be caused by two different mechanisms. First, homozygous mutations in the propeptide (D1 and D2 domains) of VWF, or patients being compound heterozygous for a propeptide mutation and a null allele, are associated with severely affected multimerization and intracellular retention and are described as both subtype 2A (IIC) and type 3 VWD.[63,68-72] Co-expression of normal and mutant VWF constructs in heterologous cell systems show a normal multimers profile and heterozygous carriers of propeptide mutations are unaffected or diagnosed with VWD type 1.[72] Secondly, several type 2A mutations have been described in the D3 domain as well, and are mostly inherited in a heterozygous fashion. These mutations are assigned as subtype 2A (IIE) and generally show some loss of HMW VWF and a decrease of proteolytic bands in the multimeric pattern in plasma. The exact mechanism behind the reduced proteolytic bands has not been elucidated yet, but could potentially be the result of increased clearance seen for these mutants, or lower sensitivity for the proteolytic effects of ADAMTS13. Mutations in the D3 domain cause a loss of HMW VWF in *in vitro* studies too, indicating an intracellular multimerization defect as well. Most mutations involve cysteine residues affecting intramolecular disulfide bonds.[67]

Several VWD type 2A mutations have been described in the A1 domain of VWF with no clear common mechanism. Many of these type 2A mutations have been assigned to VWD type 2B or 2M patients as well, and might have a combined mechanism of defective multimerization and defective GPIb binding. Overexpression of most of the A1 mutations in heterologous cell systems result in an intracellular multimerization defect, but also increased clearance rates are detected for some mutations (Table S2A-C; in supplemental data online).

Increased proteolysis by ADAMTS13

After secretion of VWF from the endothelial cells, VWF unwinds into ultra-large VWF strings making the A2 domain accessible for proteolysis by ADAMTS13.[73] This results in the release of all sizes of VWF multimers in the circulation. Normal proteolysis by ADAMTS13 in plasma can be visualized on a multimer blot by the presence of small satellite bands above and below the thick multimeric band. ADAMTS13 cleaves VWF between p.Tyr1605 and p.Met1606, two amino acids buried deep inside the A2 domain (Fig. 2C). Several mutations in the A2 domain increase the sensitivity to proteolysis by ADAMTS13, which results in a decrease in HMW VWF and the presence of broad proteolytic (satellite) bands in plasma. Intracellular multimerization is not affected for these mutations and therefore a normal multimer pattern will be generated *in vitro*. The disease-causing mechanism can therefore only be identified by multimer analysis on plasma samples or by the addition of ADAMTS13 to *in vitro* expression systems.[74-77]

VWD type 2B

The VWF A1 domain binds platelets through GPIb; this only occurs when the A1 domain is in its elongated form as induced by high shear of the microvasculature.[78] Without the presence of flow or activation of VWF by ristocetin, the VWF A1 domain is not able to interact with GPIb. However, gain-of-function mutations in the A1 domain can make VWF more active and therefore VWF can bind GPIb without activation of VWF by shear *in vivo* or by ristocetin *in vitro*. These mutations are associated with VWD type 2B. Type 2B diagnosis is based on a low VWF activity *versus* VWF:Ag level (VWF:RCo/VWF:Ag < 0.6) and increased RIPA in the presence of low concentrations ristocetin.[28] These diagnostic tools will not distinguish VWD type 2B from platelet-type VWD. As platelet-type VWD does not reflect a VWF mutation platelet-type VWD is not further considered in the review. Many type 2B patients also show increased clearance of the VWF-platelet complex and this may result in variable degrees of secondary thrombocytopenia and a loss of HMW VWF.[79,80] It was recently identified that the increased clearance is the result of a higher affinity of macrophages' LRP1 to the A1 domain for certain type 2B mutations.[12] Furthermore, blood smears of type 2B patients often show giant platelets and platelet aggregates.[81]

In total, 43 type 2B mutations have been reported and for about half of them the disease-causing mechanism was proven by *in vitro*, *in vivo* or *in silico* studies (Fig. 3A, Table S3; in supplemental data online). Although *in vitro* studies help to determine the severity of the mutation, they are not necessary for identification of the mechanism of type 2B mutations. Most mutations are located in the A1 domain, with the exception of a few mutations that have been reported at the C-terminal end of the D3 domain (Fig. 3A). Crystal structures of the VWF A1 domain and platelet GPIb have revealed two interactions between VWF A1 and GPIb (Fig.

3B).[82-84] The primary interaction is located between the β-sheet of the β-switch region of GPIb and the β-sheet of VWF, generating an extended β-sheet between the two molecules (Fig. 3B).[82-84] The second interaction is located between the N-cap of GPIb and the A1 domain of VWF, and in proximity of this interaction in the A1 domain, most VWD type 2B mutations are identified (Fig. 3C, light blue). The A1 domain at this site is stabilized by several hydrogen bonds and a disulfide bond between p.Cys1272 and p.Cys1458.[82-84] Mutations in this region can destabilize the domain, thereby inducing increased affinity to GPIb.

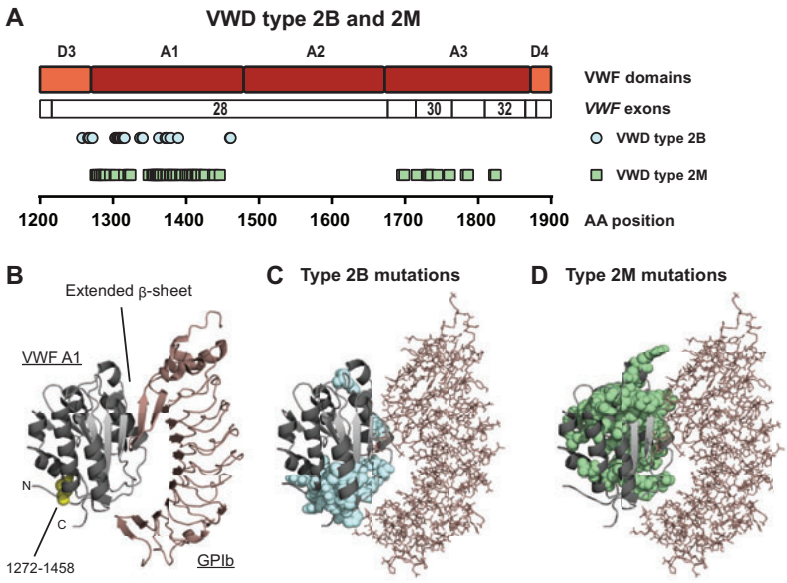

Figure 3. Overview of VWD type 2B and type 2M mutations. (A) Representation of VWD type 2B and type 2M mutations. The distribution of all VWD type 2B mutations reported so far is indicated in the upper line below the VWF domain structure and the *VWF* exon numbering. All these mutations are located in the A1 domain or at the C-terminal end of the D3 domain. In the lower line all positions of mutations are displayed that have been reported for VWD type 2M. Mutations that are associated with decreased binding to GPIb are located in the A1 domain. Mutations with an isolated collagen binding defect are located in the A3 domain. (B) Crystal structure of the VWF A1 domain (grey) together with GPIb (pink) (PDB: 1SQ0). The A1 domain interacts with GPIb through the extended β-sheet and by the N-cap of GPIb located in the bottom of this figure. The intermolecular disulfide bond in the A1 domain is indicated in yellow and is important in the structure of the domain. (C) Crystal structure of the A1 domain and GPIb in which the location of the VWD type 2B mutations are indicated in light blue. Most mutations are located in close proximity of the intermolecular disulfide bond and the interaction with the N-cap of GPIb. (D) Crystal structure of the A1 domain and GPIb in which the location of the VWD type 2M mutations are indicated in green. Mutations are spread throughout the whole domain.

Although all type 2B mutations lead to an increased binding to GPIb, clear differences have been observed between the phenotypes of certain mutations. These differences include platelet count and the ability of mutant VWF to bind platelets or immobilized GPIb in the absence or the presence of low concentrations ristocetin. A very severe phenotype can be

found in patients with for example p.Val1316Met with thrombocytopenia in resting state and a very strong binding to GPIb in the absence of ristocetin. In most patients with this mutation, also giant platelets and platelet aggregates are observed on blood smears.[85] Intermediate phenotypes are associated with thrombocytopenia only during stress situations, like surgery or pregnancy. In milder affected patients, no thrombocytopenia is observed and a low concentration of ristocetin is needed to agglutinate VWF.

VWD type 2M

VWD type 2M is usually associated with low VWF activity (VWF:RCo/VWF:Ag < 0.6), a more or less normal multimer pattern and no RIPA in the presence of low concentrations ristocetin.[28] However, patients with an isolated collagen binding defect, but normal VWF:RCo, are also assigned to VWD type 2M. In total, somewhat over 80 mutations have been assigned to VWD type 2M, of which most are located in the A1 domain (Fig. 3A, Table S4A-B; in supplemental data online).

GPIb binding defect

Most mutations associated with a decreased binding to GPIb are heterozygous mutations located in the A1 domain of VWF (Fig. 3A). These mutations can either affect the direct binding of VWF to GPIb or enhance the stability of the A1 domain, thereby reducing the rate of unfolding of the A1 domain under flow.[86,87] A lower rate of unfolding has for example been observed in VWF p.Gly1324Ala and p.Gly1324Ser.[87] A direct binding effect is usually associated with a destabilized protein, also described as molten globule, and has been seen in for example p.Ser1285Phe.[88] Whether a type 2M mutation is associated with decreased or increased stability of the A1 domain can be identified by the binding of monoclonal antibodies to specific A1 domains, or biophysical studies on for example the crystal structure of mutants or the thermodynamic stability of the protein.[86,87] Type 2M mutations that affect platelet binding are identified throughout the whole VWF A1 domain (Fig. 3A and D).

Collagen binding defect

VWF binds collagen types I and III via its A1 and A3 domains and collagen types IV and VI via its A1 domain.[89-91] Defects in collagen binding might lead to VWD or an increased risk of bleeding. Collagen binding defects could be identified by the VWF-collagen binding assay, however this assay is usually not included in diagnostic procedures.[28] And if the assay is performed, it usually only includes types I and/or III collagen.[92] Therefore, many mutations associated with collagen binding defects might remain unidentified.

Most collagen binding defects identified are restricted to binding of collagen I and III, since commercial assays are only developed for these two types of collagen.[92] Identified mutations associated with defective collagen binding are mainly heterozygous mutations located in the A3 domain of VWF (Fig. 3A).[93-98]

Collagen IV and VI binding assays are not included in regular diagnostic procedures and mutations affecting binding to these collagens have only occasionally been reported.[90,91] A combined collagen IV and VI binding defect has been reported for the A1 mutations p.Arg1399His, p.Gln1402Pro and p.Arg1392_Gln1402 del. Furthermore, a collagen IV binding defect was reported for p.Arg1315Cys (also reported as type 1 and 2A mutation) and p.Ser1358Asn, and a collagen VI binding defect for p.Ser1387Ile. It is however important to state that Arg1399His has also been identified among healthy controls, and therefore it cannot be seen as a definite VWD causing mutation, however it may be associated with an increased risk for bleeding.[90]

VWD type 2N

VWD type 2N is characterized by a loss of binding to coagulation FVIII resulting in decreased FVIII plasma levels and activity. Diagnosis is based on a normal VWF:RCo/VWF:Ag ratio (> 0.6), but a decrease in FVIII activity *versus* VWF:Ag level (FVIII:C/VWF:Ag < 0.6) and decreased VWF-FVIII binding (VWF:FVIIIB).[28] Since the stoichiometry of FVIII and VWF is 1:50 (one FVIII molecule for every 50 VWF monomers) there is an excess of FVIII binding sites in a VWF multimer and thus the type 2N phenotype will only be expressed in case of a homozygous FVIII binding defect or in case of compound heterozygosity for a FVIII binding defect and a *VWF* null allele.[99] In total, 31 different mutations in the VWF D2, D' and D3 domains have been described that affect FVIII binding (Table S5; in supplemental data online).

The major FVIII binding site in VWF is located in the D' domain of VWF.[100] The D' domain of VWF contains 2 modules, the trypsin-inhibitor-like (TIL') and E' modules.[3,100] Most type 2N mutations are located in, or in close vicinity of the TIL' module, which is a very flexible region within VWF (Fig. 4A-C).[65,100] Since the D' domain contains several disulfide bonds important in N-terminal multimerization of VWF, several type 2N mutations are associated with defective multimerization as well.[101-103] One of the most common type 2N mutation is the p.Arg854Gln mutation that, because of the high frequency, has a high prevalence of co-inheritance with other types of VWD.[104]

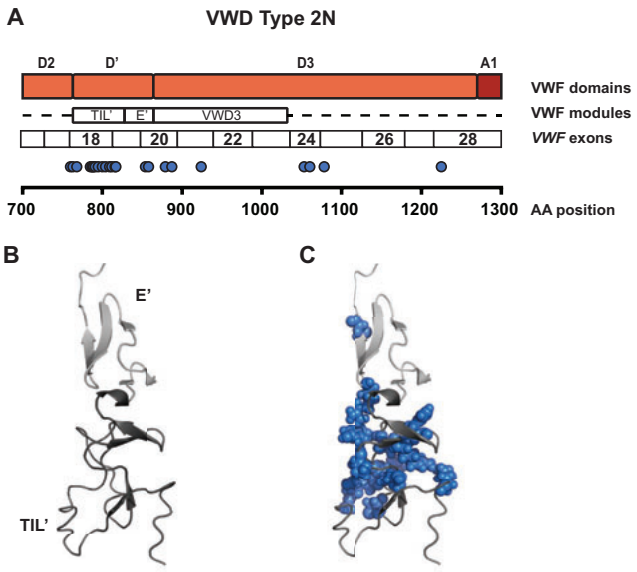

Figure 4. Overview of VWD type 2N mutations. (A) Representation of VWD type 2N mutations. The distribution of all VWD type 2N mutations reported so far is indicated below the VWF domain structure and the *VWF* exon numbering. Mutations are located in the D2, D′ and D3 domain of VWF. (B) Solution structure of the D′ domain of VWF, the major FVIII binding region (PDB: 2MHP). The D′ domain consists of two modules, the E′ and TIL′ modules. The TIL′ module is very flexible and is indicated in dark grey. The E′ module (light grey) contains a β-sheet and is less flexible. (C) Solution structure of the D′ domain of VWF with the VWD type 2N mutations indicated in blue. Most mutations are located in the flexible TIL′ module.

VWD type 3

VWD type 3 patients have undetectable VWF:Ag levels in their plasma and show a severe bleeding phenotype.[28] Most patients do not produce VWF at all, as is the consequence of homozygous or compound heterozygous null alleles. Some patients, however, are homozygous or compound heterozygous for missense mutations. These patients normally produce VWF, but the mutant VWF is not secreted from the endothelial cells, or undergoes a very rapid clearance. In total, more than 280 unique mutations have been identified with VWD type 3 (Table S6A-E; in supplemental data online).

Null alleles

Most VWD type 3 patients develop the phenotype by the presence of two null alleles caused by homozygous or compound heterozygous nonsense, frameshift or splice site mutations. Since no or very low VWF:Ag levels are detected in VWD type 3 patients, there is a high probability that the splice site mutations identified in type 3 patients also result in a frameshift and nonsense-mediated decay. Mutations associated with null alleles are located throughout the whole *VWF* gene (Fig. 5A and B). Although not frequently reported, some patients were identified having a whole *VWF* gene deletion.[35,105-107]

Null alleles could also result from a gene conversion with the pseudogene of *VWF*. This pseudogene is located on chromosome 22 and has a high sequence similarity with exons 23 until 34.[108] Depending on the length of the conversion, the conversion may be associated with mutations: p.Val1229Gly, p.Asn1231Thr, p.Ser1263Pro, p.Pro1266Leu, p.Val1279Ile, p.Leu1288Val, p.Gln1311*, p.Ile1343Val, p.Val1360Ala, and p.Phe1369Ile.[109-111] Homozygous or compound heterozygous gene conversions including p.Gln1311* will lead to VWD type 3.[111] Heterozygous gene conversions may lead to VWD type 1, depending on the variation in VWF:Ag levels as described under VWD type 1.

Homozygous/compound heterozygous missense mutations

Although most VWD type 3 is associated with null alleles, some patients have very low VWF:Ag plasma levels because of missense mutations, which are mostly inherited in a homozygous or compound heterozygous fashion (Fig. 5A; Table S6A; in supplemental data online). The effect of several missense mutations was studied in heterologous cell systems, and for all mutations tested a severe secretion defect was found confirming the plasma phenotype of very low or undetectable VWF:Ag levels (Fig. 5A).[64] Most of these mutations replace a cysteine and/or are located at the C- or N-terminal ends of VWF and are important in the multimerization process.[64,112-116]

Clearance defects cannot be detected in *in vitro* systems, however several mutations known to be associated with rapid clearance were identified in type 3 patients.[117] These patients showed increased VWFpp/VWF:Ag ratios and had a lower bleeding score compared to type 3 caused by null alleles.[117]

Conclusions

Advances in sequencing technology along with lower associated costs have resulted in an ever increasing number of patients sequenced for the *VWF* gene. This has resulted in the identification of more than 750 unique mutations in *VWF*. The disease causing mechanism behind these mutations has been proven either through *in vitro* or *in vivo* studies for approximately 220 mutations, and for several others, the mechanism became evident based on the type or location of the mutation and the patients' phenotype. However, many of the identified mutations remain candidate mutations, as the disease causing effects are yet to be elucidated.

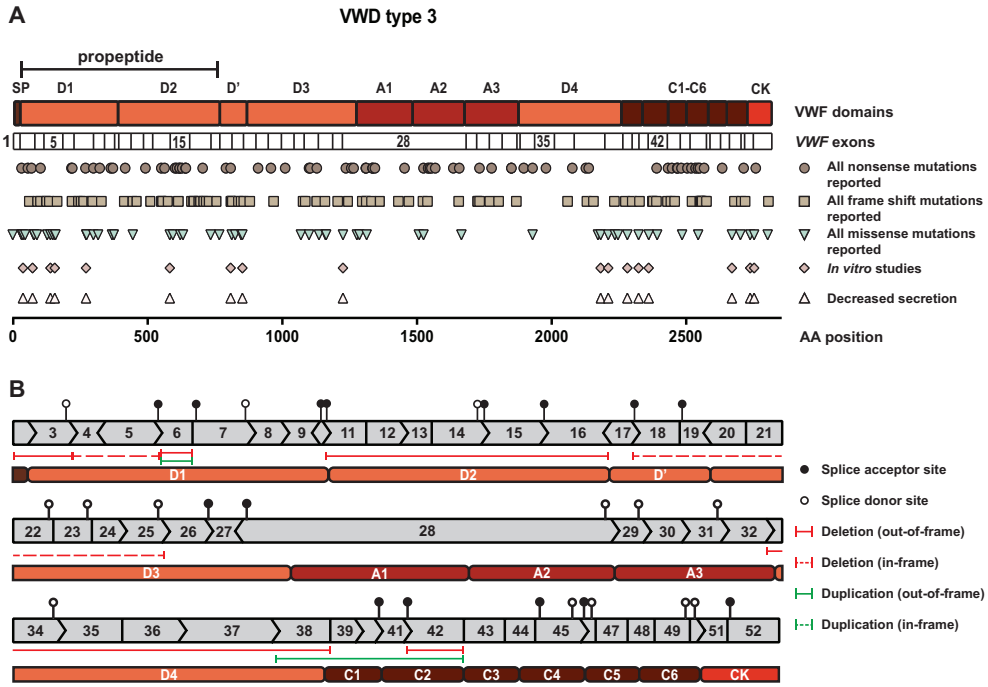

Figure 5. Overview of VWD type 3 mutations. (A) Representation of VWD type 3 mutations. The distribution of all VWD type 3 nonsense mutations reported so far is indicated below the VWF domain structure and the *VWF* exon numbering. In the second line all frameshift mutations and in the third line all missense mutations are indicated. All mutations that have been studied by *in vitro* experiments are displayed in the fourth line, and all those mutations associated with a clear secretion defect are in the lower line. (B) Representation of the open reading frame of the *VWF* exons including most of the VWD type 3 deletions and duplications described and mutations described in splice acceptor (solid circles) and splice donor sites (open circles). All exons are depicted to scale. For explanation of the open reading frame see the legend of Fig. 1.

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Supplemental information: Advanced literature search strategy

Search strategy was performed until 31 January 2017

Number of hits on PubMed: 1743

(((((“von Willebrand Diseases”[majr] OR “von Willebrand Disease”[ti] OR “von Willebrand Diseases”[ti] OR “vonwillebrand disease”[ti] OR “Willebrand disease”[ti] OR “Willebrand diseases”[ti] OR “willebrand’s disease”[ti] OR “willebrand’s diseases”[ti] OR “willebrands disease”[ti] OR “willebrands diseases”[ti] OR “VWD”[ti] OR “Von Willebrand’s Factor Deficiency”[ti] OR “Von Willebrand Factor Deficiency”[ti] OR “Von Willebrand Disorder”[ti] OR “von Willebrand Factor”[majr] OR “von Willebrand Factor”[ti] OR von Willebrand*[ti] OR vonwillebrand*[ti] OR willebrand*[ti]) AND (“Mutation”[mesh] OR mutat*[tw] OR “Allelic Imbalance”[tw] OR “Loss of Heterozygosity”[tw] OR “Chromosome Deletion”[tw] OR “Haploinsufficiency”[tw] OR “Base Pair Mismatch”[tw] OR “Chromosome Aberrations”[tw] OR “Abnormal Karyotype”[tw] OR “XYY Karyotype”[tw] OR “Aneuploidy”[tw] OR “Chimerism”[tw] OR “Chromosomal Instability”[tw] OR “Chromosome Fragility”[tw] OR “Chromosome Breakage”[tw] OR “Chromosome Duplication”[tw] OR “Tetrasomy”[tw] OR “Trisomy”[tw] OR “Chromosome Inversion”[tw] OR “Isochromosomes”[tw] OR “Chromosome-Defective Micronuclei”[tw] OR “Mosaicism”[tw] OR “Polyploidy”[tw] OR “Tetraploidy”[tw] OR “Triploidy”[tw] OR “Ring Chromosomes”[tw] OR “Sex Chromosome Aberrations”[tw] OR “Genetic Translocation”[tw] OR “Philadelphia Chromosome”[tw] OR “Uniparental Disomy”[tw] OR “Nonsense Codon”[tw] OR “DNA Repeat Expansion”[tw] OR “Trinucleotide Repeat Expansion”[tw] OR “Frameshift Mutation”[tw] OR “Gene Amplification”[tw] OR “Gene Duplication”[tw] OR “Genomic Instability”[tw] OR “Microsatellite Instability”[tw] OR “Germ-Line Mutation”[tw] OR “INDEL Mutation”[tw] OR “Insertional Mutagenesis”[tw] OR “Mutation Accumulation”[tw] OR “Mutation Rate”[tw] OR “Missense Mutation”[tw] OR “Point Mutation”[tw] OR “Sequence Deletion”[tw] OR “Chromosome Deletion”[tw] OR “Gene Deletion”[tw] OR “Sequence Inversion”[tw] OR “Chromosome Inversion”[tw] OR “Silent Mutation”[tw] OR “Genetic Suppression”[tw] OR “Polymorphism, Restriction Fragment Length”[mesh] OR “Polymorphism, Genetic”[mesh] OR Polymorphism*[tw])) OR “von Willebrand Diseases/genetics”[majr] OR “von Willebrand Factor/genetics”[majr]) AND (english[la] OR dutch[la])) OR ((((“von Willebrand Diseases”[mesh] OR “von Willebrand Disease”[tw] OR “von Willebrand Diseases”[tw] OR “vonwillebrand disease”[tw] OR “Willebrand disease”[tw] OR “Willebrand diseases”[tw] OR “willebrand’s disease”[tw] OR “willebrand’s diseases”[tw] OR “willebrands disease”[tw] OR “willebrands diseases”[tw] OR “VWD”[tw] OR “Von Willebrand’s Factor Deficiency”[tw] OR “Von Willebrand Factor Deficiency”[tw] OR “Von Willebrand Disorder”[tw] OR “von Willebrand Factor”[mesh] OR “von Willebrand Factor”[tw] OR von Willebrand*[tw] OR vonwillebrand*[tw] OR willebrand*[tw]) AND (“Mutation”[majr] OR mutat*[ti] OR “Allelic Imbalance”[ti] OR “Loss of Heterozygosity”[ti] OR “Chromosome Deletion”[ti] OR “Haploinsufficiency”[ti] OR “Base Pair Mismatch”[ti] OR “Chromosome Aberrations”[ti] OR “Abnormal Karyotype”[ti] OR “XYY Karyotype”[ti] OR “Aneuploidy”[ti] OR “Chimerism”[ti] OR “Chromosomal Instability”[ti] OR “Chromosome Fragility”[ti] OR “Chromosome Breakage”[ti] OR “Chromosome Duplication”[ti] OR “Tetrasomy”[ti] OR “Trisomy”[ti] OR “Chromosome Inversion”[ti] OR “Isochromosomes”[ti] OR “Chromosome-Defective Micronuclei”[ti] OR “Mosaicism”[ti] OR “Polyploidy”[ti] OR “Tetraploidy”[ti] OR “Triploidy”[ti] OR “Ring Chromosomes”[ti] OR “Sex Chromosome Aberrations”[ti] OR “Genetic Translocation”[ti] OR “Philadelphia Chromosome”[ti] OR “Uniparental Disomy”[ti] OR “Nonsense Codon”[ti] OR “DNA Repeat Expansion”[ti] OR “Trinucleotide Repeat Expansion”[ti] OR “Frameshift Mutation”[ti] OR “Gene Amplification”[ti] OR “Gene Duplication”[ti] OR “Genomic Instability”[ti] OR “Microsatellite Instability”[ti] OR “Germ-Line Mutation”[ti] OR “INDEL Mutation”[ti] OR “Insertional Mutagenesis”[ti] OR “Mutation Accumulation”[ti] OR “Mutation Rate”[ti] OR “Missense Mutation”[ti] OR “Point Mutation”[ti] OR “Sequence Deletion”[ti] OR “Chromosome Deletion”[ti] OR “Gene Deletion”[ti] OR “Sequence Inversion”[ti] OR “Chromosome Inversion”[ti] OR “Silent Mutation”[ti] OR “Genetic Suppression”[ti] OR “Polymorphism, Restriction Fragment Length”[majr] OR “Polymorphism, Genetic”[majr] OR Polymorphism*[ti])) OR “von Willebrand Diseases/genetics”[majr] OR “von Willebrand Factor/genetics”[majr]) AND (english[la] OR dutch[la])))