

REVIEW

The genetic basis of von Willebrand disease

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ABSTRACT

The common autosomally inherited mucocutaneous bleeding disorder, von Willebrand disease (VWD) results from quantitative or qualitative defects in plasma von Willebrand factor (VWF). Mutation can affect VWF quantity or its functions mediating platelet adhesion and aggregation at sites of vascular damage and carrying pro-coagulant factor VIII (FVIII). Phenotype and genotype analysis in patients with the three VWD types has aided understanding of VWF structure and function. Investigation of patients with specific disease types has identified mutations in up to 70% of type 1 and 100% of type 3 VWD cases. Missense mutations predominate in type 1 VWD and act through mechanisms including rapid clearance and intracellular retention. Many mutations are incompletely penetrant and attributing pathogenicity is challenging. Other factors including blood group O contribute to low VWF level. Missense mutations affecting platelet- or FVIII-binding through a number of mechanisms are responsible for the four type 2 subtypes; 2A, 2B, 2M and 2N. In contrast, mutations resulting in a lack of VWF expression predominate in recessive type 3 VWD. This review explores the genetic basis of each VWD type, relating mutations identified to disease mechanism. Additionally, utility of genetic analysis within the different disease types is explored.

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1. Introduction

von Willebrand disease (VWD) is the most common inherited human bleeding disorder. Its prevalence has been estimated to be up to 1% in some populations through epidemiological surveys of low VWF plasma level,^{1,2} but ≤ 1 in 10,000 individuals are registered with the disease at specialist tertiary care centres.³ Mucocutaneous bleeding including epistaxis, menorrhagia and prolonged bleeding after trauma and surgery results from deficient or defective plasma von Willebrand factor (VWF), a large adhesive multimeric glycoprotein. High molecular weight (HMW) VWF is essential for platelet-dependent primary haemostasis. VWF also carries procoagulant factor VIII (FVIII), protecting it from rapid proteolytic degradation and delivering it to sites of vascular damage for secondary haemostasis. Patients with VWD can be classified into one of three disease types on the basis of having a partial (type 1) or virtually complete (type 3) quantitative or qualitative (type 2) deficiency of plasma VWF.⁴

VWF is synthesised in vascular endothelial cells and megakaryocytes as a 2813 amino acid (aa) protein having repeated domains in the order D1–D2–D'–D3–A1–A2–A3–D4–B1–B2–B3–C1–C2–CK (Fig. 1). Following synthesis of the monomer and signal peptide (22 aa) cleavage, VWF dimerises through disulphide bonds between cysteine knot (CK)

domains of adjacent VWF monomers. Pro-VWF dimers are transported from the endoplasmic reticulum to the Golgi complex where short disulphide isomerase-like sequences in the D1 and D2 domains (CGLC sequences at residues 159–162 and 521–524) promote inter-dimer disulphide bonds⁵ between D3 domains, forming head-to-head multimers. The 741 aa pro-peptide (D1–D2) is cleaved by furin, producing the 2050 aa mature VWF. Multimers are packaged into endothelial Weibel–Palade bodies and platelet alpha granules. Endothelial cells secrete VWF constitutively in addition to regulated secretion following storage, whereas alpha granule VWF is only released following platelet activation.³ The protein undergoes extensive post-translational modification which includes glycosylation that adds 10–20% to its mass, along with sulphation.^{6,7} Circulating VWF multimers are composed of up to 40 subunits and range in size from 500–10,000 kDa, whereas stored VWF contains ultra-large (UL) multimers >10,000 kDa.⁸

Multimer size is an important determinant of their reactivity and is tightly regulated by the enzyme, ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). Binding to collagen in exposed subendothelium by the VWF A3 domain results in exposure of GpIb α binding sites in the A1 domain and initiates platelet tethering at sites of vascular damage. The immobilisation and extension of VWF exposes the ADAMTS13 cleavage site between aa p.Y1605–M1606 in the A2 domain. Cleavage prevents ULVWF forming platelet thrombi in the microvasculature, as occurs in thrombotic thrombocytopenic purpura.

The VWF gene (VWF) is located at the tip of the short arm of chromosome 12. It spans 178 kb of genomic DNA and comprises 52

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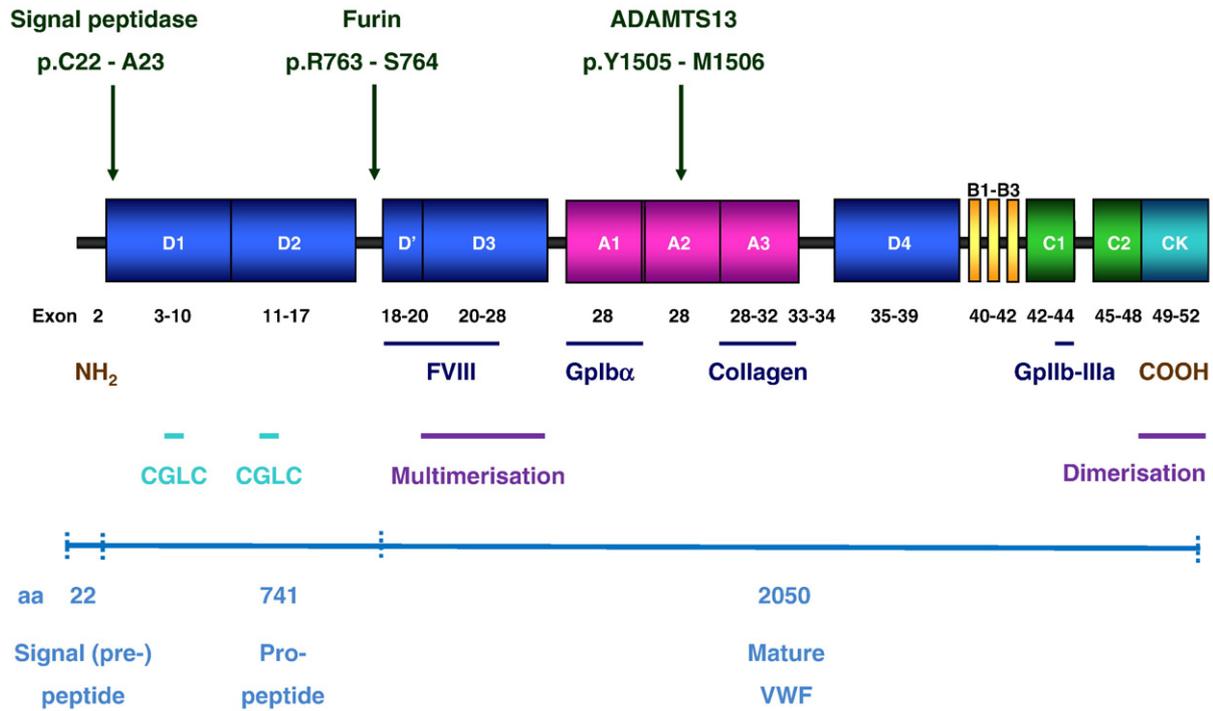


Fig. 1. Structure and function of pre-pro VWF. Exons encoding each domain are shown, along with ligand binding, cleavage and disulphide isomerase-like sites.

exons,⁹ transcribed into an 8.8 kb mRNA. Most exons are small ranging from 40–342 bp in size, but exon 28, larger at 1.4 kb, encodes several sites for essential ligand-binding and cleavage functions and is the most mutated region of VWF (Figs. 2 and 3).¹⁰ The partial VWF pseudogene on chromosome 22 is 97% similar in sequence to the coding gene.¹¹ Through gene conversion, it contributes to mutation spectrum in VWD, in addition to complicating molecular analysis.

2. Classification and nomenclature

2.1. Classification

Diagnosis and classification of a patient with suspected VWD requires a series of laboratory evaluations. These include screening tests, initial VWF analysis (VWF:Ag, VWF:RCo, FVIII:C) and tests to determine disease subtype (RIPA, VWF:FVIIIIB, VWF:CB, VWFpp, VWF multimer profile). The tests are described in Table 1. There have been three sequential classification systems for VWD, each of which has built on the previous version and incorporated the main divisions of types 1, 2 and 3. The initial classification was complex and

incorporated many categories based on multimer profile.¹² The International Society on Thrombosis and Haemostasis Scientific and Standardisation Committee (ISTH-SSC) on VWF compiled subsequent guidelines on VWD classification in 1994¹³, revised in 2006⁴. The classification aims to facilitate the diagnosis, treatment and counselling of patients diagnosed with VWD and groups together patients with similar phenotypes, even though different mutation mechanisms may be responsible. Type 2 VWD comprising the functional defects is subdivided into four subtypes; 2A, 2B, 2M and 2N. Functional defects lead to enhanced (2B) or reduced (2A, 2M) platelet interaction or impair binding to FVIII (2N) (Table 2). A third level of classification can be used to indicate specific phenotypes, such as the previous subdivisions of type 2A multimer profile that indicate different mutation mechanisms (eg IIA, IIC, IID, IIE) and the “Vicenza” rapid clearance phenotype. The 2006 update⁴ introduced two amendments. Firstly, that VWD is not restricted to VWF gene mutations; this was both to recognise that many patients do not have their causative mutation identified and also that the VWD phenotype may have other genetic causes/contributors. Secondly, in type 1 VWD, plasma VWF may include mutant subunits, but they have normal activity relative to quantity of antigen.⁴ This followed recognition of several small deviations from a normal multimer profile identified in recent studies on type 1 VWD.^{14,15}

As VWD results from the interaction between the products of both alleles and more than one sequence variant is possible on each allele, not all VWD cases fit neatly into the classification. To help recognize this, a dual classification can be given to compound heterozygous patients, for example type 2N/3 for a patient with a 2N and a null allele.⁴

2.2. Nomenclature

Several bodies have devised standardised nomenclature systems which are relevant for VWD. The ISTH-SSC on VWF recommends standard abbreviations for VWF and its activities (Table 1).¹⁶ The Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC)¹⁷ standardises gene names. Names reflect gene function and utilise only capital letters and Arabic numbers. VWF, F8 and GP1BA represent the genes encoding the VWF, FVIII and GpIb α proteins.

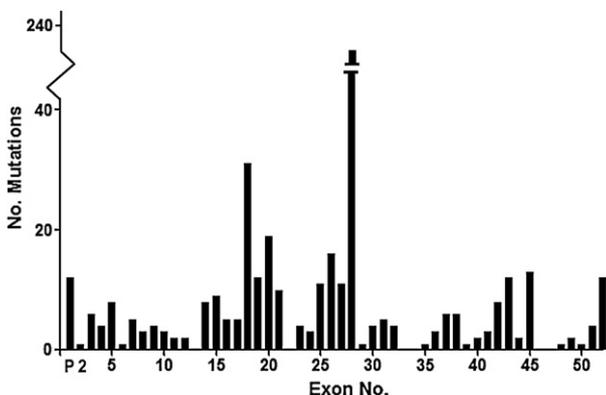


Fig. 2. Distribution of mutations in VWF. Data on point mutations from VWFdb, January 2010¹⁰. P indicates promoter. Exons are not represented to size.

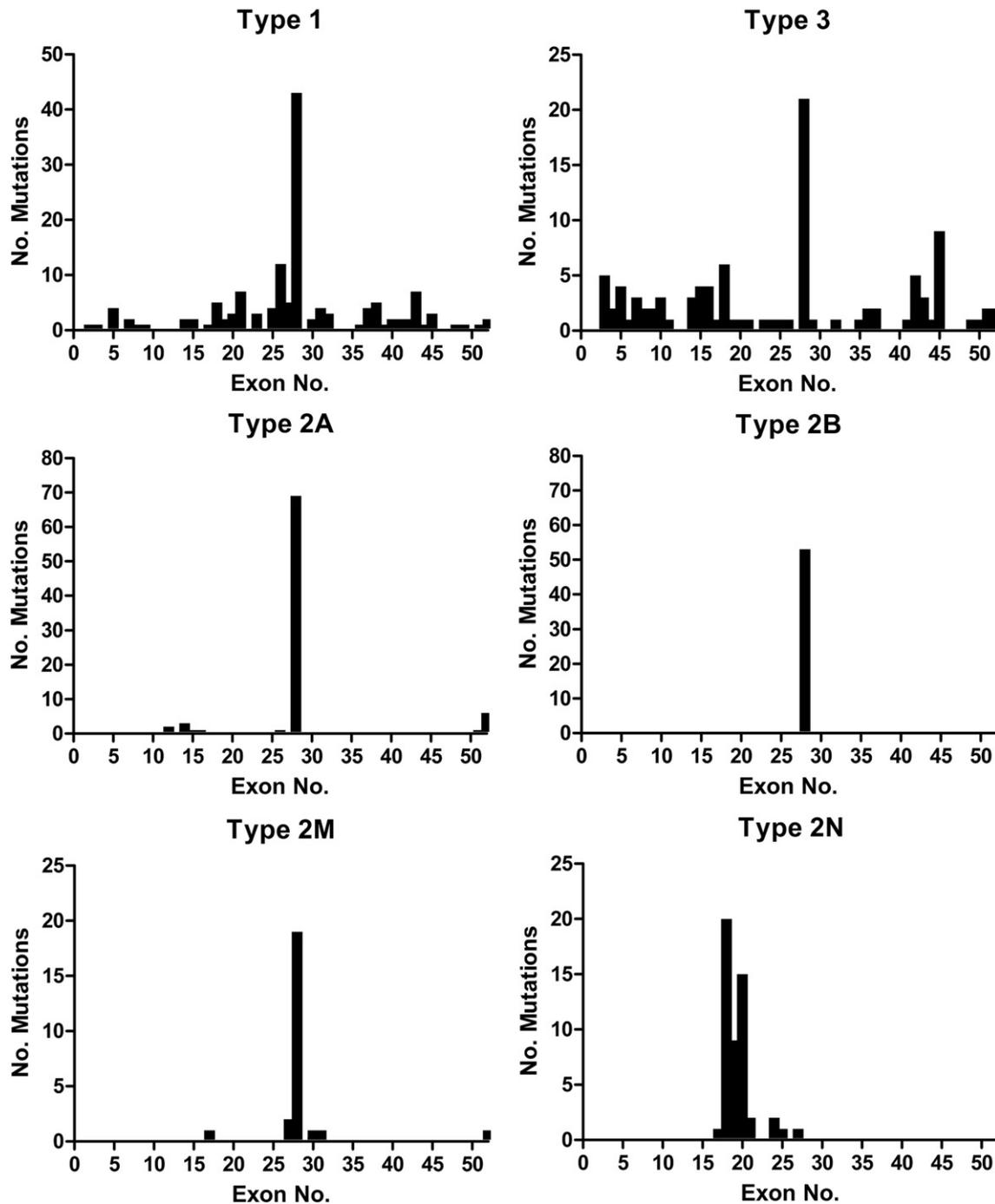


Fig. 3. Distribution of mutations in VWF by disease type. Note differences in y axis scale and that exons are not represented to size.

The Human Genome Variation Society (HGVS) has devised an extensive system to unambiguously describe genetic variants.¹⁸ All genes and proteins are numbered from the first A of the ATG initiator methionine codon ($A = +1$) at the start of every protein ($Met = +1$), with cDNA rather than genomic DNA being commonly used as reference sequence. Each sequence type has a prefix letter to denote the sequence type referred to, for example, c. is used to symbolise cDNA and p. for protein. The common “Vicenza” mutation is therefore referred to as c.3614G>A; p.R1205H. This system was instituted for VWF in 2001, but earlier publications numbered the VWF cDNA from the transcription start site, 250 nucleotides 5' to the current start site and aa from the start of mature VWF, 763 aa from the current first methionine start site. Older numbering may therefore require amending to cross-reference with the current system.

The ISTH-SSC on VWF mutation and polymorphism database (VWFdb)¹⁰ lists published and unpublished VWF variants using HGVS nomenclature. The current listing (January 2010) has 526 mutation entries, 370 of which are unique along with 202 polymorphic variants (152 unique).

3. Mutation types that contribute to VWD

Many different types of mutation are responsible for VWD. The majority of these are commonly seen in other inherited disorders whereas some result from particular features of the VWF gene and protein. Mutations are described below and summarised in Table 3.

Table 1
Phenotypic analysis of VWD.

Test	Type	Measurement
VWF:Ag ^a	Initial	Antigen; quantity of protein
VWF:RCo ^a	Initial	Ristocetin cofactor activity; ability to bind platelet GPIb in the presence of ristocetin
FVIII:C ^a	Initial	FVIII coagulant activity
VWF:Ac	Initial	Monoclonal antibody binding to a functional epitope of the A1 loop: immunoassay of GPIb binding
RIPA	Subtyping	Ability to aggregate platelets at varying doses of ristocetin. Aggregation at low doses of ~0.5 mg/mL indicates 2B VWD
VWF:FVIIIb ^a	Subtyping	FVIII binding capacity. Reduced values indicate 2N VWD
VWF:CB ^a	Subtyping	Collagen binding capacity. Reduced values correlate with reduction in HMW multimers
VWFpp ^b	Subtyping	Quantity of propeptide. Elevated VWFpp/VWF:Ag ratio indicates enhanced clearance rate from plasma
Multimer profile	Subtyping	Aberrant profiles can indicate reduction in dimerisation /multimerisation, HMW multimer loss, enhanced or reduced ADAMTS13 cleavage, enhanced clearance and mutations that replace/introduce cysteine residues, affecting disulphide bonding

^a Abbreviations recommended for VWF and its activities¹⁶.^b Abbreviations approved at ISTH-SSC on VWF 2009.**Table 2**
VWD classification.

Disease type	Description
1	Partial quantitative deficiency of VWF. Structure and distribution of plasma VWF multimers indistinguishable from normal
2	Qualitative defects
2A	Decreased VWF-dependent platelet adhesion with a selective deficiency of HMW multimers
2B	Multimer deficiency can result from defective multimer assembly or from increased sensitivity to ADAMTS13 cleavage Increased affinity for platelet GPIb. Characterized by increased RIPA at low ristocetin concentrations, resulting from enhanced interaction of mutant VWF with platelet GPIb
2M	Decreased VWF-dependent platelet adhesion without a selective deficiency of HMW multimers.
2N	Multimer assembly is approximately normal. Functional defect results from mutations that disrupt VWF binding to platelets or to subendothelium Markedly decreased binding affinity for factor VIII.
3	Results from homozygous/compound heterozygous mutations that impair FVIII binding capacity (VWF:FVIIIb). Both VWF alleles may have VWF:FVIIIb mutations, but often one allele has a FVIII binding mutation while the other expresses little or no VWF Virtually complete deficiency of VWF Generally VWF:RCo, VWF:CB and VWF:Ag <5 IU/dL and FVIII:C <10 IU/dL

Adapted from Sadler⁴.**Table 3**
Mutation types that contribute to VWD.

Mutation type	Process disrupted	Description	VWD type(s)
Transcriptional	mRNA transcription	Disrupted transcription factor binding sites (TFBS) may result in reduced or absent mRNA synthesis	1
Splice	Intron removal	Disruption of invariant GT and AG dinucleotides at 5' and 3' end of each intron or flanking nucleotides can lead to exon skipping, intron retention or other mRNA abnormalities.	Null alleles contribute to recessive type 3, 2N, 2A and type 1
Nonsense	Protein translation	Exon skipping can lead to in-frame deletion and an abnormal shortened protein Altered sequence results in a stop codon replacing an amino acid. Nonsense mediated decay can eliminate aberrant mRNA limiting production of abnormal protein	Dominant type 1 and 2A Null alleles contribute to recessive type 3, 2N, 2A and type 1
Small deletion, small insertion and small duplication	mRNA production or protein translation	Loss/gain of one or small number of nucleotides. Often affect repeated sequence motifs. Lack of protein production where amino acid coding is interrupted, similar to nonsense mutation In-frame loss/gain of amino acid(s) where multiples of 3 nucleotides are affected	Null alleles contribute to recessive type 3, 2N, 2A and type 1 Effect similar to missense mutation; types 1, 2A, 2B, 2M
Gene conversion	mRNA production or protein translation	Replacement of VWF by VWFp sequence can result in nonsense or missense changes. Sequence altered ranges from 8–335 bp	1, 2B, 2M, 3
Large deletion	mRNA production or protein translation	Single exon to > entire gene deleted. Lack of protein production where amino acid coding is interrupted, similar to nonsense mutation In-frame loss of amino acid(s) where multiples of 3 nucleotides are affected	Null alleles contribute to recessive type 3 and 2A Effect similar to missense mutation
Missense	Protein translation	Replacement of single amino acid with different residue. Effect dependent on amino acid position and nature of its' replacement	1, 2 (unclassified), 3 1, 2A, 2B, 2M, 2N, 3

3.1. Transcription

mRNA synthesis requires a number of transcription factors to bind to specific sequences in the *VWF* promoter. Mutations that disrupt these transcription factor binding sites (TFBS) may result in reduced or absent RNA transcription of the affected allele. The first mutation disrupting promoter TFBS has recently been described in type 1 VWD and appears to reduce but not eliminate mRNA production from the affected allele.¹⁹

3.2. Splice site mutations

These mutations can disrupt the highly conserved GT and AG dinucleotides at the 5' and 3' end of each intron. Replacement of these nucleotides through point mutation will often eliminate their recognition, thus disrupting normal splicing. This may result in exon skipping, whereby an exon is not recognized and is omitted from the resulting mRNA and protein. Where ends of adjacent exons are compatible, this can lead to a smaller protein being produced where the aa reading frame is maintained (in-frame mutation).

Splice mutations can also lead to retention of intronic sequence resulting in lack of normal protein production. Mutations away from the GT and AG dinucleotides may reduce but not eliminate normal splicing and can result in a variety of transcripts being produced including the wild-type (wt) and varying aberrant transcripts.

Point mutation away from splice sites can introduce a novel site recognized in preference to the wt site leading to cryptic splice site activation or creation of a novel exon within intronic sequence (cryptic exon). Mutations disrupting splicing may eliminate VWF production, contributing to the “null alleles” seen particularly in type 3 and 2N VWD. Analysis of platelet mRNA can highlight lack of expression from one or both alleles or presence of aberrant transcript(s).

3.3. Nonsense mutations

Nonsense or stop mutations can result in a lack of mRNA through nonsense mediated decay (NMD). This process eliminates aberrant mRNA with a premature termination codon, limiting production of

abnormal protein.²⁰ NMD does not occur for all nonsense mutations,²¹ and aberrant protein may be produced in some instances.

3.4. Small deletions, insertions or duplications

These mutations affecting one or a small number of nucleotides often affect repeated nucleotide sequences.²² Duplications replicate short sequence stretches. The mutations often disrupt the protein reading frame, more rarely leading to in-frame loss/gain of aa.

3.5. Large deletions

Deletions which may result from non-homologous or homologous recombination, sometimes involving Alu repetitive elements are reported infrequently in VWD,¹⁰ but are likely to be under-recognised. As above, most disrupt the reading frame, but some lead to in-frame loss of aa. The extent of large deletions and their inhibitor relationship (below) is shown in Fig. 4.

3.6. Gene conversion mutations

Mutations are likely to result from a short stretch of *VWFP* sequence replacing that of the *VWF* gene,²³ the resulting phenotype depending on the sequence replaced. Nucleotides within the 3' end of exon 27 and 5' end of exon 28 may be affected by replacement of up to 335 bp of *VWF* sequence by *VWFP*. The most common variants reported are p.P1266L in type 2B VWD, p.Q1311X in type 3 VWD and p.V1279I in type 2M and 1 VWD.^{10,15,24,25}

3.7. Missense mutations

Replacement of one amino acid by another can lead to VWF with altered structure or function. Missense changes are found throughout VWF and contribute to all disease types, being the predominant cause of type 1 and 2 VWD.

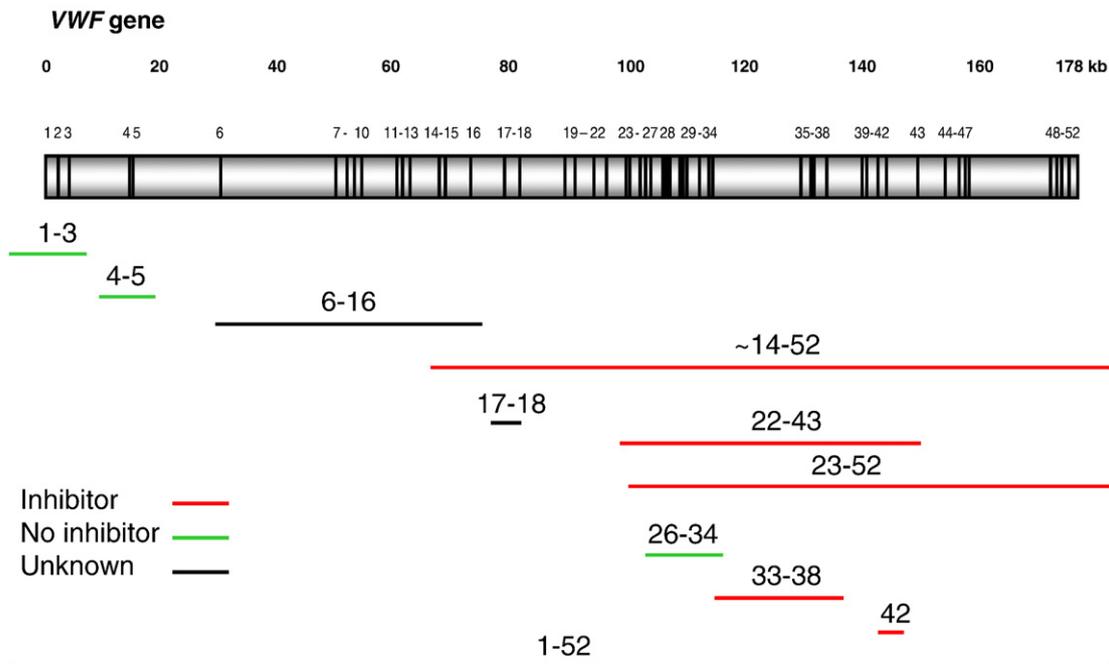


Fig. 4. Large deletions and their relationship with inhibitor development. Exon 4–5 and 26–34 deletions are in-frame.

4. Mutations responsible for different VWD types

4.1. Type 1 VWD

Three recent multicentre studies conducted in the European Union (EU),¹⁴ Canada¹⁵ and the UK²⁶ have greatly extended knowledge of the molecular basis of type 1 VWD, which prior to 2006 was extremely limited. Between them, these studies analysed over 300 patients with type 1 VWD. The studies differed in their inclusion criteria: the EU study recruiting patients with all severities of type 1 VWD diagnosed as affected by expert European clinicians, whereas the UK and Canadian studies established upper limits for VWF:Ag of 50 IU/dL and a lower limit of 5 IU/dL for the Canadian study. Additionally, the UK and Canadian studies excluded individuals with abnormal multimers, whereas the EU study retained and characterised multimer abnormalities.²⁷ Candidate mutations were identified in ~65% of index cases (IC) and despite recruitment differences, a similar range of mutations was found in IC from each of the three studies.

Changes identified comprised missense (70%), splice (9%), transcription (8%), small deletion (6%), nonsense (5%) and small insertion or duplication (2%) mutations. Only 10–15% of these may result in null alleles and lack of VWF mRNA and protein, as several splice, small deletion, insertion and duplication mutations lead to in-frame protein changes. The missense alterations included at least eight resulting from probable gene conversion (3% mutations).^{14,15} In the EU and Canadian studies, ~15% of IC had more than one candidate mutation identified. Among the EU cases, about half had two mutations on the same allele while the remainder were compound heterozygotes. Estimating the likely pathogenicity of each sequence variant in these cases can be challenging.

4.2. Disease mechanisms

Two mutation mechanisms have been characterised to date in type 1 VWD, clearance (decreased survival)^{28–30} and intracellular retention. The “Vicenza” mutation (p.R1205H), previously classified as type 2M but now incorporated among type 1 variants⁴ exemplifies the clearance phenotype. The mean half-life of VWF in plasma is 8–12 h. Clearance has been determined using ratios of levels of VWFpp to VWF:Ag or VWF recovery following desmopressin infusion.^{29,31–33} Equivalent quantities of VWFpp and mature VWF are secreted into the circulation where a 1:1 stoichiometry of their quantities in IU/dL can be determined. Increased ratios indicate reduced VWF survival. Clearance mutations, sometimes referred to as type 1C³² (not part of the ISTH nomenclature), have a robust response to desmopressin followed by a rapid return to baseline VWF levels. VWF:Ag half-life is significantly reduced to <3 h, and for p.R1205H <2 h.³³ The p.R1205H mutation was found in 6% of ~300 type 1 VWD IC.^{14,15,26} Other missense mutations identified in patients historically diagnosed with type 1 VWD also share the clearance phenotype but with slightly longer half lives. These include other missense substitutions affecting p.R1205, p.C1130 substitutions and p.W1144G (D3 domain), plus p.I1416N and p.S1279F (A1 and D4 domains).^{29,31,32}

The p.Y1584C variant was the most common change associated with type 1 VWD in the three studies, being identified in 13% of IC.^{14,15,26} It has also been shown to result in a slightly increased clearance of VWF, but this is a much more subtle effect than that described above. A combination of slightly increased clearance due to both the sequence variant and to co-inherited blood-group O along with slightly increased susceptibility to ADAMTS13 cleavage appear to contribute to the pathogenesis of this variant.^{34–36} Liver and spleen macrophages are likely responsible for clearing VWF, but the exact mechanism is not yet known.³⁷

Intracellular retention was recently demonstrated to be a common pathogenic mechanism in type 1 VWD. Missense mutations in the less commonly studied VWF regions (D1 and D4–C2) lead to markedly

impaired VWF secretion, likely due to mis-folding.³⁸ The mode of pathogenicity of several other variants awaits elucidation.

There was a trend towards greater mutation penetrance with decreasing VWF level, but many mutations demonstrated incomplete penetrance, ie they did not result in VWD in all individuals inheriting them. All three studies examined linkage of the VWF gene to symptoms of VWD by determining co-inheritance of gene and disease using polymorphic markers. Cosegregation requires the affected allele to be fully penetrant and family sufficiently large (≥ 4 members) to be informative. Less than 50% of families fulfilled these criteria. However, incomplete-cosegregation may not indicate lack of VWF involvement in VWD. Recessive inheritance of a mutation from each parent may lead to incomplete-cosegregation, as can incomplete penetrance, non-paternity or *de novo* mutations. In the EU study, only 19 of 150 families (13%) had no mutation identified plus incomplete-cosegregation and are unlikely to have a VWF contribution to their bleeding phenotype.¹⁴

In the three studies, ~35% of the 300 IC had no mutation identified. More recent work within the UKHCDO cohort has characterised an in-frame deletion of exons 4–5, removing 104 aa and resulting in a shortened VWF protein.³⁹ The deletion was found in two of 32 IC (6%). This mutation, which in the homozygous or compound heterozygous form results in type 3 VWD, may contribute to the type 1 VWD mutation spectrum in other heterozygous patients of British origin, including emigrant populations.⁴⁰

4.3. ABO blood-group

ABO blood-group O has been known to be more prevalent in type 1 than in type 2 VWD and the normal population for several years.⁴¹ This relationship was further investigated in the type 1 VWD studies. The Canadian study showed that among those with VWF levels >30 IU/dL, a significantly higher proportion (66%) of individuals had blood-group O than did those in the normal population (46%).¹⁵ The EU study examined IC with no mutation identified. 76% had blood-group O compared to 38% of the normal population. Amongst the subgroup whose VWD demonstrated incomplete-cosegregation with VWF, 89% had blood-group O.¹⁴ The effect of blood-group O appears to be through increased VWF clearance from the plasma; individuals with blood-group O having VWF plasma levels 30% lower than those with group AB. ABO glycosyltransferase alleles encode different transferase enzyme specificities. The enzyme is non-functional in blood-group O due to a null allele. A and B blood-group glycosylation protects VWF from clearance, whereas its absence in blood-group O results in more rapid clearance.²⁸ There may be a combined effect of blood-group O and other genetic factor(s) which results in low VWF level and bleeding symptoms in the patient group lacking an identified VWF mutation.

VWD symptoms in patients with VWF levels >30 IU/dL may have a multifactorial aetiology. Although VWF level was higher in IC with no VWF mutation identified, patients had a similar median bleeding severity score (BS) to those with mutations identified.¹⁴ In this group, polymorphic VWF sequence variation (below), ABO blood-group and other factors that may include platelet function defects⁴² and further blood-groups that act through VWF glycosylation such as Lewis may contribute.

Mutation analysis can be useful in patients where there is a doubt about disease subtype (Table 4), particularly in cases with VWF levels below ~30 IU/dL. Above this level, mutations have been identified in fewer cases (57% of EU IC, compared to 88% with levels ≤ 30 IU/dL). Mutation penetrance reduces with increasing VWF level, so that interpreting the contribution of a VWF variant to symptoms becomes more challenging. A UKHCDO guideline on mutation analysis in VWD recommends caution in genetic analysis of type 1 VWD.⁴³

5. Type 2 VWD

This type comprises the qualitative disorders that affect VWF function. Missense mutations and in-frame deletions, insertions or

Table 4
Genetic analysis of suspected von Willebrand disease.

Disease type and inheritance pattern	Initial analysis of exons	Further analysis of exons	Additional analysis	Common mutations	Comment
1 dominant	18–28	Promoter, 2–17 and 29–52	Dosage	p.R1205H p.Y1584C	≥1 mutation in ~15%. Often demonstrates incomplete penetrance ABO blood-group O common
1 severe recessive	18–28	Promoter, 2–17 and 29–52	Dosage		Small proportion of type 1 cases
3 recessive	18–28	2–17 and 29–52	Dosage	p.P812fs p.Q1311X p.R1659X p.R2535X p.C1272	2 “null” mutations, recessively inherited. Can be homozygous or compound heterozygous Population-specific large deletions may be common
2A dominant	28	52		p.S1506L p.R1597W p.G1609R p.C2771-C2773 substitutions Missense	Majority of mutations in the A2 and A1 domains encoded by exon 28. Rarer exon 52 substitutions can result in 2A(IID), leading to aberrant dimerisation
2A recessive	11–17				Mutations affect multimerisation. Patients either homozygous for a missense mutation or compound heterozygous with a second “null” mutation
2B dominant	28		GP1BA	p.R1306W p.R1308C p.V1316M	Missense alterations or in-frame duplications in and immediately flanking the A1 domain, encoded by exon 28. Where mutations are absent, suspect the phenocopy, platelet-type pseudo-VWD (PT-VWD)
2M dominant	28	30–31, 52	None	p.R1341Q p.V1279I p.I1425F	Very few mutations reported. Mostly missense alterations in exon 28
2N recessive	18–20	17, 24–27	F8	p.T791M p.R816W p.R854Q	p.R854Q extremely common in Caucasians. Many patients homozygous/compound heterozygous for this substitution. Second allele may be “null”. Mutations can also result in abnormal multimers (Table 6)
Phenocopies Haemophilia A X-linked recessive	F8 promoter and exons 1–26	Intron 1 inversion, Intron 22 inversion	F8 dosage	Missense	Mild haemophilia A in males and haemophilia A carriership in females may be responsible for reduced FVIII:C levels. Females may be carriers of any severity of haemophilia A
Platelet-type pseudo VWD dominant	GP1BA exons 1–2			p.G249 p.M255V substitutions	Phenocopy of type 2B. Missense or in-frame deletion mutations. Legacy numbering G233 and M239V

duplications are responsible for the majority of cases. There have been few large population studies on mutation spectrum in type 2 VWD, but two are notable. Meyer and colleagues reported mutation data on 150 French type 2 VWD patients comprising all four type 2 subtypes in 1997.⁴⁴ Recently, Federici and colleagues have determined mutations, bleeding severity and response to treatment in 67 cases of 2B.²⁴ These studies along with many on small patient groups and unpublished mutation data collated on VWFdb¹⁰ inform our understanding of type 2 VWD pathogenesis.

5.1. Type 2A

Patients demonstrate a loss of HMW multimers and reduced GpIb α binding (Table 2). Good quality multimer analysis can distinguish four main multimer abnormalities, IIA, IIC, IID and IIE along with several rarer profiles which may indicate different mutation mechanisms.⁴⁵ Classic 2A(IIA) VWD results from mutations in the A2 and A1 domains. Patients display a characteristic loss of HMW and sometimes intermediate multimers along with an increase in the outer sub-bands.⁴⁵ Mutations can result in increased intracellular retention, as the largest multimers which contain the highest proportion of mutant subunits are retained within the cell (Group I).^{46,47} Intracellular retention may result from mis-folded VWF. In the more frequent Group II, VWF is synthesised, multimerised and secreted normally, but has enhanced sensitivity to ADAMTS13 cleavage. These mutations surrounding the ADAMTS13 cleavage site may enhance access to the normally buried p.Y1605-M1606 bond, which under physiological conditions requires shear

stress to render the site accessible.^{46,47} It is not possible to predict readily whether a mutation belongs to Group I or II.

ADAMTS13 cleavage results in the characteristic triplet satellite bands seen on multimer electrophoresis. HMW multimer loss and differences in patterns of satellite bands can help to identify VWD subtype and mechanism responsible for disease.

Dimerisation defects yield VWF that is terminated by a monomer and cannot form inter-chain disulphide bonds or does so inefficiently. The characteristic 2A(IIID) multimer pattern showing HMW multimer loss and aberrant satellite bands results.⁴⁵ Dominantly inherited missense mutations, particularly those affecting p.C2771 and p.C2773 have been reported.¹⁰

D3 domain mutations can impair VWF multimerisation, which requires inter-chain and intra-chain disulphide bonding in this region.⁴⁸ Multimer profile in 2A(IIIE) demonstrates both severely reduced HMW multimers and aberrant triplet structure indicating reduced proteolytic cleavage. Reduced affinity for GpIb α and resulting impaired platelet tethering renders mutant VWF less frequently cleaved by ADAMTS13. Mutations are dominantly inherited.

D2 domain mutations (exons 11–17) can prevent full multimerisation, and are recessively inherited. Large multimers are severely reduced, proteolytic bands absent and there is an increase in dimers resulting in subtype 2A(IIC). Patients are either homozygous for a missense mutation or compound heterozygous, with a null second allele.⁴⁴

A rare mutation at the 3' end of exon 26 which appears to be a missense change altering an amino acid, but has been demonstrated to result in exon skipping and an aberrant protein was recently described.⁴⁹ This, along with reports of the less common 2A varieties,

such as a recently reported 2A(IIIH) case highlights that further mechanisms can also contribute to 2A disease.⁵⁰

In the French study⁴⁴ and on VWFdb,¹⁰ the relative proportion of type 2A mutations is A domains 82%, D2; 8%, CK; 8% and D3; 1%. Budde reports that the 2A(IIIH) phenotype, resulting from D3 domain mutations is common, comprising 34% of type 2A multimer profiles.⁴⁵ However, the mutations responsible for these profiles have yet to be described; their absence in other studies likely results from use of targeted mutation analysis.

Molecular diagnosis can be useful where there is uncertainty over VWD disease type, particularly where multimer analysis is unavailable or poor quality (Table 4).

5.2. Type 2B

Conformational changes which result from type 2B mutations stabilise the “collagen bound VWF” form and enable the A1 domain to bind Gplb α spontaneously. This can be detected through enhanced ristocetin induced platelet aggregation (RIPA) with low dose ristocetin (~0.5/mL). Patients with classic 2B may have HMW multimer loss and thrombocytopenia, in some cases only during infection/stress/pregnancy. Desmopressin may exacerbate thrombocytopenia and is contra-indicated for most 2B cases. Patients with mutations including p.P1266Q/L (New York/Malmö, resulting from gene conversion) and p.R1308L may be more challenging to identify phenotypically as HMW multimers are often present and thrombocytopenia absent. Enhanced RIPA may be the only indication of 2B VWD.^{24,51} Giant platelets with structural abnormalities are a recently recognized feature of 2B VWD. Megakaryocytopenia is modified by the enhanced VWF-Gplb α interaction which results from gain-of-function VWF mutations.⁵²

Genetic analysis of the 5' end of exon 28 (codons 1266–1461 in and flanking the A1 domain) will detect all mutations reported on the VWFdb¹⁰ and by Meyer et al.,⁴⁴ which affect only 16 different amino acids. All are dominantly inherited missense/amino acid duplication mutations, most being fully penetrant. Federici and colleagues study on 2B VWD²⁴ provides very useful genotype–phenotype correlations. BS indicates different disease severities; patients with p.V1316M had the highest BS accompanied by thrombocytopenia, worsening with stress, whilst those with p.1266Q/L and p.R1308L had the lowest BS and no thrombocytopenia in response to stress or desmopressin (Table 5).

Where genetic analysis of VWF exon 28 fails to identify a mutation, the phenocopy platelet-type pseudo VWD (PT-VWD) may be present, resulting in an indistinguishable phenotype. Mixing studies using patient and control plasma and platelets can discriminate the two disorders. Mutation analysis of *GP1BA* exons 1–2 may identify missense mutations affecting residues p.Gly249 and p.Met255 (Table 4).⁵³ An in-frame 27-bp deletion has also been described (p.Pro449_Ser457del). These mutations appear to directly or indirectly affect the confirmation of a Gplb α loop that interacts with the VWF A1 domain, leading to spontaneous binding to VWF. PT-VWD may be

Table 5
Bleeding score correlation with mutation in types 1 and 2B VWD.

The VWD type	Mutation	No. cases (no. families)	Bleeding score
			Median (range)
1	p.R1205H	18 (6)	8 (2–17)
1	p.Y1584C	23 (10)	4 (0–20)
2B	p.P1266L	6 (2)	2 (0–6)
2B	p.R1306W	15 (9)	8 (3–24)
2B	p.R1308C	13 (11)	9 (1–18)
2B	p.V1316M	4 (4)	13 (12–16)

Data from ^{14,24,73}.

responsible for 5% or more of patients diagnosed with type 2B VWD and is important to identify, as patients may require platelet transfusions in some circumstances.⁵⁴

5.3. Type 2M

This VWD type can be difficult to discriminate from 2A, 2B or type 1 VWD, without a full range of phenotypic analyses. Even with good phenotypic analysis, deciding whether patients have 2M or type 1 can be challenging and reclassification following detailed investigation may occur.

A relatively small group of patients have been reported with 2M mutations to date. Most mutations are missense changes or in-frame deletions and lie in exon 28 between codons 1266–1467 (Fig. 3).¹⁰ Isolated other mutations have been reported in the D3 (exon 24) and CK (exon 52) domains.^{10,55} Mutations are dominantly inherited and fully penetrant. James and colleagues recently reclassified a group of patients with type 2M whose initial diagnosis was type 1 VWD.³⁷ Final classification remained equivocal in some patients but the parameter that identified A1 domain mutations was a VWF:RCO/VWF:Ag ratio <0.4.

The A1 domain mutations cluster on the face which interacts with Gplb α , reducing or preventing the interaction and resulting in discrepantly low VWF:RCO/VWF:Ag ratios. Mutations lie on the opposite side of the A1 domain to those responsible for 2B VWD. Response to desmopressin is often poor in 2M VWD, leading to an inadequate rise in VWF level. Multimers are essentially normal, but relative loss of HMW forms may be seen.⁴⁵

Three missense mutations in the collagen-binding A3 domain have also been described. These result in reduced collagen-binding affinity and affect residues in exons 30–31. They fit the 2M definition through their interference with binding to subendothelium (Table 2), but authors describing the mutations have argued against their classification as 2M VWD, partly as the patients do have a clinically useful response to desmopressin.^{56,57}

Genetic analysis of VWF exon 28 detects the majority of previously described missense mutations. Further analysis of exons 29–32 may be necessary to identify mutations which could interfere with binding to subendothelium (Table 4).

5.4. Type 2N

This VWD subtype (Table 2) in which VWF binds FVIII poorly or not at all mimics mild haemophilia A. Symptoms largely result from reduced FVIII level. VWF:RCO and VWF:Ag levels can both be within the normal range, while FVIII:C is typically 5–40 IU/dL. The multimer profile is normal in the majority of cases. Inheritance pattern may highlight whether 2N VWD or haemophilia A is more likely, but in some cases, bleeding history in family members is insufficiently widespread. Differential diagnosis can be achieved using the VWF:FVIII assay, but the assay is not widely available nor well standardised, partly due to lack of external quality assessment, and

Table 6
Type 2N VWD mutations and their association with multimer profile.

Exon no.	Mutation	Multimer profile	Reference
17	R760C ^a	Supranormal, smeary	82
18	R763G	Supranormal, smeary	83
18	Y795C	Supranormal, smeary	60
24	Q1053H ^a	Supranormal	84
18	C788R	Absent HMW	85
18	C788Y	Absent HMW, smeary	44
18	C804F	Slightly reduced HMW	86
20	D879N	Reduced HMW	60
24	C1060R	Slightly reduced HMW	84
27	C1225G	Absent HMW	85

^a Dominant inheritance.

false positive indication of 2N VWD has been reported.⁵⁸ Mixed historic diagnoses of both haemophilia A and VWD within a family may be reported; molecular analysis can reveal that a single disorder is responsible for bleeding. Patients with reduced FVIII:C plus a multimer abnormality are challenging to subtype using phenotypic analysis alone. Several 2N missense mutations have been described to result in either UL multimers or reduced HMW multimers, some of the latter resulting in a mixed 2N-2A(IIE) phenotype (Table 6).

Recessive inheritance of two mutations is necessary for 2N VWD and a range of mutation types contributes to disease. The study by Meyer et al.⁴⁴ compiled mutations in 51 French patients. 49% were homozygous for a single missense mutation, 12% compound heterozygous for two different 2N missense mutations and 39% compound heterozygous for a 2N missense change plus a null (or unidentified) second mutation. The non-2N mutations nearly all result in lack of VWF expression and are the same null alleles seen in type 3 VWD. A single 2N allele rarely lowers FVIII:C level sufficiently for bleeding to occur and heterozygous relatives are rarely symptomatic. Mutation of both alleles dramatically reduces or abolishes FVIII binding ability. VWF mutation can abolish normal electrostatic interaction with FVIII, which can eliminate FVIII binding capability.⁵⁹ Cysteine mutations in the D' domain may abrogate intra-molecular disulphide bonds necessary for normal secondary structure on which FVIII binding depends.⁶⁰

Mutation analysis of exons 18–20 will identify D' domain mutations, which comprise about 85% of reports.^{10,44} Remaining 2N missense mutations are reported in exons 17, 24, 25 and 27 (Fig. 3). Once mutations are identified, genetic analysis can be used to examine relatives, particularly siblings at risk of the disorder.

The p.R760C substitution encoded by exon 17 disrupts the furin cleavage site and leads to persistence of the propeptide. This may sterically hinder binding of FVIII to VWF resulting in a 2N picture in addition to UL multimers. Unusually for 2N VWD, this mutation is dominantly inherited. The missense change p.R854Q occurs at a polymorphic frequency in Caucasian populations and is often found in the homozygous form in 2N cases (39% of 51 French 2N patients). Additionally, the frequent heterozygous occurrence of p.R854Q results in it being co-inherited with many other VWD mutations, resulting in mixed 2N/other VWD phenotypes.

Where no missense mutations are identified following analysis of exons 17–20 and 24–27, 2N VWD is unlikely and reduced FVIII level may result from a F8 mutation. Males in this category often have mild haemophilia A resulting from a FVIII missense change. Females may be symptomatic carriers of a similar mutation, or sometimes carry a moderate/severe haemophilia A mutation. Skewed X-chromosome inactivation (Lyonisation) may be responsible for FVIII:C <50 IU/dL and bleeding symptoms. DNA sequence analysis of the F8 promoter and 26 exons detects mutations in most cases. Where point mutations are not identified, the two intra-chromosomal inversions affecting introns 22 and 1 may be analysed and dosage analysis undertaken to identify partial/complete gene deletions or duplications.^{61,62}

5.5. Unclassified or type 2U

There is no “unclassified” category in the 2006 ISTH classification.⁴ However, 6% mutations submitted to VWFdb have not been classified into a VWD subtype and are listed as unclassified. These include several type 2 VWD mutations; D3 domain missense changes (exons 25–27) that may fit the 2A(IIE) category,⁶³ missense mutations affecting p.R1315C and p.R1374 which lead to a pleiotropic phenotype with features of more than one VWD subtype⁶³ and a large in-frame deletion of exons 26–34 (Fig. 4).⁶⁴

5.6. Type 3

This virtually complete VWF deficiency (Table 2) results from homozygosity or compound heterozygosity for two mutations

resulting in lack of VWF expression. Phenotype analysis is generally sufficient for diagnosis of the disorder, although discriminating from severe type 1 VWD can be dependent on assay sensitivity. Molecular analysis may be useful where carrier status determination or pre-natal diagnosis (PND) is required (Table 4).

Four cohort studies comprising at least 20 cases each have been reported and between them have identified mutations in an average of 92% of 111 cases^{65–68} and 100% in one study of 40 patients.⁶⁸ 80–90% of mutations on VWFdb and in these studies are predicted to result in null alleles. VWFdb entries comprise nonsense (31%), small deletions (18%), large deletions (12%), splice (11%) and small insertion (10%) mutations. Missense alterations comprise ~18% mutations and may result in VWF that cannot dimerise or multimerise resulting in mis-folded VWF retained within the cell. About 20% of reported mutations are in the large exon 28 (Fig. 3), but the remainder are found throughout the gene, reported from exon 3–52. To reduce the analysis required, the central region of the gene can be analysed first for the most frequent mutations. Some laboratories stop analysis once two obviously disease-causing mutations are identified. Deletions of ≥1 exon, up to more than the full gene in length are readily detected in the homozygous form, but often require a mutation-specific gap PCR^{69–71} or dosage analysis (quantitative PCR or multiplex ligation-dependent probe amplification (MLPA)⁷²) for their detection in the heterozygous form. They may result in either a disrupted protein and null allele, or in-frame aa loss and intracellular retention.⁶⁹

PND may be requested where a couple already have an affected child. The process often relies on characterisation of both mutated alleles in an affected case, their confirmation in heterozygous carrier parents, followed by analysis of a potentially affected foetus by chorionic villus analysis at 11–13 weeks gestation. If both mutations responsible for type 3 VWD cannot be identified, linkage analysis using intragenic polymorphisms may be an alternative.⁴³

6. Phenotype-genotype correlation

6.1. Extent of bleeding

The BS^{73,74} provides a standardised tool to determine whether extent of bleeding is abnormal and to compare bleeding between VWD patients. Examples shown in Table 5 illustrate BS for common mutations in type 1 and 2B VWD. This information is likely to become more widely available and may be useful in indicating the predicted extent of bleeding associated with particular mutations.

6.2. Response to desmopressin

The EU type 1 VWD study determined desmopressin response in 77 patients with varying mutations. The majority had a clinically useful response whereas partial/non-responders largely had mutations in the D3 and A1–A3 domains.³³ Further understanding of the mutation-desmopressin response may enable prediction of desmopressin utility upon identification of the patient's mutation(s).

6.3. Inhibitors

Inhibitory antibodies directed against VWF arise in a small proportion of type 3 VWD patients. There are no systematic surveys on prevalence, but case reports suggest that large deletions and some nonsense mutations⁷⁵ occur in inhibitor patients. Anaphylaxis may occur following concentrate treatment in a proportion of the same patient group.⁷⁶ Fig. 4 illustrates the relationship of large deletions to inhibitor development.

7. Founder effect

A proportion of mutations seen repeatedly are likely to result from inheritance from a common ancestor. This can be referred to as identity by descent or founder effect. Demonstration of a shared polymorphic haplotype in affected individuals can highlight where this is the case. Founder haplotypes have been identified for p.Y1584C⁷⁷ and p.R924Q and are likely for the c.2435delC mutation in exon 18, common in northern European type 3 VWD.⁶⁵ Shared ancestry is more readily demonstrated for large deletion mutations were the same break-points are unlikely to occur by chance. “Common” large deletions contribute to type 3 VWD in Hungary (exons 1–3),⁷⁰ Germany and Italy (253 kb complete gene deletion)⁷¹ and the UK (exon 4–5).⁶⁹ Where these mutations are common in a population, they should be the first mutation sought when undertaking genetic analysis.

8. De novo mutations

The relative frequency of *de novo* mutations is not well reported in VWD. The Canadian and EU type 1 VWD studies each identified cases of *de novo* mutations. These occurred in 2/123¹⁵ and 4/98⁷⁸ families, suggesting that the new mutation rate in type 1 VWD may be at least 2–4% of cases.

9. Mutation or polymorphism?

It is often difficult to decide whether a particular sequence variant is associated with disease or is part of normal variation. In addition to potentially causing VWD, sequence variants may also more subtly modulate phenotype and this area is only just starting to be explored in VWD. “Polymorphisms” can be defined as variants that occur in at least 1% of the general population. However, presence within the normal population does not exclude an effect on VWF. p.R854Q, p.R924Q and p.Y1584C all occur in the Caucasian population at ≥1% frequency and all affect VWF level, being associated with type 2N and 1 VWD. Some variants may affect only laboratory determinations and have no associated phenotype, for example p.P1467S.⁷⁹ In addition, polymorphisms p.V1565L and p.G1643S have an association with increased ADAMTS13 proteolysis and p.D1472H, p.Q1571H and p.P1601T with resistance to proteolysis.^{80,81} Further associations of some of the ≥150 reported polymorphic variants in the exons, closely flanking intronic sequence plus 5′ and 3′ untranslated regions of VWF with phenotypic modulation are likely.

10. Conclusion

Genetic analysis of patients with known or suspected VWD can be a useful facet of diagnosing and sub-typing disease. Genotype–phenotype correlations will increasingly inform patient management and genetic counselling decisions. However, knowledge of VWF and non-VWF variation that contributes to phenotypic variation between individuals with the same mutation and to those diagnosed with type 1 VWD but with no VWF mutation currently identified remains rudimentary and much further work is required.

Conflict of Interest

The author declares no conflict of interest.

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