

The Molecular Analysis of von Willebrand Disease: a Guideline from the UK Haemophilia Centre Doctors' Organisation Haemophilia Genetics Laboratory Network

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Summary

von Willebrand Disease (VWD) is a common autosomally inherited bleeding disorder associated with mucosal or trauma-related bleeding in affected individuals. VWD results from a quantitative or qualitative deficiency of von Willebrand factor (VWF), a glycoprotein that is essential to primary haemostasis and that carries and protects coagulation factor VIII (FVIII) in the circulation. Through characterisation of the phenotype and identification of mutations in the VWF gene in patients with VWD, understanding of the genetics and biochemistry of VWF and VWD has advanced considerably. The importance of specific regions of VWF for its interaction with other components of the vasculature has been revealed, and this has facilitated the formal classification of VWD into three subtypes based upon quantitative (types 1 and 3) and qualitative (type 2) deficiency of VWF. The underlying genetic lesions and associated molecular pathology have been identified in many cases of the qualitative type 2 VWD variants (2A, 2B, 2M, 2N) and in the severe quantitative deficiency, type 3 VWD. However in the partial quantitative deficiency, type 1 VWD, the picture is less clear: there is a variable relationship between plasma levels of VWF and bleeding, there is incomplete penetrance and variable expressivity within affected families, the causative molecular defect is unknown in a substantial number of cases, and even in those cases where the causative mutation is known, the associated molecular pathology is not necessarily understood. This guideline aims to provide a framework for best laboratory practice for the genetic diagnosis of VWD, based upon current knowledge and understanding.

Keywords

von Willebrand factor, genetics, guideline, von Willebrand disease, mutation analysis, UKHCDO

Introduction

von Willebrand factor (VWF) is a large multimeric glycoprotein which is essential to platelet-dependent primary haemostasis, particularly in the microvasculature where high fluid shear forces are present. VWF also acts as a carrier for procoagulant factor VIII (FVIII) in the circulation, protecting FVIII from proteolytic degradation and transporting it to the site of vascular injury (overviewed in [1]). The VWF gene (*VWF*) is located on the short arm of chromosome 12 at 12p13.3 [2,3], resides within 178 kb of genomic DNA and consists of 52 exons, the largest, exon 28, spanning 1.4 kb [4]. *VWF* gives rise to a 9kb mRNA that encodes a 2,813 amino acid protein [5], designated pre-pro-VWF [6]. This precursor polypeptide consists of a 22 amino acid signal (pre) peptide, a 741 amino acid propeptide and a 2,050 residue mature subunit. VWF has a repeating domain structure (Fig. 1). A partial pseudogene (*VWFP*) located on the long arm of chromosome 22 at 22q11.22 to 22q11.23 is highly homologous with exons 23-34 of *VWF* [7,8]. *VWF* is highly polymorphic, at the time of writing there are over 150 reported polymorphic variations in the *VWF* exons and closely flanking intronic sequences recorded on the ISTH SSC on VWF Database (VWF Database, URL <http://www.vwf.group.shef.ac.uk/>). The highly polymorphic nature of *VWF* has implications for genetic analysis and may complicate some investigations.

von Willebrand disease (VWD) is caused by quantitative or qualitative deficiencies of VWF [9,10]. It is the most common of the inherited bleeding disorders, with a reported prevalence derived from epidemiological studies of up to 1% in the general population [11,12] although only approximately 1 in 10,000 individuals has clinically significant bleeding [13]. VWD is classified into three main sub-types [9,10]: type 1 - a partial quantitative deficiency of VWF, type 2 - a qualitative abnormality of VWF, and type 3 - a virtually complete absence of VWF. Type 2 VWD is further divided into four sub-categories with specific

functional defects: type 2A shows a selective loss of high molecular weight (HMW) VWF multimers and an associated decrease in platelet-VWF interaction; type 2B is associated with an increased affinity of VWF for platelet glycoprotein Ib; type 2M is associated with a VWF multimer pattern that is

similar to normal, but with reduced VWF-platelet interaction; type 2N variants show a reduced ability to bind FVIII. An enormous diversity of mutations has been characterised in VWD (VWF Database). The molecular basis of VWD has recently been reviewed [10,14].

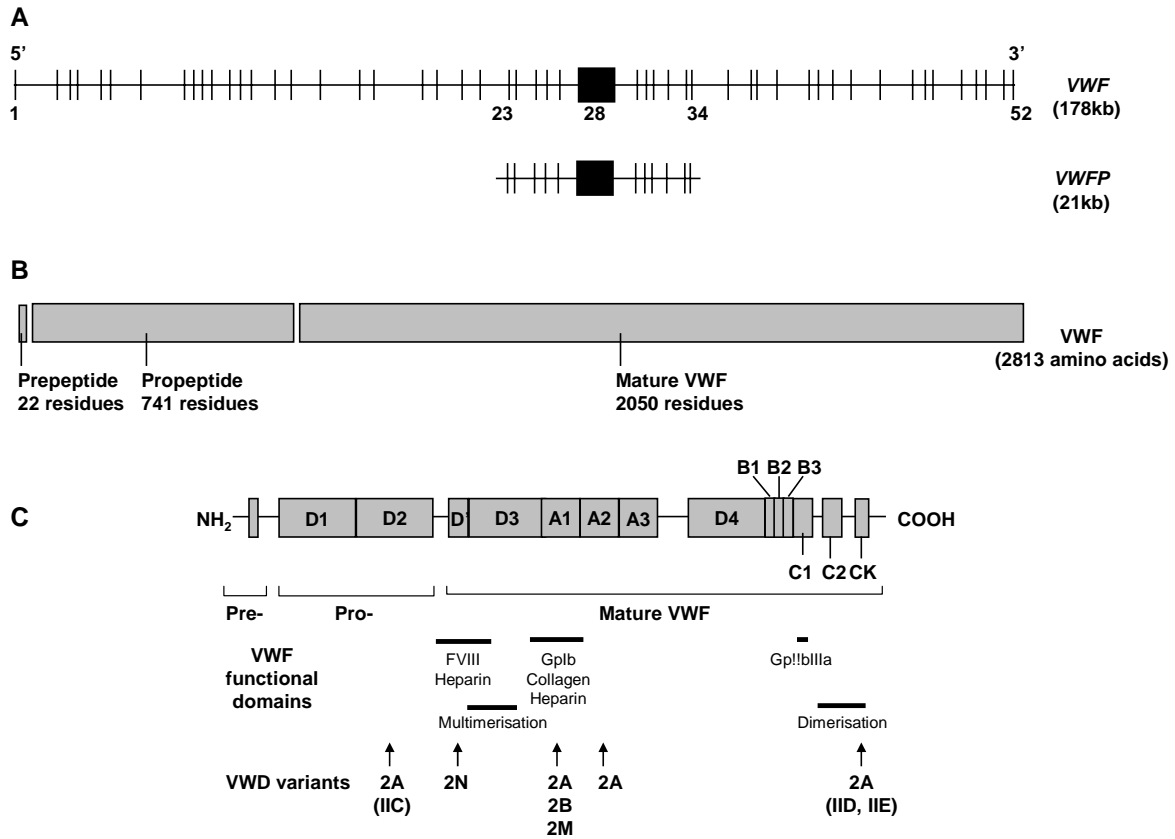


Fig. 1 VWF organisation and functional domains. Schematic showing: (A) The structure of human VWF and VWFP; (B) Pre-pro-VWF; (C) VWF functional domains and locations of common type 2 VWD variants. Lettered boxes denote homologous domains within VWF. Panels A to C are not to scale.

The initial diagnosis of VWD is based on clinical and phenotypic information [10,15,16]. Genetic analysis may provide information to confirm or support the initial diagnosis, and additionally to permit family studies and counselling, which are of particular value in type 3 VWD. Genetic investigation also plays a role in differential diagnosis, for example in distinguishing VWD type 2N and mild haemophilia A, or VWD type 2B and platelet type pseudo-VWD [17,18]. In addition, identification of the genetic basis of the disorder may inform treatment choices and improve our understanding of the mechanisms underlying different VWD subtypes. It is, however, apparent that genetic testing is likely to have limited clinical utility in cases where the phenotype clearly reveals the VWD sub-type. This guideline aims to provide a rational approach to genetic testing in VWD, based on present understanding, using currently available laboratory techniques and best practice.

Methods

This guideline document was prepared on behalf of the UKHCDO Haemophilia Genetics Laboratory Network (UK-HGLN) following a Best Practice workshop meeting held on November 1st 2007 in Manchester, UK. The workshop was attended by UK based health care scientists working in the field of VWD genetic testing. Diagnostic approaches and issues were discussed within the group. The guideline writing group, consisting of UK based scientists with expertise in the field of VWD genetic testing, used the discussion during the meeting as a basis for the preparation of a draft guideline document. This draft was prepared with reference to relevant literature reports, reviews and web based resources relating to the subject. The draft was circulated to the membership of the UK-HGLN for comment and the final document was approved by the UKHCDO Advisory Committee.

Genetic Testing in VWD

Initial classification of VWD is currently based on clinical and phenotypic information, which is therefore central to the investigation of this disorder. Genetic testing must be integrated and interpreted together with all other relevant information gathered in the investigation of a patient with suspected VWD [19,20,21].

The requirement for molecular genetic studies in VWD is variable because the utility of genetic testing varies for different VWD subtypes. It is a prerequisite of a clinical diagnostic service that full genetic counselling is available to patients and families with VWD. Counselling should be undertaken as a matter of course, and before any genetic studies are carried out. This will ensure that the individuals concerned have an appreciation of the inheritance of VWD in their family and will understand, with regard to providing informed consent, exactly what information may be obtained by any proposed investigations and the implications of that information for the individual and other family members. Further counselling should be carried out to explain the results of genetic tests when they become available. Genetic counselling must always be non-directive and should be undertaken by suitably qualified health professionals, preferably in conjunction with individuals who have both significant experience in the clinical management of VWD and a good understanding of the molecular basis of the disorder [15,22].

In the UK, it is recommended that genetic testing for VWD should be performed in a member laboratory of the UK-HGLN, details of which are hosted by the UKHCDO (URL <http://www.ukhcdo.org/>). The UK-HGLN is a consortium of laboratories, mostly within Comprehensive Care Haemophilia Centres, which work to defined, peer-reviewed standards [15], thereby providing an equivalent quality of testing nationally.

General diagnostic strategy

Type 3 VWD

Although the incidence of this type of VWD is very low, ranging from 0.5 to 6.0 per million, depending on the population studied [23,24,25], genetic testing and family studies are valuable in type 3 VWD due to the severity of the bleeding diathesis and autosomal recessive pattern of inheritance. Genetic diagnosis may facilitate family studies and counselling as a precursor to possible prenatal diagnosis (PND), and also may provide valuable information to affected families for family planning decisions.

No mutation hotspots have been recognised in type 3 VWD [26,27, VWF Database] and mutation analysis in type 3 VWD is carried out in UK-HGLN laboratories by nucleotide sequence analysis of the essential regions of *VWF* following PCR amplification. The essential regions are defined as the promoter region, exons 1 to 52 together with their splice junctions and flanking sequences, and the 3' polyadenylation signal region. Mutation pre-screening methods have not been found to be an efficient use of resources in VWD analysis due to the highly polymorphic nature of *VWF*: polymorphisms are detected in the pre-screen and this can result in the redundant nucleotide sequence analysis of up to 30% of amplicons for each patient [28].

If, in a type 3 VWD patient, no mutations are found in the essential regions of *VWF*, or if a patient appears to be homozygous for a given mutation, the possibility of an

insertion, rearrangement or whole or partial gene deletion on the other *VWF* allele should be considered. These can be explored using Southern blot analysis, however this is no longer a mainstream routine diagnostic technique and is likely to require considerable time and effort. Other options are described in the section, "**Detection of large scale deletions in heterozygous carriers**".

The recessive inheritance of type 3 VWD implies homozygosity or compound heterozygosity for causative gene defects. Mutation identification may take a considerable amount of time, therefore, in the context of the timescale required for PND, it is preferable that the causative gene defects within a family have been characterised previously. Where this is not possible, linkage studies (see below) offer a valuable alternative.

Type 2 VWD

In most cases of type 2 VWD, a clinical diagnosis is made based on phenotypic parameters, however there is now considerable understanding of the molecular basis for type 2 VWD and genetic testing can provide valuable confirmation of the phenotypic diagnosis. Molecular analysis may be of particular benefit in the following circumstances:

- Genetic diagnosis of type 2N VWD
- Differentiation of type 2B VWD and platelet-type pseudo VWD
- Confirmation of type 2M VWD

In patients where identification of the mutation would help in obtaining a definitive diagnosis and classification of their VWD sub-type, precise molecular diagnosis in the qualitative VWD variants (types 2A, 2B, 2M and 2N) may be achieved by screening for common mutations in specific regions of *VWF*. However, the absence of routine genetic analysis makes it difficult to obtain an accurate estimate of the pick up rate for mutations in all type 2 cases and this is exacerbated by the variability in diagnostic strategies and phenotypic testing for type 2 variants between diagnostic laboratories. Personal communication from the French INSERM Network on Molecular Abnormalities on von Willebrand disease suggests that the detection rate for type 2 VWD mutations is around 75%. However, the precise detection rate will be influenced by the stringency of the diagnostic criteria applied. The proportion of patients diagnosed with 2B VWD who may actually have platelet type pseudo VWD is not known.

For genetic characterisation of types 2A, 2B, 2M, and 2N VWD, direct mutation analysis by DNA sequencing of specific regions of *VWF* is the method currently in use by members of the UK-HGLN. In addition, type 2N VWD analyses may include phenotypic measurement of the ability of *VWF* to bind FVIII (*VWF*:FVIII_B). A number of patients with type 2N VWD, the result of a defect in the FVIII binding site of their *VWF*, have previously been misclassified as having mild haemophilia A or haemophilia A carriership. In type 2N VWD the phenotype in a homozygous or compound heterozygous individual is one of a partial deficiency of FVIII, as in mild or moderate haemophilia A, but with an autosomal recessive inheritance pattern [29,30]. Type 2N VWD and haemophilia A can be discriminated by an ELISA-based FVIII binding assay [31,32], which determines the FVIII binding capacity of a patient's *VWF*. This assay may be particularly useful to screen for type 2N VWD in cases where

FVIII deficiency does not show clear X-linked inheritance, before proceeding to factor VIII gene (*F8*) mutation screening. A diagnosis of type 2N VWD based upon VWF-FVIII binding studies does not exclude the need for genetic analysis, family members may be carriers of a recessive type 2N allele and genetic diagnosis provides a means to clearly establish inheritance.

Type 1 VWD

There is currently considerable debate on the definition and diagnosis of type 1 VWD, relating to the underlying pathophysiology of the disorder [10,14,33]. The historical variability in phenotypic data on which an individual diagnosis of type 1 VWD may have been based further complicates this picture. Therefore the role of genetic diagnosis in type 1 VWD in a routine clinical setting remains to be determined. "Classical" type 1 VWD is considered to show autosomal dominant inheritance linked to *VWF*. It is however, recognised that the normal variation in VWF levels in the general population and bleeding risk in VWD is influenced by genetic variability not linked to *VWF*. Additional genetic components, such as ABO blood group [34], *VWF* sequence alterations such as p.Tyr1584Cys [35,36], and platelet integrin polymorphisms [37,38], together with circumstantial influences on VWF level such as stress and pregnancy [15], may strongly influence bleeding symptoms in type 1 VWD. In many cases, therefore, the condition may be considered to be a complex multi-factorial disorder, with inter-relating genetic and environmental components. Up to 50% of cases diagnosed to have type 1 VWD may not have an identifiable mutation within the essential regions of *VWF* and the phenotype is not always linked to the gene [39,40,41,42,43,44]. At the present time there is limited *diagnostic* utility for genetic analysis in type 1 VWD, either by direct mutation detection or linkage studies. Possible exceptions to this include mutation screening in the more severe forms of type 1 VWD. For example, cases with a VWF:Ag level of <20 i.u. dL⁻¹ where over 90% may have penetrant *VWF* mutations [45], screening for the VWD Vicenza variant (p.Arg1205His) or other *VWF* mutations associated with a reduced VWF survival time in the circulation, for which a confirmed diagnosis may alter management [46], or reclassifying patients as having type 2 VWD [42].

Mutation detection strategies

Detection of previously characterised mutations

Because a number of mutations are recurrent in certain VWD subtypes (in particular in type 2 VWD), some laboratories are now directly screening patients for relevant characterised mutations, or for previously identified familial mutations in *VWF* (see the UKHCDO Directory of Molecular Diagnostic Services for Inherited Bleeding Disorders at URL <http://www.ukhcdo.org/>). Direct mutation detection typically involves PCR amplification of the relevant regions of *VWF* followed by analysis of the amplification product using a method that demonstrates the presence or absence of the mutation, such as bi-directional nucleotide sequence analysis.

Detection of unknown mutations

Strategies to detect unknown mutations require PCR amplification of the relevant regions of *VWF* in an affected

individual, followed by analysis of the amplification product using nucleotide sequence analysis or a method to screen for sequence changes. Several primer sets have been described to achieve this [42,44,47].

Direct DNA Sequencing

DNA sequencing is considered to be the gold standard for mutation detection. Using streamlined procedures, either the essential regions of *VWF*, or large stretches of the gene sequence are now amenable to direct DNA sequence analysis in a rapid and cost effective fashion. Streamlined methods, including automated or semi-automated procedures can generate full sequence data for *VWF* within the rapid timescale often required in a diagnostic setting.

Primer design

There is no standard primer set recommended for amplification of the essential regions of *VWF*, but it is important to consider the following when designing primers or using those previously published:

1. Primer sequences should be regularly checked for underlying SNPs (Diagnostic SNPcheck tool available at URL <http://ngri.man.ac.uk/SNPCheck/index.html>) to avoid allele specific amplification, a particular concern because *VWF* is highly polymorphic.
2. Tailed primers (for example with m13 sequence) are recommended to facilitate downstream sequencing protocols and standardisation of sequence quality across all amplicons screened.
3. Care must be taken in PCR primer design to ensure that there is no possibility of amplification of pseudogene sequence.
4. The design of primers that function in identical thermocycling conditions permits multiple targets to be amplified on a single PCR run.

DNA Sequencing Best Practice

DNA sequencing should have a very high degree of sensitivity. Since in most cases heterozygous changes are being sought, sequence quality is paramount. It is recommended that the following points be given particular attention:

1. Software analysis tools (those which facilitate comparative sequence analysis such as the open source Staden Package (URL <http://staden.sourceforge.net/>)) should be employed when analysing large quantities of DNA sequence data.
2. It is recommended that sequence analysis is performed on both forward and reverse strands and any sequence change that is used for genetic diagnosis should be confirmed by repeat sequencing in an independent sample from the same individual, and preferably by sequence analysis of a second affected family member. As a minimum, confirmation should be done by recourse to the original DNA (or stored blood) sample and re-amplification from the original sample. Some centres may wish to issue an interim report until they have been able to verify a base change in an independent sample. If deemed necessary, e.g. where DNA sequence quality is sub-standard, a secondary confirmatory assay for a particular mutation may be employed, such as restriction endonuclease digestion. It is important to note that the confidence of restriction analysis depends upon whether a mutation creates or destroys a restriction site. The loss of a restriction site can

arise from a change in any one of the nucleotides in that site therefore digestion is not truly specific for a given nucleotide change. In contrast, the creation of a restriction site is highly likely to arise as a result of only a single possible nucleotide change; therefore the diagnostic confidence for analysis by restriction digestion in this case is considerably greater.

DNA sequence analysis of subtypes

Qualitative (type 2) VWD variants

For the qualitative VWD types 2A, 2B and 2M, screening of exon 28 by nucleotide sequence analysis is recommended. A small proportion of mutations underlying type 2A VWD are found outside exon 28, in the regions encoding the D2 or CK domains [48,49,50,51]. Therefore, these regions should be screened if the exon 28 sequence is normal. The D2 and CK type 2A mutations result in recessive rather than the more common dominant inheritance. In the case of patients with an apparent type 2B VWD phenotype and no obvious mutation in *VWF*, the possibility of platelet-type pseudo-VWD caused by gain of function mutations in platelet *GPIBA* should be considered [17,18,52]. Such *GPIBA* mutations are listed on the Cardiff Human Gene Mutation Database (URL <http://www.hgmd.cf.ac.uk/ac/index.php>).

For type 2N VWD, the minimum sequencing requirement should cover exons 18 to 20 encoding the VWF-FVIII binding domain. Phenotype (VWF level (VWF:Ag) and FVIII/VWF:Ag ratio) may indicate whether the patient is expected to have two type 2N mutations or one affecting VWF:FVIII plus a null (non-expressed) allele. A mutation responsible for the latter can be anywhere in *VWF*. Identification of a well characterised type 2N VWD mutation may prove useful in diagnosis where VWF-FVIII binding studies have not been carried out. It should be noted that there are isolated reports of mutations associated with a VWF:FVIII defect in other regions of *VWF* (exons 17 and 24-27) [53,54,55,56,57], therefore targeted sequence analysis of exons 18-20 alone can not exclude this disorder.

Quantitative (types 1 and 3) VWD variants

To date, mutations associated with type 3 VWD have been found in 35 of the 52 *VWF* exons (VWF Database). Therefore direct sequencing of the essential regions of *VWF* should be performed. Some laboratories carry out this analysis in a specific order, but in all cases full analysis of the essential regions of *VWF* is recommended. Sequence analysis of a single amplicon can be applied where a familial mutation has been identified previously. Gene conversion events between *VWF* and *VWFP* have been demonstrated in type 3 patients [58,59,60,61] and these are readily identified by the presence of multiple substitutions in *VWF*.

For type 1 VWD, the current understanding of the genetics underlying this disorder makes definitive recommendations for mutation analysis difficult. Due to the heterogeneous nature of the disorder, if analysis is being performed in a diagnostic context and no prior causative mutation has been characterised, full sequence analysis of the essential regions of *VWF* is indicated. This approach may be warranted when applied to more severe type 1 VWD cases with a VWF level <30 i.u. dL⁻¹ which are likely to be associated with a more penetrant mutation [44,47], however, it is of questionable clinical utility in milder cases. If a prior causative mutation

within a family has been characterised, direct mutation detection is an appropriate first line investigation and the results should be interpreted in the context of the phenotypic and clinical data, and the possibility of variable penetrance or additional modifiers outside *VWF*. A number of VWF amino acid alterations may act as risk factors for bleeding. This has been proposed for the p.Tyr1584Cys change in VWF, which clearly shows variable penetrance [35,36], in part dependent on modifiers such as ABO blood group [62,63]. In general, where genetic studies are performed, criteria including phenotypic and clinical data, variable penetrance and modifiers outside *VWF* must be taken into account, both when interpreting results and when carrying out genetic counselling. Furthermore, unless linkage of the type 1 VWD phenotype with *VWF* can be clearly demonstrated, the results of any genetic family studies should be interpreted with caution.

Mutation Validation

The CMGS has produced practice guidelines for the interpretation and reporting of unclassified variants (UV Guideline,

http://cmgsweb.shared.hosting.zen.co.uk/BPGs/Best_Practice_Guidelines.htm)

. A standard questionnaire is used to assess whether or not the variant is predicted to affect protein function, principally by means of cross-species amino acid alignment algorithms, evaluation using splice-site prediction tools and documented searches to demonstrate that the variant has not been previously reported in the literature or on mutation databases. In the case of targeted mutation screening for the qualitative VWD variants, the VWF Database should be consulted to see if a particular candidate mutation has been associated previously with a particular VWD subtype. When a novel nucleotide change is found, caution should be exercised before deciding that it is the one responsible for disease (see below).

Whereas termination, deletion and insertion mutations may obviously be pathogenic, missense and other changes may not. The VWF Database acts as an online repository of information of interest to those involved in *VWF* genetic analysis. This should be consulted to determine whether the change has been previously reported. Note that entries on the VWF Database are not always peer-reviewed and should be interpreted with a degree of caution. Minimum mutation validation checks should include the following questions:

1. Has the candidate mutation been reported previously as a polymorphism?
2. Is the previously reported disease phenotype consistent with that in the patient being investigated?
3. For missense mutations, does the nature and location of the amino acid substitution confer a high risk of it being detrimental to protein structure/function?
4. Is the changed amino acid conserved across species? At present there is no standard cross species alignment of the VWF protein available as a resource for this purpose and laboratories are advised to refer to the UV Guidelines for guidance when using this approach.
5. Could the candidate mutation affect splicing?
6. Could the ethnic origin of the patient affect interpretation of polymorphism/candidate mutation status for a given base change?

Interpretation of the significance of a DNA base change, particularly amino acid substitutions, should draw upon all information resources available, including interpretation of VWF life cycle and structure/function data. Prediction software, such as that referred to in the UV Guideline, can be used to make predictions on the likely effect of each amino acid substitution. Software tools can be used to allow alternative splice site prediction. The UV Guideline recommends that at least four such predictions are run before making a conclusion on pathogenicity. If an effect on splicing is predicted or suspected, a fresh blood sample from the patient should be requested, and cDNA amplified from platelet RNA [32,42] to confirm the predicted effect. Lack of expression from one allele can also be demonstrated by absence from the platelet RNA of one allele of a SNP known to be heterozygous in genomic DNA and occurring in exonic, protein coding sequence.

Further corroboration may be obtained by genotyping a panel of normal DNA samples of the same ethnic origin, where available, to rule out a polymorphic change (a minimum of 100 alleles). Wherever possible, candidate mutations should be confirmed in affected and excluded in unaffected members of the family.

Additional genetic analysis procedures

VWF mRNA studies

The analysis of VWF mRNA may prove useful in cases where no mutation is readily apparent at the genomic DNA level and to confirm expression of both VWF alleles. Platelet derived VWF mRNA represents a relatively convenient source of material for such analyses, should they be required [64].

Linkage Analysis

Linkage studies and VWD

Wherever possible, mutation detection should be used for genetic studies in VWD families. However, this is not always practicable. Where direct mutation detection is not feasible, linkage analysis provides an acceptable alternative which offers a high degree of diagnostic confidence. In this regard linkage analysis may be particularly useful in type 3 VWD, especially for PND in a family in which the causative mutation(s) has not been identified.

If linkage analysis is used, the potential for variations in the VWD phenotype resulting from variable penetrance should always be taken into account when interpreting the results and when carrying out genetic counselling. Unless linkage of VWD with VWF can be clearly demonstrated, the results of genetic family studies should be interpreted with caution. It is important that, whenever possible, the likely informativity of genetic testing is assessed in advance of any request for PND, for example by reference to the pedigree and the family members available for the study. Identification of informative markers can be challenging in consanguineous partners from multiply consanguineous families. Linkage is most often used to identify the affected allele(s) in a child affected by type 3 VWD, and to include or exclude inheritance of this allele from a foetus at risk of inheriting it. Linkage analysis has limited application in other areas of family studies in VWD.

Intron 40 of VWF contains three tetra-nucleotide short tandem repeat (STR) sequences that are useful for PCR-based linkage studies in VWD. They can be amplified separately

[65,66,67,68], or as multiplexes [69], and can be analysed on polyacrylamide gels or using an automated sequencer for allele sizing [70]. Sequencing of 54 VWF alleles has revealed 14 different sequence combinations in this region while the size variability in the region only amounts to a 7-allele system, thus not all alleles of identical length based on PCR are the same [71]. A (GT)_n repeat in the VWF promoter is multiallelic and also highly informative for linkage studies [72]. More than 90% of individuals are heterozygous for at least one of the STRs in intron 40 plus the VWF promoter [70]. The investigator should however be aware of the potential for instability in the number of STR repeat sequences between generations [73,74].

If STRs prove uninformative, or further informative markers are required, the many SNPs in VWF can also be utilized. The VWF Database lists currently known VWF SNPs together with their frequencies in different populations and this may prove useful in designing a linkage strategy. The informativity of SNPs is, however, limited by their biallelic nature and, in many cases, sub-optimal allele frequencies. Genotyping of multiple VWF SNPs can be achieved by sequencing regions with several SNPs, such as parts of exon 28, or by individual PCR followed by restriction endonuclease digestion. PCR primers corresponding to VWF regions homologous with VWF_P must be designed to avoid amplification of the pseudogene. A 3bp CTT duplication in intron 15 (c.1946-19_1946-17dupCTT) can be readily genotyped by PCR and sizing by polyacrylamide gel electrophoresis [75].

No reports of recombination between a mutation in VWF and a polymorphism used to track inheritance of the gene have been made and a recombination risk of <1% per meiosis across VWF can be assumed.

Although linkage studies are being superseded by direct mutation analysis protocols, intragenic markers may still be of particular value under certain circumstances, for example:

1. Where a family has previously been investigated by intragenic markers
2. Where a mutation(s) has not been verified
3. Where a mutation(s) has not been found
4. In families with a large deletion, where a mutation-specific PCR product cannot readily be amplified, but intragenic markers in the remainder of the gene may permit inheritance of the deletion allele to be tracked.

Only intragenic markers should be considered for use in linkage studies. Table 1 details some of the genetic markers used for linkage studies in VWD within the UK-HGLN.

Linkage Analysis Pitfalls

Linkage analysis may fail in a number of families for one of the following reasons:

1. Lack of prior/known family history
2. Key pedigree members not available
3. Polymorphisms uninformative in key family members (rarely)
4. Non-paternity

In these families, mutation detection should be used.

Prenatal Diagnosis

Ideally families should be investigated, and the causative mutation identified in advance of any PND testing. PND in

type 3 VWD is generally performed by chorionic villus sampling (CVS) at between 11 and 13 weeks gestation. Confirmation is required that no maternal contamination is present in the sample. This can be achieved by PCR amplification using commercially available kits that amplify, for example, multiple autosomal STRs to demonstrate the absence of maternal material in the fetal sample. Such kits may additionally include primers that allow foetal sexing. A Best Practice Guideline on determination of maternal contamination has been ratified by the CMGS

http://cmgsweb.shared.hosting.zen.co.uk/BPGs/Best_Practice_Guidelines.htm. VWD status can be determined by analysis of a previously determined familial mutation or informative marker(s) from a linkage study. For analyses which involve PCR amplification, results should be provided within 2-3 days of the CVS sample being taken. Analyses requiring initial mutation detection may take considerably longer and it is essential to assess that the timescale is consistent with that required for the clinical situation.

Intron/Exon No.	Nucleotide No	Amino Acid No	Alleles	Restriction Enzyme	Allele Frequency	Population	Source
Promoter	-2144 to -2105	Not Applicable	(GT) ⁿ	Not Applicable	Multi-allelic	Swedish	Zhang et al., 1992 [72]
Intron 15	17_1946-19	Not Applicable	dupCTT	Not Applicable	0.42/0.58	Swedish	Zhang et al., 1994 [75]
Intron 40		Not Applicable	(TCTA) ⁿ	Not Applicable	Multi-allelic	South Wales	Peake et al., 1990 [65]
Intron 40		Not Applicable	(TCTA) ⁿ	Not Applicable	Multi-allelic	South Wales	Ploos van Amstel and Reitsma, 1990 [66]
Exon 18	2365	Thr789Ala	T/A	RsaI	0.65/0.35	Several Nationalities	Kunkel et al., 1990 [80]
Exon 28	4141	Thr1381Ala	A/G	HphI	0.35/0.65	Several Nationalities	Sadler and Ginsburg, 1993 [81]

Table 1. VWF polymorphisms used for linkage studies with the UK Network. PCR primers must be designed to avoid amplification of *VWFP* (Figure 1) for polymorphisms that occur in the region of homology between *VWF* and the pseudogene.

Wording of reports

Reports must be clear, concise, accurate, fully interpretive, credible and authoritative. For general guidance on report writing refer to example Report Writing Best Practice Guidelines, such as those hosted by the EMQN (<http://www.emqn.org/emqn/>).

Mutation Analysis Reporting

1. Reports should clearly state the clinical question being addressed.
2. Wording will depend on the confidence placed in the interpretation of the significance of any candidate mutation(s) and the VWD sub-type, e.g. does it/do they represent an autosomal dominant, recessive, heterozygous, homozygous or compound heterozygous and unaffected family members to seek co-segregation of candidate mutation and disease.
3. The report should state the gene analysed, the exon/intron in which a change was identified, the nucleotide change and the predicted effect on the protein. The mutation nomenclature recommended by the Human Genome Variation Society (HGVS, see below) should be used and recommendations referenced in the report.
4. So that the information can be transcribed as accurately as possible from the report if required, the mutation information should be written as continuous text. For example, “c.3916C>T predicted to result in p.Arg1306Trp”, rather than the worded descriptor, “a substitution of C by T at nucleotide 3916 in the VWF cDNA, predicted to result in the replacement of arginine

mode of inheritance? What degree of penetrance is/are the candidate mutation(s) thought to exhibit?

3. Due to the variety of phenotypes involved in VWD, it is of utmost importance that the disease phenotype, plus any relevant phenotypic data (levels of VWF:Ag, VWF ristocetin cofactor activity (VWF:RCo), VWF:RCo or other activity measurement, VWF collagen binding activity (VWF:CB), FVIII coagulant activity (FVIII:C), VWF:FVIIIIB, comment on multimer normality/abnormality) is included on the report and it is concluded whether or not this is consistent with the genetic result. Where uncertainty remains about the pathogenicity of a sequence alteration, samples should be requested from other affected at codon 1306 by tryptophan”. It may, however be helpful to the clinician to include the worded descriptor in the report in addition to the formal HGVS descriptor.
7. It should be highlighted in the report that the mutation(s) can be sought in other family members to confirm or to diagnose/exclude VWD in them.
8. Reference sequences used (RefSeq) for nucleotides and amino acids should be stated, along with the version number (Table 2). These can be given as a footnote to the report.

Gene Name (HGNC nomenclature)	von Willebrand factor (<i>VWF</i>)
OMIM Number	193400
GeneCards ID	VWF
Ensembl Gene ID	ENSG00000110799
GenBank cDNA reference sequence	NM_000552.3
GenBank protein reference sequence	NP_000543.2
Chromosomal location	12p13.3
Entrez Gene ID	7450
Pseudogene Name (HGNC nomenclature)	von Willebrand factor pseudogene (<i>VWFP</i>)

Table 2. Key nomenclature and reference sequence identifiers for the *VWF* gene

Mutation nomenclature

The HGVS provides a standard nomenclature for mutations and should be referenced in genetic reports that follow that nomenclature (URL <http://www.hgvs.org/mutnomen/>). General guidance based on the HGVS convention is given below:

1. Use cDNA nucleotide numbering; +1 is the first base (“A”) of the initiator methionine codon. Previous nucleotide numbering was from the transcription start site, 250nt 5’ to the current start site for numbering. The accession number for the reference VWF cDNA is given in table 2.
2. Amino acid numbering starts at +1 for the initiator methionine. Previous numbering of amino acids was from the start of the mature VWF sub-unit, corresponding to Ser764 in the standard nomenclature. *This older numbering system should not be used, but where a family member has been previously reported using “old numbering”, both old and new should be referred to in subsequent reports on the family. (eg p.Arg1374His, previously referred to as Arg611His).*
3. Three letter amino acid codes should be used in the report to avoid confusion between amino acids and DNA bases.
4. Intronic numbering should be based on the cDNA nucleotide number of the closest end of the adjacent exon and: “-” for intronic mutations upstream of an exon, where “-1” is the intronic nucleotide immediately 5’ to the first exonic nucleotide; “+” for intronic mutations downstream of an exon end, where “+1” is the intronic nucleotide immediately 3’ to the last exonic nucleotide.
5. In some circumstances, the partial VWF genomic sequence of Mancuso (Mancuso et al, 1989) may prove useful when describing certain sequence variants but it should be made clear that this does not follow HGVS genomic sequence numbering convention.

Detection of large scale deletions in heterozygous carriers

Large deletion mutations are particularly difficult to detect in autosomal conditions, where the failure to amplify one of the two *VWF* alleles must be detected in heterozygotes. Possible methods for detection of mutation status in these families include;

1. Use of linkage analysis, which may reveal loss of heterozygosity for markers in the deleted region.
2. Analysis of platelet derived VWF mRNA, which may reveal an aberrantly sized RT-PCR product.

3. Methods based on gene dosage analysis, such as multiplex ligation-dependent probe amplification (MLPA) [76].
4. Gap or Long PCR protocols may be developed where deletion boundaries are partially or fully mapped.

Neither of the last two methods is in routine diagnostic use and may need to be developed specifically for a given family investigation.

Mosaicism

Germline and somatic mosaicism may complicate any genetic diagnosis in VWD, however reports of mosaicism in VWD are limited [77,78]. The recent EU study of type 1 VWD identified 4 families of 98 with apparently *de novo* mutations [79]. Particular attention should be given to the possibility of mosaicism in such families.

Reference samples for test optimisation and validation

An EQA scheme has been established for VWF genetic investigation. Details are available from UK NEQAS for Blood Coagulation (<http://www.ukneqasbc.org/content/PageServer.asp?S=932234149&C=1252&ID=32>). Participation in EQA is recommended and is obligatory for UK-HGLN laboratories. There are currently no commercially available reference materials for genetic investigation of *VWF*. These may become available in the future through the National Institute for Biological Standards and Control (NIBSC) (URL <http://www.nibsc.ac.uk/>).

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