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Phenotypic and Genotypic Diagnosis of von Willebrand Disease: A 2004 Update

Reinhard Schneppenheim[a] and Ulrich Budde[b]

In the last two decades, progress in the diagnosis of von Willebrand disease (VWD) came from the rapidly developing field of molecular techniques that allowed the first phenotype-genotype correlations. In particular, structural and functional defects of von Willebrand factor (VWF) that underlie VWD type 2 and their molecular basis not only helped to understand the pathophysiology of VWD but also the complex post-translation processing of VWF and the multiple VWF functions. In contrast to the dramatic development of molecular techniques, improvement of methods for phenotypic description, a prerequisite for phenotype-genotype comparisons, has been neglected. The gold standard to differentiate VWD type 2 from type 1 and between diverse type 2 subtypes is the electrophoretic analysis of VWF multimers, a demanding technique that itself is not easily standardized but of crucial relevance for correct classification. This article summarizes the current knowledge on phenotype-genotype correlations as well as up-to-date phenotypic and genotypic methods in the diagnosis of VWD.
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Functional Properties of von Willebrand Factor

The modular structure of von Willebrand factor (VWF) forms the basis of its multifunctional properties. Several repeated domains provide distinct functions (Fig 1), for example, platelet glycoprotein (Gp)Ib binding at the A1 domain,[1] platelet GpIIb/IIIa binding at the RGDS site in the C1domain,[2] factor VIII (FVIII) binding at the D′ domain,[3] collagen binding at the A1 and A3 domains,[4] and heparin binding at the A1 domain.[5] These binding sites are essential for the function of VWF in primary hemostasis, its bridging function between subendothelial receptors like collagen and platelets under conditions of high shear.[6] The important function of VWF in secondary hemostasis is the binding of coagulation FVIII, thereby protecting it against proteolytic degradation.[7] This is demonstrated by the very low FVIII levels of patients with severe VWF deficiency, called von Willebrand disease type 3 (VWD 3).

[a]University Hospital Hamburg-Eppendorf, Department of Pediatric Hematology and Oncology, Hamburg, Germany.
[b]Lab Association Prof., Arndt and Partners, Coagulation Laboratory, Hamburg, Germany.
Address correspondence to Reinhard Schneppenheim, MD, PhD, University Hospital Hamburg-Eppendorf, Department of Pediatric Hematology and Oncology, Martinistrasse 52, D-20246 Hamburg, Germany. E-mail: schneppenheim@uke.uni-hamburg.de

Structural Requirements of von Willebrand Factor Function

VWF is composed of multiple copies of its mature subunit ranging in size from 500 kd of the dimer to greater than 10,000 kd of polymers of different sizes, so-called VWF multimers. Whereas FVIII binding of VWF is size-independent,[8] VWF function in primary hemostasis (binding to GpIb, GpIIb/IIIa, and collagen) strongly correlates with VWF multimerization,[8] with the largest multimers being the most active (Fig 2). VWF multimers are formed first by dimerization at the carboxy-terminal cysteine-rich CK domain and then by further polymerization of VWF dimers at the sub-aminoterminal cysteine-rich D3 domain. The 763–amino acid VWF pre-pro-peptide that is cleaved from the mature peptide during the posttranslation modification process and which does not contribute to the biologic function of VWF plays an essential role in VWF multimerization, probably by catalyzing disulfide bonding of VWF dimers between their D3 domains. The presence of two disulfide isomerase consensus sequences (CGLC) in the D1 and D2 domains (Fig 1) is in accordance with this hypothesis.[9] Besides intermolecular disulfide bonds, a number of intramolecular disulfide bonds are present in VWF. It has been shown that no free sulfhydryl groups exist in mature VWF.[10] This implies that each single cysteine is involved in cysteine bridges.

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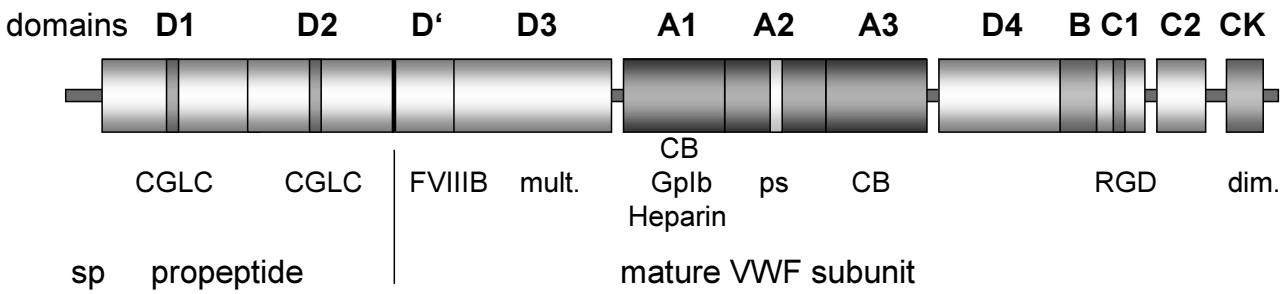

Figure 1 Structural and functional domains of pre-pro-VWF. sp, signal peptide; CGLC, amino acid consensus sequence of disulfide isomerases; FVIIIB, factor VIII binding site; mult., multimerization site; CB, collagen binding site; GpIb, platelet GpIb binding site; Heparin, Heparin binding site; ps, proteolytic site; RGD, GpIIb/IIIa binding site; dim., dimerization site.

Structural Defects of von Willebrand Factor

The complex posttranslation processing of VWF offers multiple opportunities for failure. Defects of dimerization at the CK domain and multimerization at the D3 domain have been identified repeatedly.[11-13] Given the involvement of the D1 and the D2 domains in the multimerization process, defects of multimerization can be expected either in the D1, D2, or D3 domain of VWF and examples of these defects have been published.[13-18] In general, dimerization and/or multimerization defects result in the lack or relative decrease of high molecular weight multimers (HMWM) with the consequence of impaired VWF function in primary hemostasis. The structure of VWF also may be impaired by mutations that result in either loss or creation of single cysteines, creating aberrant inter- or intra-VWF subunit disulfide bonds or free sulfhydryl groups. These events may give rise to an unstable VWF structure.

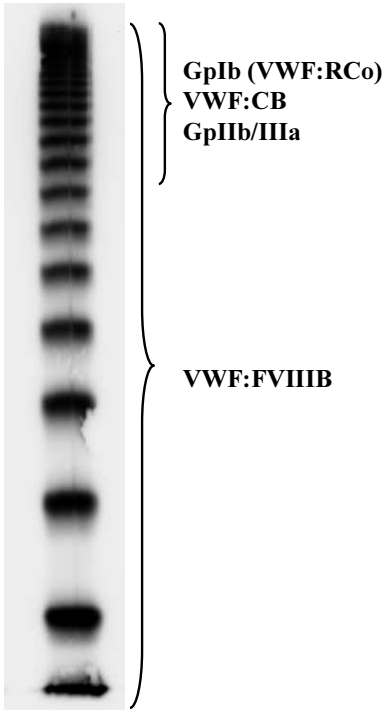

Figure 2 Recombinant VWF multimer pattern. Only the largest multimers provide the function in primary hemostasis like binding to GpIb, GpIIb/IIIa, collagen binding, and ristocetin cofactor activity. Note the lack of a triplet structure of individual multimers due to absence of the VWF specific protease ADAMTS13 (see Fig 3).

Functional Defects of von Willebrand Factor

Since particular functions of VWF are located in particular VWF domains the identification of isolated functional defects was an exciting but not surprising finding. The first such identified defect was the gain of function condition observed in VWD subtype 2B (VWD 2B), where enhanced affinity of mutant VWF to platelet GpIb may cause spontaneous agglutination of platelets by HMWM-VWF resulting in the loss of both HMWM and platelets.[19] This reaction may be triggered by acute-phase situations, by pregnancy, and by the hemostatic drug desmopressin, and it may occur spontaneously in newborns.[20-22] The lack of HMWM and possibly also the low platelet count are responsible for the bleeding tendency in this subtype.

The deficiency of VWF in binding FVIII[23] corresponds to the clinical condition of true "pseudohemophilia," the term originally chosen by Erik Adolf von Willebrand for the disease that later bore his name.[24] Defective VWF:FVIII binding was subsequently identified in many other families from different countries.[25-28] It may mimic hemophilia A completely by FVIII deficiency being the only diagnostic laboratory parameter. The clinical severity is variable depending on the residual VWF:FVIII binding and may range from almost no bleeding tendency to symptoms in the range of moderate hemophilia.[27] This VWD subtype is called VWD 2N or "Normandy."

Impaired platelet-dependent function was found in patients with defects in VWF:GpIb binding that seemed not due to the lack of HMWM but to an intrinsic functional deficiency of the GpIb binding region residing in the A1 domain. VWF: ristocetin cofactor (RCo) activity is impaired. This condition contributes to VWD subtype 2M (VWD 2M).[29]

Isolated deficiency of VWF:collagen binding (CB) activity

Table 1 Diagnostic Procedures for Diagnosis of von Willebrand Disease and Recommended Abbreviations

Screening tests
Bleeding time (BT)
Tests with high shear forces
Partial thromboplastin time (aPTT)
Platelet count
Confirmatory tests
FVIII activity (FVIII:C)
VWF antigen (VWF:Ag)
Ristocetin cofactor activity (VWF:RCo)
VWF collagen binding capacity (VWF:CB)
Tests performed by specialized laboratories
Ristocetin-induced platelet agglutination (RIPA)
Bothrocetin-induced platelet agglutination
Platelet VWF
VWF propeptide (VWF:Ag II)
VWF multimeric structure
VWF subunits
FVIII-binding capacity (VWF:FVIIIB)

not associated with the lack of HMWM was observed in a French family with a bleeding tendency in two members.[30] In contrast, this condition, even more pronounced, was identified in three unrelated German probands without bleeding tendency.[31] The clinical impact of isolated VWF:collagen binding deficiency has still to be established.

Decreased Half-life of von Willebrand Factor

Variants of VWD with low VWF:antigen (Ag) are increasingly recognized as due to a decreased half-life time of mutant VWF. This condition may be suspected subsequent to a response to desmopressin of only short duration. It can also be inferred from a higher than usual ratio of VWF propeptide to VWF:Ag. One well-known example of decreased half-life seems to be VWF in VWD 2M Vicenza, characterized by VWF:Ag less than 10 U/dL and a good response to desmopressin, though of short duration.[32-34]

Phenotypic Characterization of von Willebrand Disease

Due to the complexity of the disease, diagnosis of VWD is one of the most challenging of the coagulation disorders. Depending on the functional epitope mutated, only subsets of tests may be abnormal. Thus the laboratory investigation requires a battery of tests. Depending on the experiences of individual laboratories, their equipment, and their status as routine or reference laboratory, the test panel may differ considerably. Generally the tests can be subdivided into screening procedures, confirmatory tests, and tests performed by special laboratories (Table 1).

Diagnosis of von Willebrand Disease and Identification of Type

Previously VWD type 2 variants were classified according to their abnormal multimer pattern[35] and comprised, for example, the subtypes IB, I platelet discordant, IIA, IIA-1, IIA-2, IIA-3, IIC, IIE, IIF, IIG, IIH, and II-I (Figs 3, 4, and 5). This classification resulted in more than 50 variants and was impractical even for experts. Since 1994, VWD type 2 is classified into four subtypes.[29] However, because the mechanisms that result in the decrease or lack of HMWM are rather different and the mode of inheritance also varies, further subtyping may still be necessary. According to a consensus by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (SSC/ISTH), in this case the old nomenclature can be used in parentheses.

Quantitative Deficiency of von Willebrand Factor

The diagnosis of VWD is relatively easy whenever the VWF is absent (type 3) or moderately reduced (type 1). Usually the severity of bleeding correlates with the residual protein concentration. The distinction between mild cases of type 1 and normals is difficult because of the broad normal range, the blood group dependency of VWF, and its behavior as an acute-phase reactant.[36] The determination of platelet VWF may be helpful if it is shown to be decreased, because it reflects synthesis of the VWF molecule. According to provisional consensus criteria established by a working party of the SSC/ISTH (1996 annual report of the SSC/ISTH Subcommittee on VWF) VWD type 1 is proven if the patient has a significant mucocutaneous bleeding history, decreased VWF, as compared to reference values for the same ABO

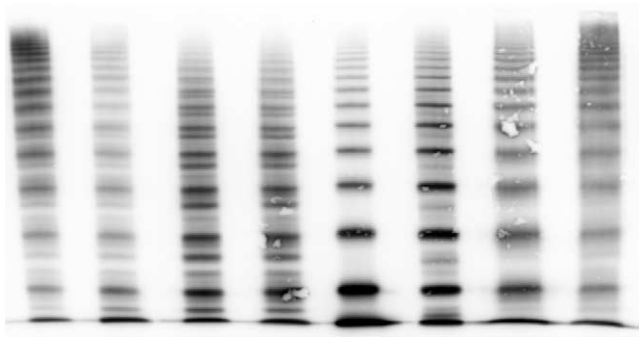

Figure 3 Well-characterized inherited variants in a discontinuous medium resolution gel (1.6% low gelling temperature [LGT]-agarose). The dye front is at the bottom of the gel. Lane 1, normal plasma (pool of 30); lane 2, type 2A with all multimers, but a relative loss of the largest multimers (subtype IB); lane 3, type 2A classical pattern (IIA); lane 4, type 2B; lane 5, type 2A, subtype IIC, characterized by the pronounced protomer; lane 6, type 2A, subtype IID, note the less intensive intervening band between 2 major bands; lane 7, type 2A, subtype IIE with broad central bands, amorphous material instead of a triplet structure and a normal protomer; lane 8, type 2A with a smeary appearance. The "VWF-free" space between the oligomers is filled with amorphous material.

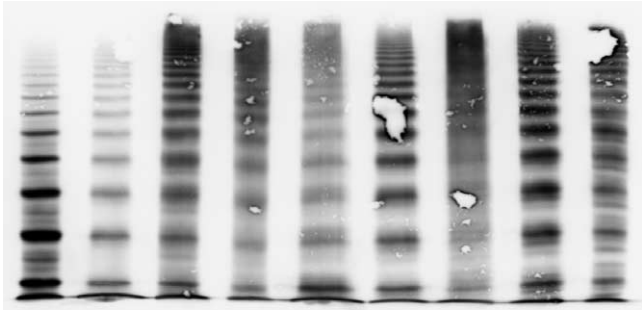

Figure 4 Well-characterized inherited variants (lanes 1, 2, 4, 6-8) compared with acquired abnormalities of the VWF molecule (lanes 3 and 5) in a discontinuous medium resolution gel (1.6% LGT-agarose). The dye front is at the bottom of the gel. Lane 1, dimerization defect (subtype IID); lane 2, multimerization defect (subtype IIC); lane 3, congenital lack of VWF-CP, Upshaw Shulman syndrome; lane 4, type 2M with a smeary multimer pattern; lane 5, decrease of large multimers in a sample from a patient with a lymphoproliferative disorder; lane 6, multimerization defect, subtype IIE; lane 7, type 2M with a smeary multimer pattern; lane 8, multimerization defect, subtype IIE; lane 9, a normal plasma (pool of 30). The acquired abnormalities cannot readily be distinguished from inherited variants; rather the rate of interaction between the VWF, its receptors and the VWF-CP is reflected by the multimer pattern. Note the progressive appearance of proteolytic sub-bands from no visible sub-bands (lanes 1-4), severely decreased sub-bands (lanes 5 and 6), clearly visible, but still abnormal sub-bands (lanes 7 and 8), to a normal pattern (lane 9).

blood group, without qualitative abnormalities, and a positive family history with dominant inheritance. Because these stringent criteria are met by a small fraction of patients only, the term "possible VWD" has been introduced.

Another approach is that proposed by Sadler.[37] Because of the blood group and age dependency and its behavior as an acute-phase reactant, a grey zone exists between 25% and 70%. Below 25% in almost all families a specific gene defect will be found with a much lower incidence of gene defects between 25% and 70% where the occurrence of gene defects levels off. Sadler proposed to consider patients with confirmed VWF levels within this grey zone as having a risk for excessive bleedings in risk situations (and perhaps some protection from thromboembolic events) without establishing a firm diagnosis of mild VWD type 1. He compared the situation of this population with that of a population having a mild increase of cholesterol.

To be on the safe side, FVIII coagulation activity (FVIII:C), VWF:Ag, VWF:RCo (and VWF:CB), and the RIPA test, together with the ratios FVIII:C/VWF:Ag and VWF:RCo/VWF:Ag (VWF:CB/VWF:Ag), should be determined whenever the clinical history is compatible with a disorder of primary hemostasis. For the proper diagnosis of patients with a very low VWF concentration, the measurement of platelet VWF concentration is the best way to distinguish between type 3, severe type 1, and homozygous type 2.[38]

Because of the shorter residence time of VWF in patients with blood group 0,[39] a desmopressin challenge test may distinguish between patients with impaired production (gene defect) or accelerated degradation (extragenic defect). To possibly overcome the problems in diagnosis of VWD type 1, three studies (two in Europe and one in Canada) addressed the identification of precise laboratory and clinical markers for type 1 VWD and understanding of the molecular basis for type 1 VWD in relation to the involvement of the *VWF* gene. The results will hopefully clarify most of the pending issues.

Qualitative Deficiency of von Willebrand Factor

Whenever the above-mentioned battery of tests gives evidence of a qualitative abnormality (such as a lower than normal ratio between a functional test and the VWF concentration), multimer analysis, VWF:FVIIIB, and the RIPA test will allocate the different subtypes. Subtyping of VWD 2 is the most difficult aspect in the classification of VWD due to the enormous heterogeneity of the functional and structural defects. Among them, mere functional defects, such as the deficient FVIII binding in VWD type 2N, are easy to distinguish and qualify this phenotype as its own subtype. Other subtypes, sharing only some common features, are classified together as VWD 2A, although the molecular mechanisms and even the inheritance are rather heterogeneous.

Loss of function because of lack of large multimers, VWD 2A includes all patients displaying a lack or decrease of HMWM paralleled by a decrease of platelet-dependent functions. For those subtypes of type 2A with a clear phenotype–

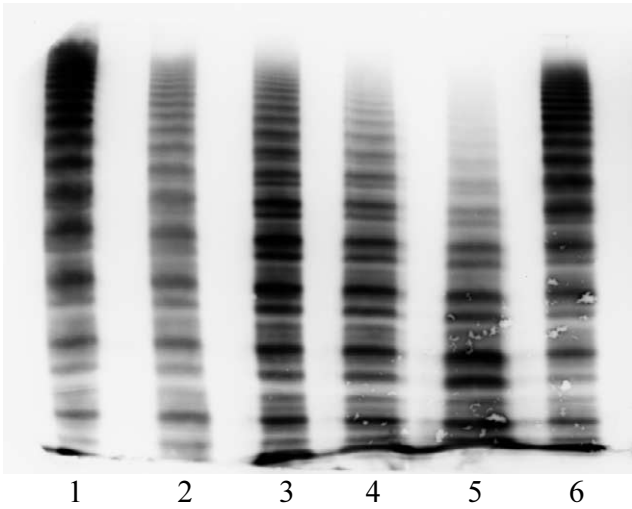

Figure 5 Well-characterized inherited variants (lanes 4 and 5) compared with acquired abnormalities of the VWF molecule (lanes 2 and 3) in a discontinuous medium resolution gel (1.6% LGT-agarose). The dye front is at the bottom of the gel. Lane 1, normal pattern, pool of 30; lane 2, aortic stenosis (enhanced shear stress); lane 3, thrombocythemia (expanded receptors); lane 4, type 2B (gain of function); lane 5, type 2A, group II (enhanced susceptibility to VWF-CP). The acquired abnormalities cannot readily be distinguished from inherited variants; rather the rate of interaction between the VWF, its receptors and the VWF-CP is reflected by the multimer pattern. Note the progressive increase of proteolytic subbands from lane 1 to lane 5.

genotype relation, we will still use the classification from 1987[35] in parentheses.

Lack of Large Multimers Because of Defective Dimerization (subtype IID)

Besides the lack of the large multimers, a missing triplet structure and a minor band located between the oligomers is characteristic.[40]

Lack of Large Multimers Because of Defects in the Prosequence (subtypes IIC and IIC Miami)

A hallmark of these multimerization defects (IIC) is the increase of the protomer (Figs 3 and 4). Furthermore, the large multimers are severely decreased and no proteolytic bands are visible; rather a fine smear can sometimes be detected around the central bands.[41] The Miami subtype shows a much higher VWF concentration in the upper normal or increased range; thus it is at least five times higher than in patients with the classical IIC pattern. In addition, some very faint proteolytic bands are detectable.[42]

Defects in the D3 Domain With a Lack of Large Multimers

The phenotypic pattern of patients with defects in this region is that of the 2A subtypes IIE and IIC Miami.[43,42] In addition to a decrease or absence of HMWM, the multimer patterns of the subtype IIE show an aberrant sub-banding structure with a lack of the outer bands but with pronounced inner bands of the "triplet" (quintuplet is the correct description of the sub-banding pattern, but triplet is the more common term; Figs 3, 4, and 6).[13] The characteristic structure is caused by a reduced proteolytic cleavage.[43] Although rarely described in the literature after the first description by Zimmerman et al,[43] in our laboratory the IIE phenotype is rather common and makes up one third of patients with VWD type 2A.

Type 2A With the "Classical" IIA Pattern

The hallmark of this type is a more or less severe loss of the large and in many patients intermediate multimers together with an increase of the outer flanking sub-bands (Figs 3 and 5).

Loss of Large Multimers and a Smeary Structure of the VWF Multimers

A relatively common variant of type 2A shows a relative loss of the large multimers with sometimes faint supranormal multimers. The triplet structure in most patients shows a decrease or even absence of sub-bands and it is overlaid with amorphous material, giving the electrophoresis lane a smeary appearance.

Relative Loss of the Large Multimers With a Preserved Triplet Structure (subtype IB)

The IB phenotype (Fig 3) with a relative loss of the largest multimers is represented by about 15% of our 2A patients.

Qualitative Variants With Increased Affinity to Platelet GpIb: VWD Type 2B

Due to higher affinity of the VWF to GpIb, the large multimers are bound and proteolyzed by ADAMTS-13 (Figs 3 and 5). The paradox result of the gain of function is a loss of the large multimers and bleeding diathesis.[19] In some of these patients, however, the enhanced affinity of VWF to GpIb is not correlated with loss of HMWM and a normal triplet structure, for example, in VWD 2B New York/Malmö.[44,45]

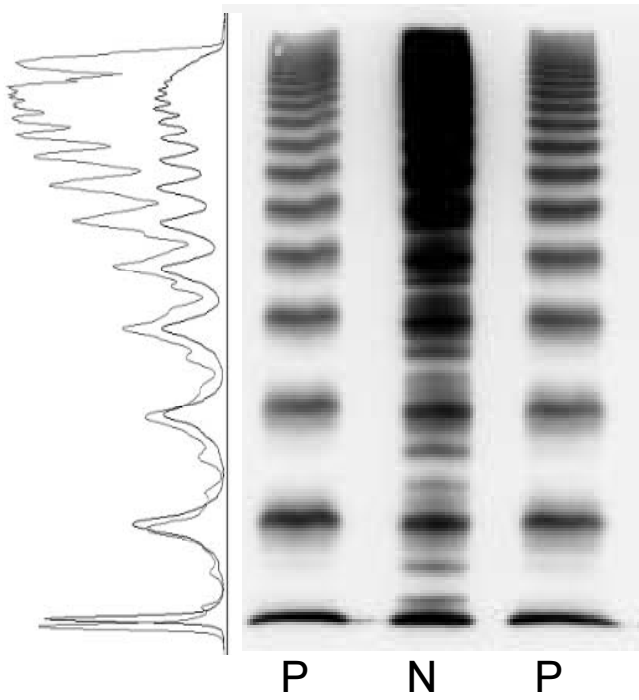

Figure 6 VWF multimers from two family members of a kindred with VWD type 2A (subtype IIE, P) compared to normal plasma (pool of 30, N). Note the severe lack of sub-bands in the patient samples and the relative decrease of the large multimers. The distribution of multimers is illustrated on the left site by densitometric tracing (light curve, normal; dark curve, patient).

von Willebrand Disease Type 2M

This type, which is inherited in a dominant fashion, includes patients with decreased VWF platelet-dependent functional parameters in the presence of HMWM disregarding further issues of differentiation like an aberrant structure of individual multimers or the presence of ultralarge HMWM (supranormal multimers) as seen in the subtype VWD type 2M Vicenza. The Vicenza type seems to be much more abundant than previously thought.[46] However, the most prevalent variant of type 2M (92% of all 2M patients and 15% of type 2 patients) shows the presence of all multimers, and in many cases greater than normal (supranormal) multimers. The triplet structure in most patients shows a decrease or even absence of sub-bands and it is overlaid with amorphous material, giving the electrophoresis lane a smeary appearance (Fig 7).

von Willebrand Disease Type 2N

Whenever FVIII:C is disproportionally low, the VWF:FVIIIB is the essential test for the diagnosis of type 2N patients. This type comprises patients with defects in the

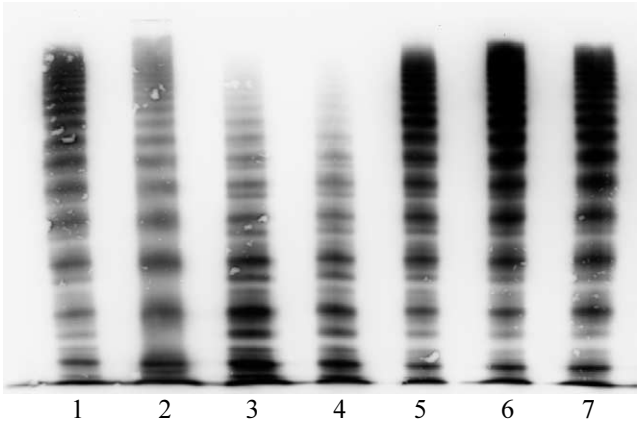

Figure 7 VWF multimers from patients with no qualitative abnormalities (lanes 1, 5-7), a type 2A pattern (lanes 3 and 4) and a patient with type 2M (subtype with a smeary pattern, lane 2) compared to normal plasma (pool of 30, lane 5). Note the blurred appearance of individual multimers without a distinct triplet structure and the presence of supranormal multimers of high molecular weight in lane 2.

FVIII binding region of VWF.[23,25,27,28,47] Accordingly, the phenotype may either mimic hemophilia A exactly or can be combined with decreased VWF:Ag.[27,47] As patients with an additional aberrant multimeric pattern are not uncommon, analysis of the multimeric structure should be included.[28,48-50]

Molecular Basis of von Willebrand Disease

The considerable heterogeneity of VWD correlates with the wide spectrum of underlying mutations. Published mutations, either in journals or in abstract form, are listed in a VWD mutation database maintained at the University of Sheffield, UK. (http://www.ragtimedesign.com/vwf/mutation/).

VWF Quantitative Deficiency

VWD 1 and VWD 3 are defined as pure quantitative deficiencies of VWF with relative deficiency in type 1 and absolute deficiency in type 3. Although VWD 1 is considered the most common type of VWD, only few candidate mutations have been reported. This may change in the near future when the results of two large studies on VWD 1, the European study "Molecular and clinical markers for diagnosis and management of type 1 von Willebrand disease" (MCMDM-VWD1) and the "Canadian VWD type 1 study," will be available.

The mutation spectrum in VWD 3 is very heterogeneous (Fig 8). In most populations there is no mutation with a higher frequency, except a single nucleotide deletion (2435delC) identified on VWD 3 chromosomes with a frequency of 50% in the Swedish population,[51] of up to 20 % in the German population[52] (unpublished data), and even 75% in patients from Poland,[53] whereas it is absent from most other populations.

According to the severe deficiency of VWF in VWD 3 the mutation spectrum comprises mutations predicting

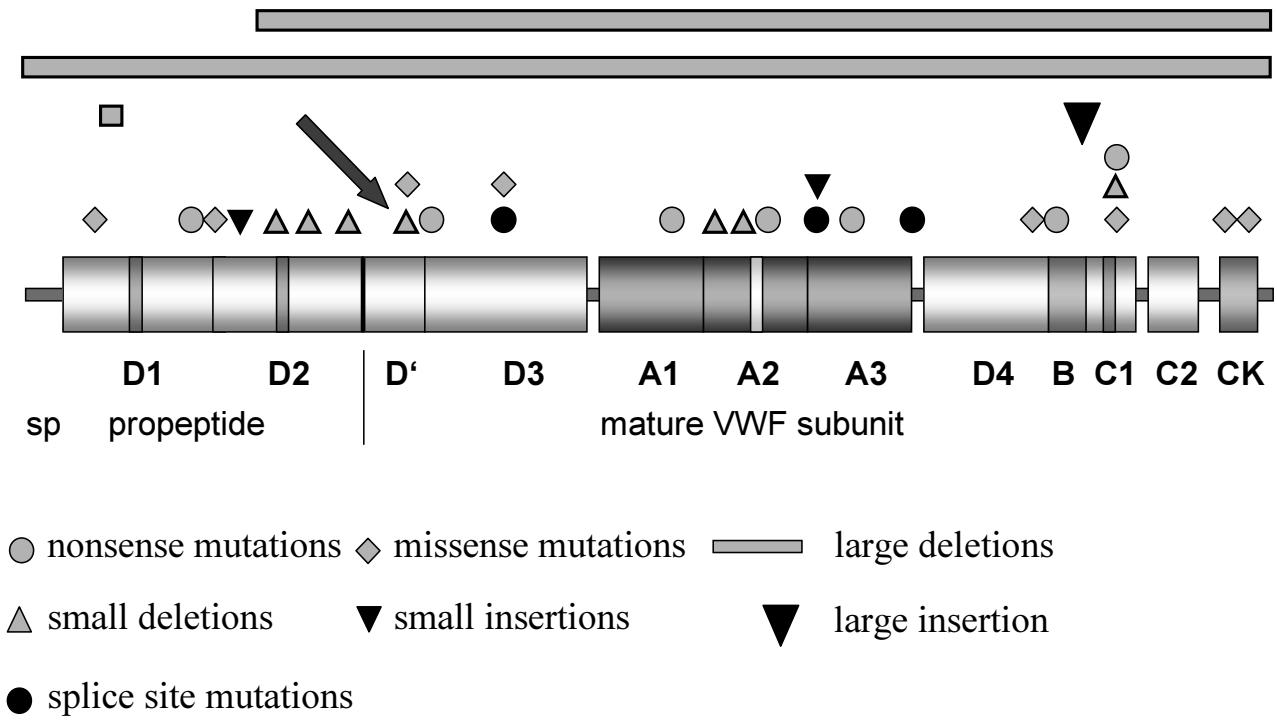

Figure 8 Mutation spectrum of VWD type 3 patients in Germany. Arrow, most common VWD type 3 mutation 2435delC (Germany 20%, Sweden 50%, Poland 75% of VWD type 3 chromosomes).

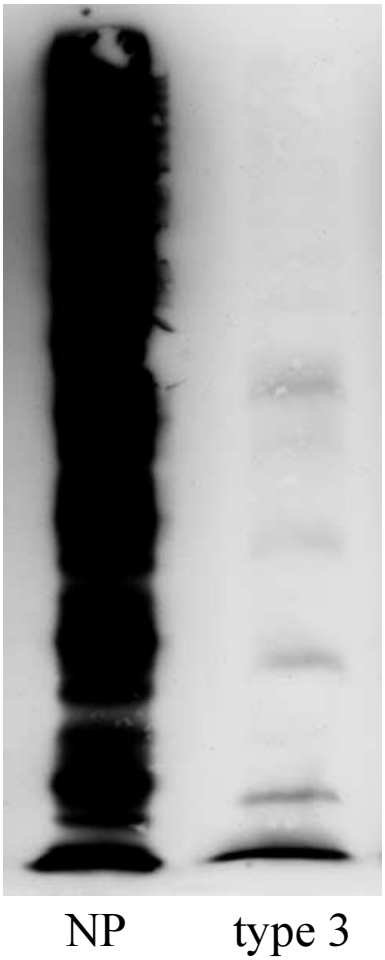

Figure 9 Multimers of a patient with VWD type 3 due to a missense mutation compared to normal plasma. Small multimers can be visualized by extended measuring of luminescence by means of a photo imager and electronic enhancement.

absent synthesis (large deletions) and truncated protein or no protein (nonsense mutations, small insertions/deletions, and splicing mutations). However, a considerable number of missense mutations have also been identified (http://www.ragtimedesign.com/vwf/mutation/). Most affect dimerization and multimerization and are located in the VWF prosequence (D1 and D2 domains), at the multimerization site (D3 domain), and at the dimerization site (CK domain). In patients with missense mutations small quantities of VWF may still be detected with sensitive methods. In most cases, however, only the small oligomers (dimers, tetramers, and hexamers) are present in the multimer pattern (Fig 9). Clinically, such patients cannot be distinguished from patients with nonsense mutations or insertion/deletions. On the molecular level, however, they could also be regarded as severe VWD 2.

VWF Structural and Functional Defects

The considerable posttranslation processing of the VWF pre-pro-peptide offers several occasions for deviations and errors that may compromise the final structure of VWF. Whereas inherited extrinsic reasons for such aberrations are not known yet, many respective different intrinsic deviant properties of VWF were identified. These may affect structure and function of VWF.

Structural Defects

The first structural VWF defect, identified by Ruggeri and Zimmerman,[54] corresponds to a particular VWD phenotype called VWD type IIA. This phenotype is characterized by the lack of HMWM and the corresponding loss of platelet-dependent function. The observed pronounced proteolytic sub-bands of individual VWF oligomers suggested enhanced proteolysis[43] (Figs. 3 and 5), a property that has been confirmed by (1) the identification of a specific proteolytic site in the VWF A2 domain,[55] (2) particular mutations correlating with enhanced proteolysis flanking this site[56] (Fig 10), and (3) recently by identification and functional characterization of the VWF specific protease ADAMTS13.[57,58] Whereas proteolytic sub-bands are the specific result of ADAMTS13 proteolysis, the loss of HMWM can either be due to the action of ADAMTS13 and depending on shear or to defects in dimerization, multimerization, intracellular transport, and secretion, respectively. Specific mutations can be assigned to these diverse mechanisms. In contrast to the enhanced proteolysis in the VWD phenotype IIA due to mutations flanking the VWF proteolytic site in its A2 domain, other phenotypes like IIC, IID, and IIE display decreased or absent ADAMTS13 proteolysis[43] (Figs 3, 4, 6, and 7). It has been shown that mutations causing phenotype IID are located at the carboxy-terminal end of VWF in the CK domain[11,59] (Fig 10). This cysteine-rich domain provides the initial dimerization of the VWF protomer. Heterozygous cysteine mutations in the CK domain prevent the formation of dimers, which results in the transport also of VWF monomers from the endoplasmic reticulum to the Golgi compartment where they can participate in multimer formation, with an odd number of monomers that are usually not present in normal multimers (Fig 3).

Mutations in the D1, D2, or D3 domain may affect multimer formation of dimers by cysteine residues in their D3 domains. Two major pathogenetic mechanisms have been suggested.

(1) Previous studies showed that D1-D2 domain-deleted VWF cannot form multimers, although the multimerization site in the D3 domain was present.[60] This was explained by the possible function of the D1 and D2 domains as disulfide isomerases catalyzing the formation of multimers through intermolecular disulfide bonding between D3 domains. The D1 and D2 domains each contain the disulfide isomerase consensus sequence CGLC. Expression studies with an altered CGLC sequence in the D2 domain showed that this sequence is necessary to allow multimer formation.[9] The multimerization defects caused by mutations in the D1 or D2 domain are inherited recessively and correlate with a particular phenotype, namely, IIC (Fig 10). Although VWF multimers in some unaffected heterozygous carriers are not completely normal, likely half-normal disulfide isomerase activity is sufficient to allow formation of functional VWF multimers.

(2) A cluster of mutations has been identified in the D3 do-

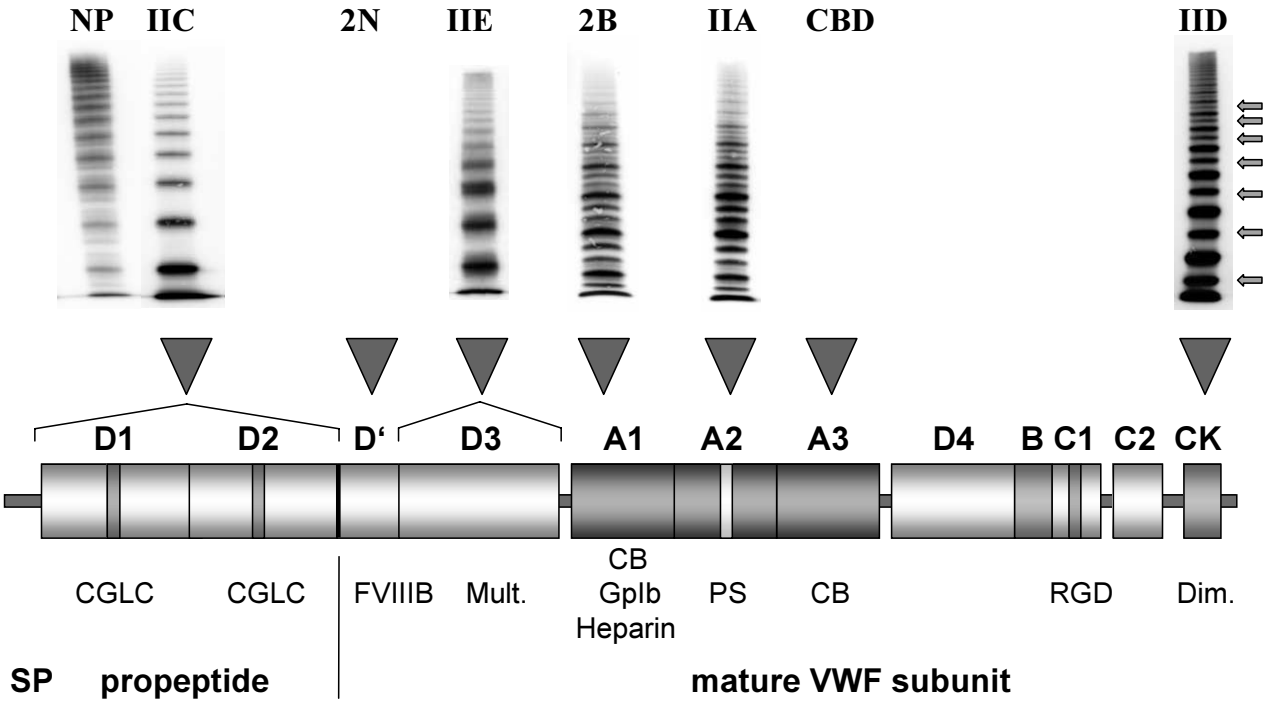

Figure 10 Phenotype–genotype correlation between particular multimer patterns, functional defects with normal multimers (2N, CBD) and location of the underlying defects. Triangles point to mutation clusters in individual domains. Arabic numbers refer to the current classification, roman numbers to the previous classification proposed by Sadler[29] and Ruggeri,[35] respectively. CBD, collagen binding defect.

main.[13,49,50,61-63] Most mutations correlate with the VWD phenotype IIE (Fig 10). Although it is not quite clear which cysteines are directly involved in intermolecular disulfide bonding between VWF D3 domains, the fact that the D3 mutation cluster comprises mainly cystein mutations (Fig 11) suggests that these mutations may directly or indirectly affect multimerization.[13]

There is a distinct multimer pattern seen in a significant number of patients characterized by presence of all multimers, some with and some without a relative decrease of HMWM but a smeary appearance of the individual oligomer that cannot be resolved into a triplet structure (Fig 3). We identified mainly cysteine mutations that were, however, not

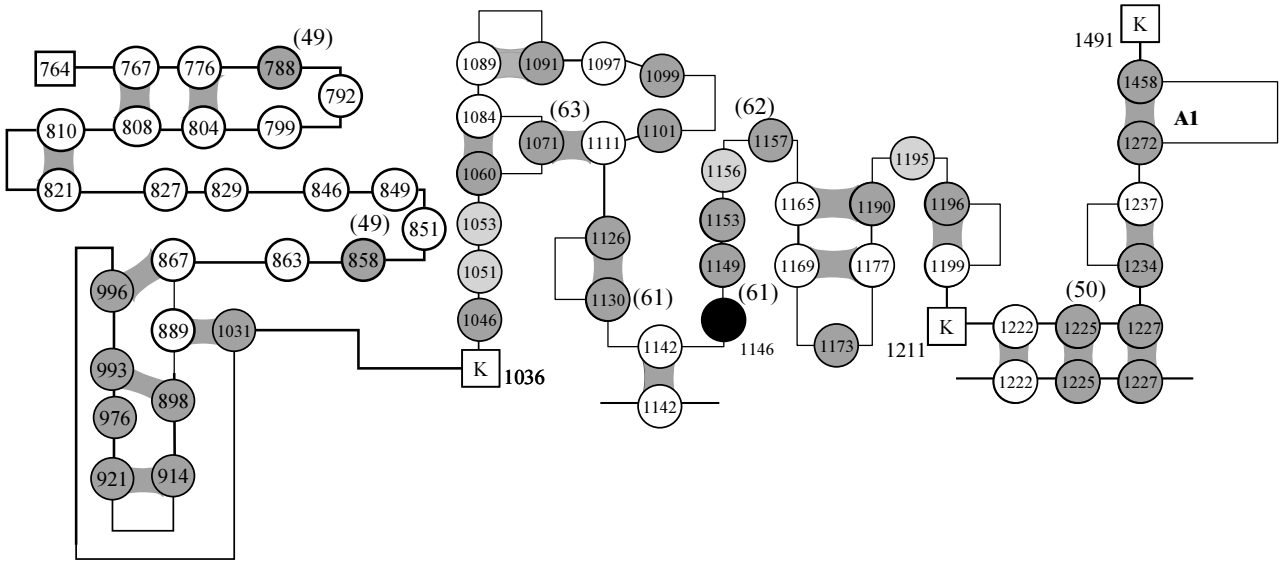

Figure 11 Cluster of mutations in the VWF D3 domain that correlate with a specific subtype of VWD. Dark grey circles: mutation eliminates a cysteine, black circle: mutation creates a cysteine, light grey circles: other mutations, unfilled circles: no mutation identified to date, in brackets: references for mutations identified by others. Cysteine residues and proposed disulfide bonds in the amino-terminal of mature VWF are according to Marti et al[10] and Dong et al.[64]

confined to particular domains (unpublished data). The multimer pattern of this phenotype suggests an unstable VWF molecule.

In addition to mutations residing in the A2 domain causing enhanced proteolysis (so-called group 2 mutations), other mutations in the same domain seem to affect intracellular processing and/or secretion of large multimers (group 1 mutations).

Functional Defects

VWD type 2B. Generally, the above-mentioned structural defects also cause VWF functional deficiency since particular functions like binding to GpIb or to collagen depend on the intact structure of HMWM. In contrast, in VWD type 2B a functional defect—the enhanced interaction of the VWF A1 domain with platelet GpIb—causes consumption of HMWM, with their subsequent deficiency resulting in a multimer pattern that cannot be distinguished from the structural defect in phenotype IIA with enhanced proteolysis (Fig 3). Mutations causing VWD type 2B cluster in a very small region of the A1 domain between amino acids 1266 and 1341 with two outliers at amino acids 1460 and 1461, respectively (Fig 10), (http://www.ragtimedesign.com/vwf/mutation/). In cases where the RIPA test with low ristocetin concentrations has not been performed to distinguish patients with VWD type 2A from type 2B, it is nevertheless possible to correctly classify patients with VWD 2B by molecular analysis only. A particular type 2B phenotye, the formerly designated type 1 New York/Malmö,[44,45] is caused by the mutation P1266L, located at the amino terminal of the A1 domain. This mutation is generated together with others by gene conversion between the *VWF* gene and pseudogene.[65] In contrast to classical VWD type 2B, multimers in such patients are normal and the bleeding tendency is usually mild.

Additional isolated functional defects exist without any evidence of an aberrant VWF structure. Two examples are isolated VWF-FVIII binding and collagen binding defects, respectively.

VWD type 2N (FVIII binding defects). One of the most interesting functional defects is the deficiency of VWF in binding FVIII. The majority of mutations are located in the D′ domain, harboring the VWF-FVIII binding site, a few others in the D3′ domain (Fig 10). They cause a specific VWD subtype, namely, VWD type Normandy or 2N (VWD 2N).[25-28,48-50] This subtype is inherited in an autosomal recessive manner: homozygosity or compound heterozygosity for VWD 2N mutations or compound heterozygosity for a VWD2N mutation and a null allele cause VWD 2N, a condition that can hardly be differentiated from sporadic hemophilia A without the appropriate tests. The severity of the functional defect depends on the particular mutation. A specific mutation (R854Q) causes only mild FVIII deficiency due to residual VWF-FVIII binding activity, whereas mutation E787K may cause severe pseudohemophilia with FVIII:C down to 1 IU/dL.[27] Specific mutations may also cause structural defects of VWF multimers,[28,48-50] but generally multimer structure is normal in patients with VWD 2N. Several cases of VWF-FVIII binding defects combined with a null allele on the other chromosome (small deletions or nonsense mutations) have been reported.[27,28] VWF:Ag may be below or in the low normal range, in contrast to homozygosity or compound heterozygosity for VWF-FVIII binding defects only, where FVIII deficiency is the only deviant screening parameter.

VWD type 2CB (collagen binding defects). Recently, a mutation in the A3 domain (S1731T) that interferes with binding to collagens type I and type III has been described and its causative role confirmed by expression studies.[30] We have detected three different heterozygous mutations (Q1734H, I1741T, and Q1762R) in the same region of unrelated patients with defective VWF:CB.[31] The causative role of these mutations could be confirmed by expression in 293 EBNA cells and subsequent analysis of binding curves for VWF:Ag, VWF:CB, and VWF:RCo and evaluation of the VWF multimers. All four hitherto described naturally occurring mutations are in close proximity to three artificially introduced mutations that interfere with binding to collagen type III in vitro[66] (Fig 10). However, our patients were either asymptomatic (an unaffected mother of a patient with VWD 3 bearing a stop codon and the VWF:CB mutation), had other reasons for bleeding (aplastic anemia), or were investigated due to familial thrombophilia. Thus the evident paucity of VWD patients with an isolated collagen binding defect and the detection of such defects in obviously unaffected probands challenges the hypothesis of the biologic significance of collagen binding to the A3 domain.

Functional defects of GpIb binding. Several mutations have been identified in the VWF A1 domain that decrease GpIb-dependent function of VWF.[67-69] Some of these mutations apparently do not alter VWF multimer structure and are accordingly designated as type 2M. However, in other cases it is not clear if the "normal" multimer structure is possibly due to technical problems with multimer analysis. A relative decrease of HMWM in some of these patients (Fig 12, Budde and Schneppenheim, unpublished data) may be responsible for their decrease in VWF functional activity, rather than an isolated functional deficiency.

Phenotype–Genotype Correlation in the Diagnosis of von Willebrand Disease

Defects Solely Determined by Genotype

These are properties of the recombinant molecule that can be proven in vitro and are never (or very rarely) detectable in the acquired form of von Willebrand syndrome (aVWS). The first abnormality described was the enhanced interaction with GpIb (pathologic RIPA test) in patients with VWD type 2B. The enhanced interaction can be proven in the recombinant molecules. Only one patient with aVWS and an enhanced RIPA test has been described in the literature.[70] Defective VWF:FVIIIB in patients with type 2N can be

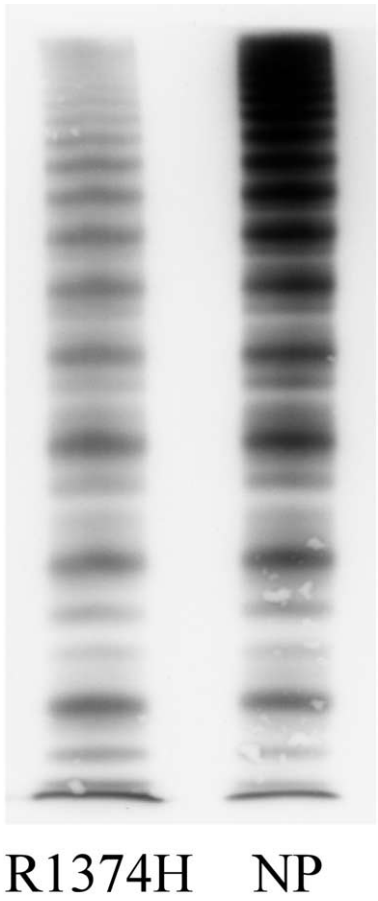

Figure 12 Plasma VWF multimers of a patient with the mutation R1374H compared to normal plasma (NP). Additional patients with this mutation have been classified as type 2M earlier. The relative decrease of large multimers corresponding to reduced VWF:RCo suggests VWD type 2A.

reproduced in recombinant form and has never been shown in aVWS. The same holds true for the isolated defective VWF:CB in rare patients with type 2M, and the odd numbered multimers in VWD type 2A (IID). In patients with type 2A and group 1 mutations, the enhanced outer flanking sub-bands speak clearly in favor of an additional increased susceptibility to proteolysis because a gain of function can be ruled out.

Defects Determined by Both Genotype and the Fate of the Molecule in the Circulation

Wild-type VWF is secreted from the cells in multimeric form with very large (supranormal) multimers and without any evidence of proteolytic processing. After incubation with its physiologic proteolytic enzyme (ADAMTS13), all multimers are degraded in a stepwise process. Limited proteolysis with different amounts of ADAMTS13 and a fixed time (Fig 13) first generates smear around the bands without a visible loss of the multimers; thereafter bands close to the central bands appear, and finally the main outer flanking bands of the triplets arise. During the generation of these bands, a gradual loss of the large multimers is clearly visible with the end product of completely cleaved VWF. These in vitro–generated multimer and triplet structures remarkably resemble those found in patients. The less functional the patient's VWF, the fewer sub-bands are generated (Fig 4), while any gain of function generates enhanced outer flanking sub-bands (Fig 5). These considerations are corroborated by the fact that VWF needs activation and binding to its receptor GpIb to be processed by ADAMTS13.[71,72]

Keeping these considerations in mind, one can construct a hierarchy of function (Figs 4 and 5) from no function (no sub-bands), minimal function (smear just around the central bands), decreased function (only inner to more inner than outer sub-bands), normal function (normal triplet structure with hardly visible inner sub-bands), and a gain of function (enhanced outer flanking sub-bands). However, although in-

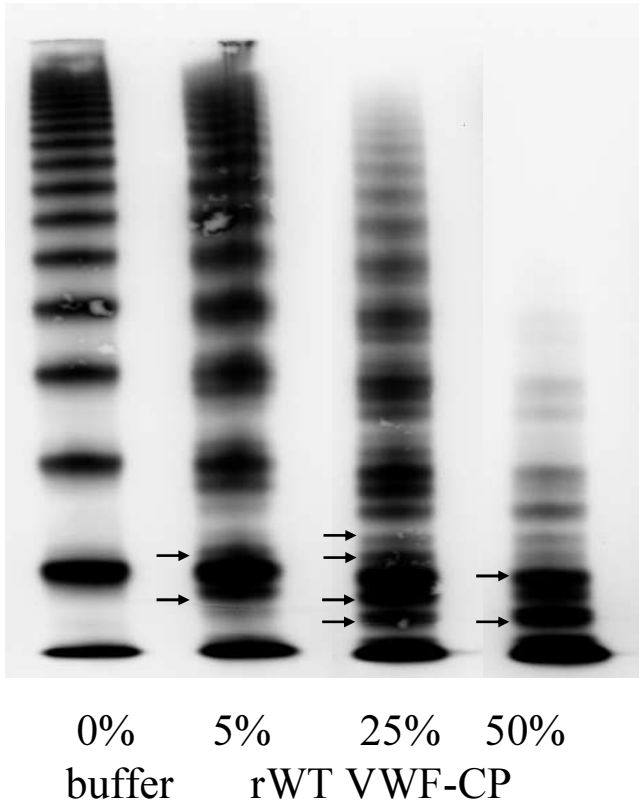

Figure 13 Progressive cleavage of supranormal multimers and generation of proteolytic sub-bands after incubation of recombinant wild-type (rWT) VWF and rWT VWF-CP in defined dilutions for 5 hours under static conditions. Together with the loss of large multimers, proteolytic bands appear in a defined and reproducible fashion. With a low (5%) concentration of VWF-CP, only the inner sub-bands of the "triplet" appear (arrows), followed by the outer flanking bands at higher concentrations. At the end of this process the inner sub-bands are used up and fastest sub-band is the prominent band. These sub-bands are at the same positions as those detected in plasma samples. Not only the generation of sub-bands, but also the well-known proteolytic cleavage of the large multimers can be shown in medium resolution gels (1.6% LGT-agarose).

Table 2 Pathogenic Mechanisms That Operate in Different Disorders

Specific autoantibodies or nonspecific autoantibodies that form circulating immune complexes and enhance the clearance of VWF
Lymphoproliferative disorders
Neoplastic diseases
Immunologic disorders
Adsorption of VWF onto malignant cell clones or other cell surfaces
Lymphoproliferative disorders
Neoplastic diseases
Myeloproliferative disorders
Enhanced shear stress
Increased proteolytic degradation of VWF
Specific
Myeloproliferative disorders
Enhanced shear stress
Uremia
Ciprofloxacin
Nonspecific (plasmin)
Primary hyperfibrinolysis
Secondary hyperfibrinolysis
Lysis therapy
Enhanced shear stress
Congenital cardiac defects
Aortic stenosis
Endocarditis
Malformation of vessels (M. Osler, Kasabach-Merritt syndrome)
Severe atherosclerosis
β-thalassemia
Decreased synthesis
Hypothyroidism
Unknown
Valproic acid
Virus disease
Liver transplantation

herited variants are the prototypes of these dysfunctional VWF molecules, for each inherited variant we can find an aVWS with remarkably similar phenotypic features (Figs 4 and 5) and these multimeric abnormalities can be easily deduced from the different pathogenetic mechanisms operating in disorders associated with aVWS (Table 2). Thus, aberrant triplet structures are not directly related to the genotype but are the end product of the abnormal VWF molecule (genetically determined) and its interplay with the specific receptors and the regulating enzyme(s). Prototypic inherited and acquired forms of VWD are listed in Table 3. Clearly, a normal triplet structure proves that the VWF and the actors in its function are working to a normal extent and that any abnormal triplet structure is the product of a malfunction in one of the steps (Figs 14 and 15). Thus a prerequisite of diagnosing VWD type 1 is to prove that the multimers show a normal triplet structure besides the presence of all multimers and any abnormality of this structure challenges the diagnosis of VWD type 1.

The Problem of Diagnosing VWD Types 1 and 2M

The problem in diagnosing VWD 2M is similar to the situation in VWD 1, since the quality of the multimer analysis leaves some range of interpretation. Mutations in the A1 domain in previously misdiagnosed or misinterpreted VWD type 1 (or type 2M) on reappraisal were found to correlate with a relative decrease of HMWM, suggesting that the correct classification should be VWD 2A.[73] Careful re-evaluation of patients with VWD type 2M and patients with VWD type 1 who present with a discrepancy between VWF:Ag and either VWF:RCo or VWF:CB seems necessary. The only way to define large multimers objectively is quantitative evaluation by densitometry (Fig 16) of the multimer fraction $\geq$10 in a low-resolution gel, create a 95% confidence range, and include only those patients with a normal array of large multimers into the type 2M fraction. In addition to the issue of the definition of the presence or absence of large multimers,

Table 3 Prototypic Inherited and Acquired Forms of von Willebrand Disease With Characteristic Tripet Patterns and Their Pathophysiologic Mechanisms (Figs 6 and 7)

No proteolysis
Severe loss of large multimers due to gene defects: types 2A (subtypes IIC and IID)
No function of ADAMTS-13: congenital Upshaw-Shulman syndrome, acquired thrombotic thrombocytopenic purpura
Severely decreased proteolysis
Severe loss of large multimers due to gene defects: type 2A (subtype IIC Miami)
Severe loss of function without loss of multimers due to gene defects: type 2M (smeary pattern)
Severe loss of large multimers due to auto antibodies or adsorption onto cell surfaces: lymphoproliferative disorders
Less decreased proteolysis
Gradual loss of large multimers due to gene defects: type 2A (subtype IIE)
Less severe loss of function without loss of multimers due to gene defects: type 2M (smeary pattern)
Less severe loss of large multimers due to auto antibodies or adsorption onto cell surfaces: lymphoproliferative disorders
Normal proteolysis
Normals
Genetic forms: VWD type 1, VWD type 2B (subtype New York/Malmö)
Acquired form: hypothyroidism (decreased production of proteins)
Enhanced proteolysis
Gain of function after treatment with desmopressin
Gain of function because of gene defects: VWD type 2B
Enhanced susceptibility to proteolysis: VWD type 2A (group 2 mutations)
Enhanced physiological cleavage due to expanding receptors: thrombocythemia
Activation and enhanced binding due to enhanced shear stress: cardiovascular disorders, severe angiodysplasia

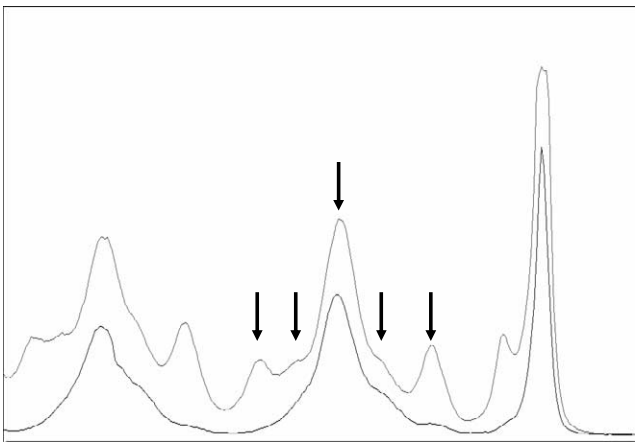

Figure 14 Densitometric evaluation of the quintuplet pattern of the smallest oligomers in a normal plasma (top curve, light) compared to a plasma from a VWD type 2A, subtype IIE patient (bottom curve, dark). Note the almost complete lack of the outer flanking bands (arrows) in the type IIE patient.

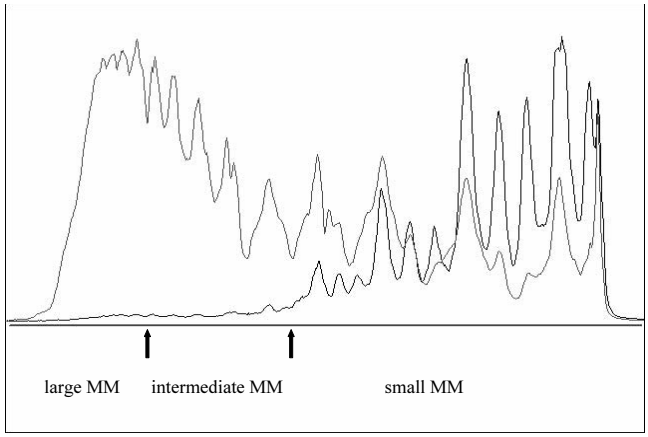

Figure 16 Quantitative evaluation of the distribution of the small (1-5), intermediate (6-10), and large (>10) multimers. A normal distribution (upper curve) bears an equal amount (~33%) of each fraction. The lower curve shows an example of a type 2A plasma with absence of large and intermediate multimers.

careful evaluation of the triplet structure is of utmost importance for the allocation of patients in type 1 and type 2M. Sometimes the aberrant phenotype is unmasked after infusion of desmopressin (unpublished observation). The safest way to confirm the causative role of a candidate mutation is its expression in homozygous and heterozygous states and testing its behavior in the appropriate function studies (see Phenotype–Genotype Relations).

Future Perspectives

Although many aspects of VWF and VWD have been addressed during recent years, the burden of unresolved issues is still high. However, some aspects might be clarified soon.

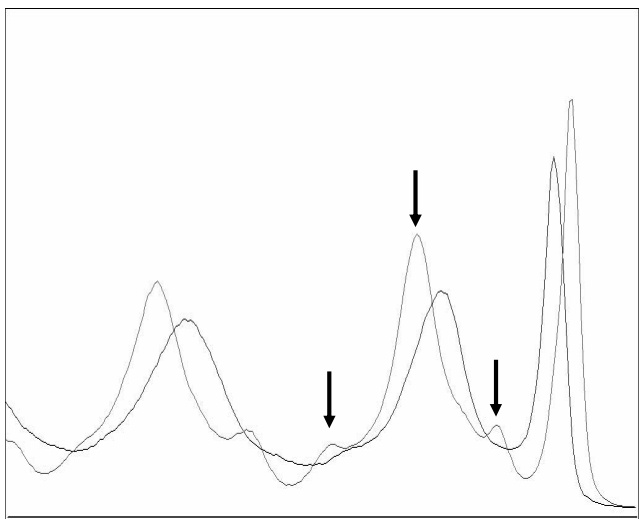

Figure 15 Densitometric evaluation of the quintuplet pattern of the smallest oligomers in a normal plasma (light curve) compared to plasma from a VWD type 2M subtype with a smeary pattern (dark curve). Note the almost complete lack of all sub-bands (arrows) and the faster running central bands in the type 2M patient.

Ongoing studies will help to better characterize VWD type 1. The classification from 1994[29] based on phenotypic data only could be revised and extended towards a classification that combines molecular and phenotypic data in the near future.

We now have convincing data that in many cases, and particularly for patients with type 2M, a correct diagnosis is difficult without multimers of good quality. Thus multimers are not just an instrument for the fine tuning of classification, but should become a first-line test for this purpose as long as no other tests can resolve the issues of classification. However, even with the best available methods, acquired and inherited forms of VWD are indistinguishable in most cases if diagnosis relies on laboratory tests only. Without the patient's medical history, the danger of misdiagnosis is great.

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