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Phenotypic classification of von Willebrand disease

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A B S T R A C T

Von Willebrand disease (VWD) is caused by quantitative and/or qualitative defects of the von Willebrand factor (VWF), a multimeric high molecular weight glycoprotein. Typically it affects the primary hemostatic system, which is reflected by a mucocutaneous bleeding tendency simulating a platelet function defect. VWD is the most common inherited coagulation disorder. The current classification comprises the main types 1, 2 and 3. The qualitative type 2 variants are differentiated into the subtypes 2A, 2B, 2M and 2M. Among these, type 2A is very heterogeneous, consisting of several phenotypically different disease entities with the diagnostic hallmark of a loss of the large multimers. Up to now, the quantitative type 1 variants were considered the most prevalent with frequencies of 70% to 80%, compared to type 2 (15-25%) and type 3 (<5%). Phenotypic characterisation of our patients was done by a battery of tests that included VWF:AG, VWF:CB, VWF:FVIIIB, the RIPA-test, and multimer analysis combined with luminescent visualisation of the electrophoretic bands by means of a sensitive video system. Among 634 patients with inherited VWD, studied during 2003, 51% had VWD type 1 compared to 47% with type 1 and 2% with type 3. Most of the type 2 variants were type 2A (75%), while type 2M (16%), type 2B (6%) and type 2N (3%) were infrequent. Thus, many patients with presumed VWD type 1 and bleeding symptoms have VWD type 2 instead. The diagnosis of VWD type 2A subtypes depends on a standardised multimer analysis as the critical method.

Key words: von Willebrand disease, von Willebrand factor, multimeric structure, classification.

Due to the complexity of the disease, diagnosis of VWD is one of the most challenging of any coagulation disorder. Depending on the functional epitope mutated, only subset 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 speaking 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 the type

Previously VWD variants were classified according to their abnormal multimer pattern[1] and comprise for instance the subtypes IB, I platelet discordant, IIA, IIA-1, IIA-2, IIA-3, IIC, IIE, IIF, IIG, IIH and II-I (Figures 1,2,3). This classification resulted in more than 50 variants and was impractical even for experts. Therefore since 1994 VWD is classified into 3 types.[2] However, because the mechanisms that result in the decrease or lack of HMWM are rather different and the mode of inheritance also varies, subtyping may still be necessary. According to a consensus by the SSC 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.[3] 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 Scientific and Standardiza-

Table 1. Diagnostic procedures for VWD diagnosis 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
F VIII-binding capacity (VWF:FVIIIB)

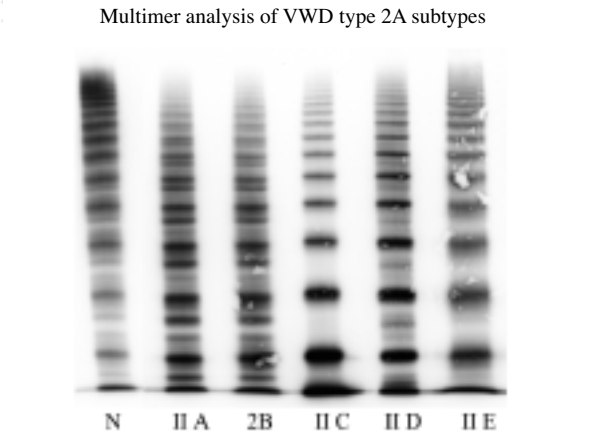

Figure 1. Well characterized inherited variants in a discontinuous medium resolution gel (1.6% LGT-agarose). The dye front is at the bottom of the gel. Lane 1 = normal plasma (pool of 30), lane 2 = type 2A, classical pattern, lane 3 = type 2B, lane 4 = type 2A, subtype IIC, characterized by the pronounced protomer, lane 5 = type 2A, subtype IID, note the less intensive intervening band between 2 major bands, lane 6 = type 2A, subtype IIE with broad central bands, amorphous material instead of a triplet structure and a normal protomer

tion Committee of the International Society on Thrombosis and Haemostasis (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 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.

To be on the safe site, FVIII:C, VWF:Ag, VWF:RCo, (and VWF:CB) and the RIPA test together with the ratios F VIII:C/VWF:Ag, VWF:RCo/VWF:Ag (VWF:CB/ VWF:Ag) should be determined, whenever the clinical history is compatible with a disorder of primary hemostasis. To possibly overcome the problems in diagnosis of VWD type 1 three studies, two in Europe and one in Canada addressed the following topics: Identification of precise laboratory and clinical markers for type 1 VWD and an understanding of the molecular basis for type 1VWD in relation to the involvement of the VWF gene. The results from these studies will hopefully help to 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 (e. g. a lower than normal ratio between a functional test and the VWF concentration), multimeric analysis, VWF:FVIIIB and the RIPA test will allow the allocation to the different subtypes. Subtyping of VWD 2 is the most difficult aspect in the classification of VWD. This is emphasized by the enormous heterogeneity of the functional and structural defects.

Loss of function because a lack of large multimers, von Willebrand disease type 2A

VWD 2A includes all patients displaying a lack or decrease of HMW multimers paralleled by a decrease of platelet dependent functions. For those subtypes of type 2A with a clear phenotype – genotype relation we will still use the classification from 1997[1] in parentheses.

Lack of large multimers because of defective dimerization (subtype IID)

Characteristic is besides the lack of the large multimers, a missing triplet structure and a minor band located between the oligomers.[4]

Lack of large multimers because of defects in the prosequence (subtype IIC)

A hallmark of these multimerization defects (IIC) is the increase of the protomer (Figures 1,4). Furthermore, the large multimers are severely decreased and no proteolytic bands are visible. Rather sometimes some fine smear can be detected around the central bands.[5]

Defects in the D3-domain with a lack of large multimers

The phenotypic pattern of patients with defects in this

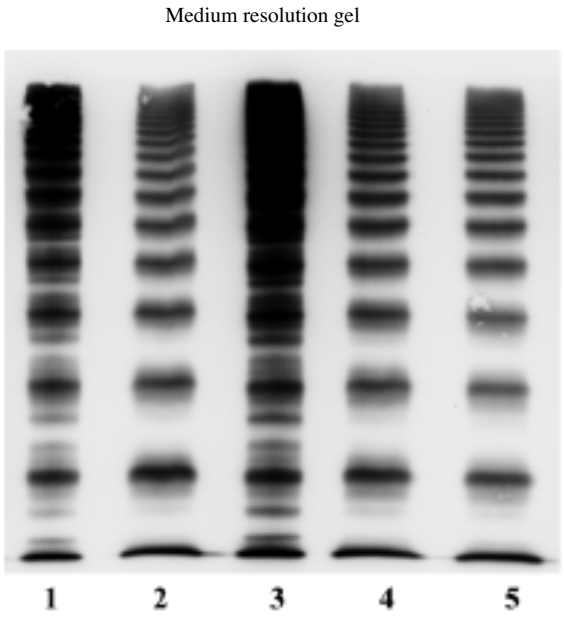

Figure 2. VWF multimers from 3 family members of a kindred with VWD type 2A (subtype IIE, lanes 2,4,5) compared to normal plasma (lanes 1,3). Note the severe lack of subbands in the patient samples and the relative decrease of the large multimers.

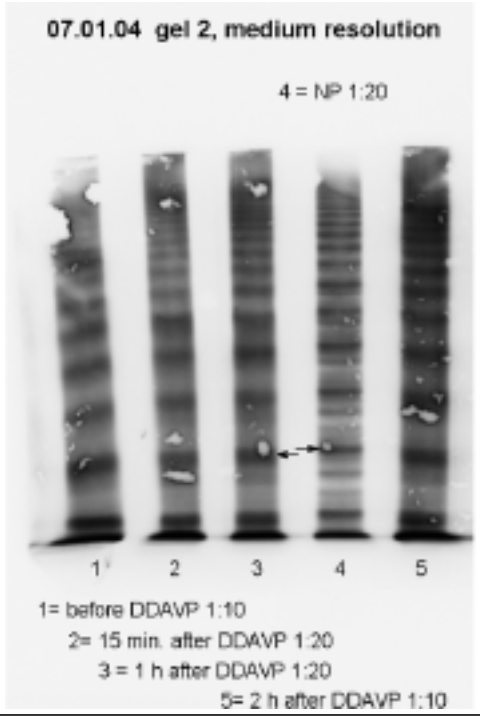

Figure 3. VWF multimers from patient with type 2M (subtype with a smeary pattern, before and after DDAVP, lanes 1-3, 5) compared to normal plasma (pool of 30, lane 4). Note the blurred appearance of individual multimers without a distinct triplet structure and the presence of supranormal multimers of high molecular weight. DDAVP does not change the abnormal pattern.

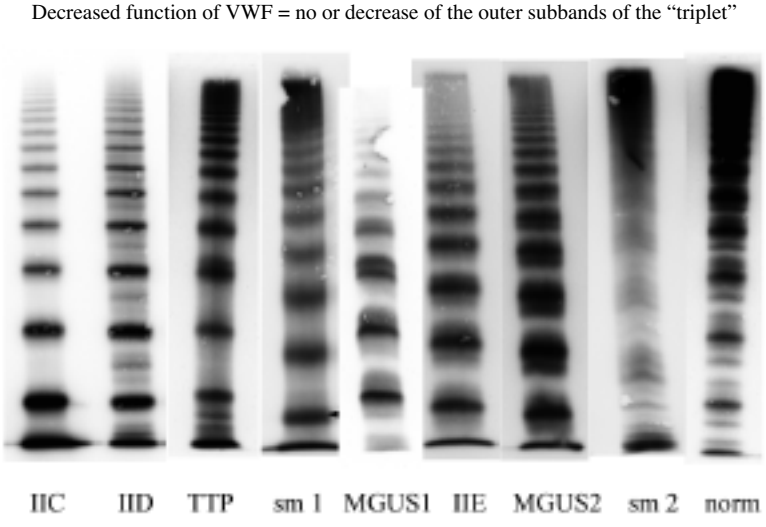

Figure 4: Well characterized inherited variants (IIC, IID, sm1, IIE, sm 2) compared with acquired abnormalities of the VWF molecule (TTP, MGUS1, MGUS2) in a discontinuous medium resolution gel (1.6% LGT-agarose). The dye front is at the bottom of the gel. Lane 1 = dimerization defect (subtype IID), lane 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 = decrease of large multimers in a sample from a patient with a lymphoproliferative disorder, lane 8 = type 2M with a smeary multimer pattern, lane 9 = a normal plasma (pool of 30). The acquired abnormalities cannot readily be distinguished from inherited 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 subbands from no visible subbands (lanes 1-4), severe decreased subbands (lanes 5 and 6), clearly visible, but still abnormal subbands (lane 7 and 8) to a normal pattern (lane 9).

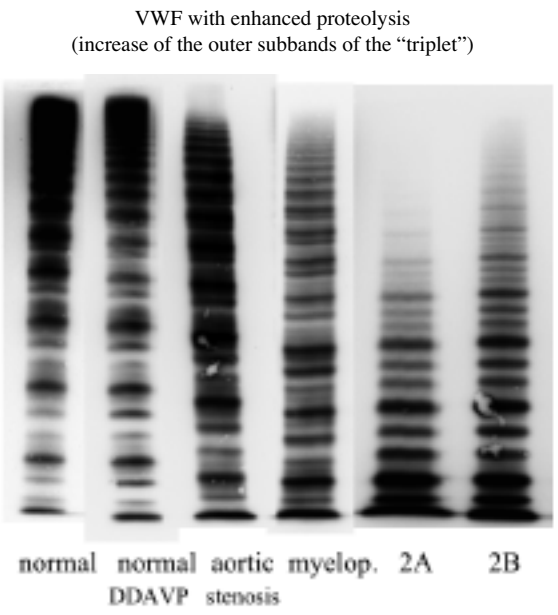

Figure 5. Well characterized inherited variants (lanes 5 and 6) compared with acquired abnormalities of the VWF molecule (lanes 2 - 3) in a discontinuous medium resolution gel (1.6% LGT-agarose). The dye front is at the bottom of the gel. Lane 1 = normal pattern, pool of 30, lane 2 = gain of function after DDAVP, lane 3 = aortic stenosis (enhanced shear stress), lane 4 = thrombocythemia (expanded receptors), lane 5 = type 2B (gain of function), lane 6 = type 2A, group II (enhanced susceptibility to VWF-CP). The acquired abnormalities cannot readily be distinguished from inherited 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 6.

region is that of the 2A subtype IIE.[6] In addition to a decrease or absence of HMWM, the multimer patterns of the subtype IIE show an aberrant subbanding structure with a lack of the outer bands but with pronounced inner bands of the triplet (Figures 1,2,4).[7] The characteristic structure is caused by a reduced proteolytic cleavage that has been described already with the first description of this subtype by Zimmerman et al. (6). Although rarely described in the literature after the first description, in our laboratory the IIE phenotype is rather common and makes up one third among 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 subbands (Figures 1,5).

Qualitative variants with increased affinity to platelet glycoprotein Ib. VWD type 2B

Due to higher affinity of the VWF to GP Ib, the large multimers are bound and proteolysed by ADAMTS-13. The paradox result of the gain of function is a loss of the large multimers and bleeding diathesis (Figures 1,5).[8]

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 ultra large HMWM (supranormal multimers) as seen in the subtype VWD type 2M Vicenza. The Vicenza type seems to be much more abundant than previously thought.[9] However, the most prevalent variant of Type 2M (92% of all 2M patients and 15% of the 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 subbands and it is overlaid with amorphous material giving the electrophoresis lane a smeary appearance (Figure 3).

VWD type 2N

Whenever FVIII:C is dysproportional low, the VWF:FVIIIB is the essential test for the diagnosis of type 2N patients. This type comprises patients with defects in the FVIII binding region of VWF.[10-15] Accordingly, the phenotype may either mimic hemophilia A exactly or can be combined with decreased VWF:Ag.[10,11] As patients with an additional aberrant multimeric pattern are not uncommon, analysis of the multimeric structure should be included.[12-15]

Phenotype – genotype correlation in the diagnosis of VWD

Defects solely determined by the genotype

These are properties of the recombinant molecule that can be proven *in vitro* and are never (or very rarely) detectable in acquired form of von Willebrand syndrome (aVWS). The first abnormality described was the enhanced interaction with GP Ib (pathologic RIPA-test) in patients with VWD type 2B. Defective VWF:FVIIIB in patients with type 2N can be 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, the odd numbered multimers in VWD type 2A (IID).

Defects determined by both, the 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.

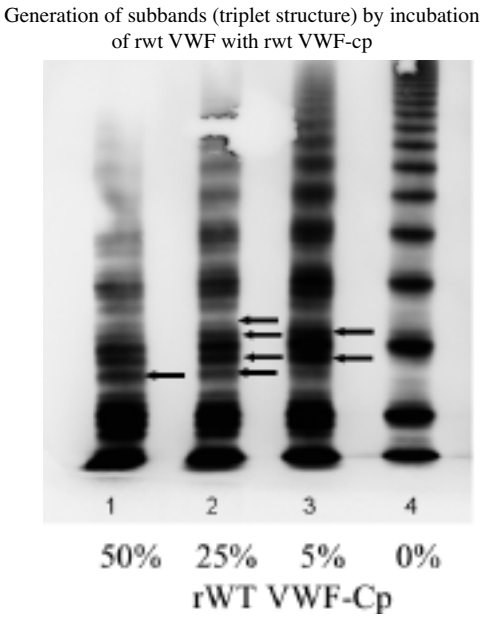

Figure 6. Progressive cleavage of supranormal multimers and generation of proteolytic subbands after incubation of rWT VWF and rWT VWF-CP in defined dilutions for 5 hours under static conditions. Together with the loss of large multimers proteolytic bands appear in a defined and reproducible fashion. With a low (5%) concentration of VWF-CP only the inner subbands of the "triplet" appear (arrows), followed be the outer flanking bands at higher concentrations. At the end of this process the inner subbands are used up and fastest subband is the prominent band. These subbands are at the same positions as those detected in plasma samples. Not only the generation of subbands, but also the well known proteolytic cleavage of the large multimers can be shown in medium resolution gels (1.6% LGT-agarose).

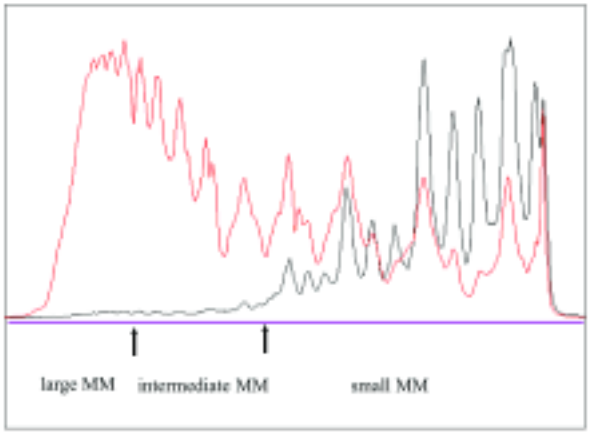

Figure 7. Quantitative evaluation of the distribution of the small (1-5), intermediate (6-10) and large (>10) multimers. A normal distribution (upper curve) bears an equal amount (~33%) of each fraction. The lower curve shows an example of a type 2A plasma with absence of large and intermediate multimers.

After incubation with its physiologic proteolytic enzyme (ADAMTS - 13) all multimers are degraded in a stepwise process. Limited proteolysis with different amounts of ADAMTS - 13 and a fixed time (Figure 6) generates first bands close to the central bands appear and the main outer flanking bands of the triplets appear later. 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 patients VWF is, the less subbands are generated (Figure 4) while any gain of function generates enhanced outer flanking subbands (Figure 5). These considerations are corroborated by the fact that VWF needs activation and binding to its receptor GP Ib to be processed by ADAMTS -13.[16,17]

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

However, although inherited variants are the prototypes of these dysfunctional VWF molecules, for each inherited variant we can find an acquired von Willebrand syndrome (aVWS) with remarkable similar phenotypic features (Figures 4,5) and these multimeric abnormalities can easily deducted from the different pathogenetic mechanisms operating in disorders that are associated with aVWS. 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 given in Table 2. Thus one can clearly state that 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. 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 a type 1.

The problem of diagnosing VWD type 1 and type 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. The only

Table 2. Prototypic inherited and acquired forms of VWD with characteristical tripet patterns and their pathophysiological mechanism (Figures 7, 8).

No proteolysis
Severe loss of large multimers due to gene defects:
Types 2A (IIC and IID)
No function of ADAMTS-13: congenital = Upshaw-Shulman syndrome, acquired = TTP

Severely decreased proteolysis:
Severe loss function without loss of multimers due to gene defects: Type 2M (sm)
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 (IIE)
Less severe loss function without loss of multimers due to gene defects: Type 2M (sm)
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
Acquired form: hypothyroidism (decreased production of proteins)

Enhanced proteolysis:
Gain of function after treatment with DDAVP
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

way to objectively define large multimers is quantitative evaluation by densitometry (Figure 7) of the multimer fraction ≥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. Besides the issue of the definition of the presence or absence of large multimers, we could show that the careful evaluation of the triplet structure is of utmost importance for the allocation of patients in type 1 and type 2M (Figure 8). The safest way to confirm the causative role of a candidate mutation is its expression in homo- and heterozygous states and testing its behavior in the appropriate function studies (see phenotype-genotype relations).

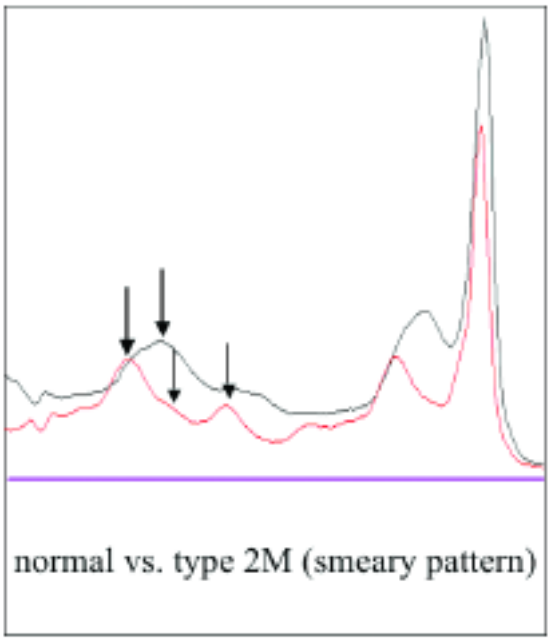

Figure 8. Densitometric evaluation of the quintuplet pattern of the smallest oligomers in a normal plasma (red) compared to a plasma from a VWD type 2M, subtype with a smeary pattern in black). Note the almost complete lack of all subbands (small arrows) and the faster running central bands (large arrows).

Concluding remarks and 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 in the near future. The above mentioned type 1 projects will help to better characterize VWD type 1. The classification from 1994[2] that relies on phenotypic data only, will possibly 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 most obvious for patients with type 2M a correct diagnosis is difficult without multimers of good quality.

Thus multimers are no instrument for the fine tuning of classification, but should become a first line test 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. Thus without the medical history of the patients the danger of misdiagnosis is great.

References

1. Ruggeri ZM. Classification of von Willebrand disease. In: Thrombosis and Haemostasis 1987. Ed. Verstraete M, Vermylen J, Lijnen R and Arnout J. Leuven: Leuven University Press. p. 419 - 45.
2. Sadler EJ. A revised classification of von Willebrand disease. Thromb Haemost 1994;71:520-5.
3. Rodeghiero F, Castaman G. Congenital von Willebrand disease type I: definition, phenotypes, clinical and laboratory assesment. Bailliere´s Clin Haematol 2001; 14:321-35.
4. Kinoshita S, Harrison J, Lazerson J, Abildgaard CF. A new variant of dominant type II von Willebrand's disease with aberrant multimeric pattern of factor VIII-related antigen (type IID). Blood 1984; 63:1369-71.
5. Ruggeri ZM, Nilsson IM, Lombardi R, Holmberg L, Zimmerman TS. Aberrant multimeric structure of von Willebrand factor in a new variant of von Willebrand's disease (type IIC). J Clin Invest 1982; 70: 1124-27.
6. Zimmerman TS, Dent JA, Ruggeri ZM, Nannini LH. Subunit composition of plasma von Willebrand factor. Cleavage is present in normal individuals, increased in IIA and IIB von Willebrand disease, but minimal in variants with aberrant structure of individual oligomers (types IIC, IID and IIE). J Clin Invest 1986; 77: 947 - 51.
7. Schneppenheim R, Obser T, Schneppenheim S et al. Von Willebrand disease type 2A with aberrant structure of individual oligomers is caused by mutations clustering in the von Willebrand factor D3 domain. Blood 2000; 96: 566a (abstr.).
8. Ruggeri ZM, Pareti FI, Mannucci PM, Ciavarella N, Zimmerman TS. Hightened interaction between platelets and factor VIII/von Willebrand factor in a new subtype of von Willebrand's disease. N Engl J Med 1980; 302:1047-51.
9. Zieger B, Budde U, Jessat U et al. New families with von Willebrand disease type 2M (Vicenza). Thromb Res 1987; 87:57-64.
10. Nishino M, Girma JP, Rothschild C, Fressinaud E, Meyer D. New variant of von Willebrand disease with defective binding to factor VIII. Blood 1989; 74: 1591-9.
11. Mazurier C. Von Willebrand disease masquerading as haemophilia A. Thromb Haemost 1992; 67: 391-6.
12. Jorieux S, Fressinaud E, Goudemand J Gaucher C, Meyer D, Mazurier C. Conformational changes in the D´ domain of von Willebrand factor induced by CYS25 and CYS95 mutations lead to factor VIII binding defect and multimeric impairment. Blood 2000; 95: 3139 - 45.
13. Schneppenheim R, Obser T, Lenk H et al. Characterization of a combined defect of FVIII binding and multimerization in a patient with von Willebrand disease type 2N. Thromb Haemost 2004;29:36-41
14. Allen S, Abuzenadah AM, Blagg JL et al. A novel type 2N von Willebrand disease-causing mutation that results in defective factor VIII binding, multimerization, and secretion of von Willebrand factor. Blood 2000;95:2000-7.
15. Jorieux S, Gaucher C, Goudemand J, Mazurier C. A novel mutation in the D3 domain of von Willebrand factor markedly decreases its ability to bind factor VIII and affects its multimerization. Blood 1998;92:4463-70.
16. Moake JL. Thrombotic microangiopathies. N Engl J Med 2002;347:589-600.
17. Nishio K, Anderson PJ, Zheng XL, Sadler JE. Binding of platelet glycoprotein Ib (·) to von Willebrand factor domain A1 stimulates the cleavage of the adjacent domain A2 by ADAMTS13. Proc Natl Acad Sci USA 2004;101:10578-83.