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Research Article

Molecular Etiology and Laboratory Phenotypes of Recessive Von Willebrand Disease 2N Due to Mutations in the D'D3 Factor VIII-Binding Domain of the Von Willebrand Factor Gene: A Critical Appraisal of the Literature and Personal Experiences

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Abstract

Introduction: The FVIII binding site on von Willebrand factor (VWF) is located in the D' (766-864) and D3 (1054-1060) regions of the VWF gene. The cysteine residues in the D' domain form disulfide bridges within the D' trypsin-inhibitor-like (TIL') and E' regions, and these are of critically importance for the binding between TIL'E' and FVIII.

We analyzed the molecular etiology and laboratory phenotype of von Willebrand disease (VWD) 2N patients reported in the literature and added personal experiences from three European VWF VWD Research Centers.

Critical appraisal of the literature and personal experiences: Homozygous R854Q/R854Q and double heterozygous R854Q, E787K, T791M and R816W non-cysteine mutations in the D' domain result in a FVIII binding defect (FVIII:BD) featured by mild to moderate hemophilia A with normal bleeding time and normal VWF functions and multimers consistent with VWD type 1. The FVIII:BD is mild in R854Q (about 30%) and markedly decreased (less than 10%) in E787K, T791M, R816W, D879N and C1060W mutations either homozygous or double heterozygous with a null allele. VWF-FVIII binding defect due FVIII mutations in the C1

and C2 domain of the FVIII gene mimic VWD 2N and show low FVIII:C/VWF ratios below 0.50 with normal secretion and multimerization of VWF after DDAVP. The double heterozygous cysteine/null Y795C/null and cysteine/noncysteine Y795C/R854Q mutations produced VWD 2N with aberrant VWF multimers similar as seen in heterozygous Y795C/WT asymptomatic carriers. The VWF multimers in VWD 2N due to homozygous cysteine or cysteine/null mutations are abnormal. VWF multimers in double heterozygous cystein/non-cysteine C804F/R854Q and C858F/R854Q mutations are corrected to normal by the R854Q allele. Homozygous R760W/R760W (D2 domain) and C788R/C788R (D' domain) induce a recessive VWD type 2 secretion and multimerization defect with a mild FVIII:BD of about 35% without features of VWD 2N, whereas double heterozygous R854Q/R760W mutation in the D'D2 domains produce hybrid phenotype 2N/2A VWD with a smeary pattern of VWF multimers due to a mixture of normal mature VWF and proVWF. Patients double heterozygous R854Q/R763G (Furin cleavage site) produce a hybrid VWD phenotype 2N/2A with a smeary VWF multimeric pattern due to a mixture of normal VWF and pro-VWF protein that is also seen in the heterozygous R763G/WT carriers. The homozygous C1060R/C1060R mutation in the D3 domain, and the double heterozygous D879N/null and C1060R/null mutations are associated with a hybrid phenotype of 2N/2E VWD.

Conclusion: Classical VWD 2N caused by homozygous non-cysteine or double heterozygous non-cysteine/null mutations R854Q and R816W due to a FVIII binding defect (FVIII-BD) in D' Domain of the VWF gene is featured by low FVIII;C, normal VWF levels and normal multimeric structure of VWF. Double heterozygous cysteine/noncystein mutations produce VWD type 2N with normal VWF multimers. The C760C in the D2 domain and the R763G Furin cleavage site mutations combined with the R854Q mutation produce a hybrid 2N/2A VWD phenotype associated with aberrant multimerization due to a mixture of mature VWF and pro VWF. Homozygous C1060R/C1060R and the double heterozygous D879N/null, C1060R/R854Q or C1060R/null mutations in the D3 domain are associated with a hybrid 2N/2E VWD phenotype.

Keywords: Von Willebrand Disease 2N; Laboratory Phenotype; Factor VIII Binding Defect; Von Willebrand Factor Gene and Protein; Personal Experiences

Introduction

FVIII circulates in complex with the multimeric glycoprotein bound to the D' and D3 FVIII binding domain of VWF (Figure 1) that protects FVIII from premature clearance and proteolytic degradation. Factor VIII (FVIII) consists of 2332 amino acids with distinct domain structure: A1-a1-A2-a2-B-A3-C1-C2 (figure 1) [1]. Intracellular processing of the B-domain of FVIII is co-localized with VWF in WPB in blood outgrowth endothelial cells (BOEC) [2]. FVIII in BOEC colocalizes with VWF in Weibel-Palade bodies (WPB) in controls and in VWD 2N, even when FVIII carries mutations in the light chain that are associated with defective binding of VWF to FVIII [5,6]. The mutation of sulfated tyrosine on position Tyr1680 in the a3-domain of FVIII results in a FVIII-VWF binding defect (Figure 1) [3-6]. A second mutation Arg2150His in the C1 domain of FVIII has been recognized as a cause of mild/moderate hemophilia A due to a FVIII-VWF binding defect (figure 1) [8].

The FVIII binding site on von Willebrand factor (VWF) is localized in the D' (766-864) and D3 (1054-1060) regions of the VWF gene (Figure 1). Mazurier., *et al*. described 14 different missense mutations in D' factor VIIII binding domain of the VWF gene located in exons 18, 19, 20: R782W, G785E, E787K, C788R, C788Y, T791M, Y795C, M800V, R816W, R816Q, H817Q, R854Q, R854Q, and C858F (Table 1). In 73 unrelated type 2N VWD patients, 37 homozygous and 35 compound heterozygous for VWD 2N mutations located in the D' domain (Table 1) [9]. Homozygous muta-

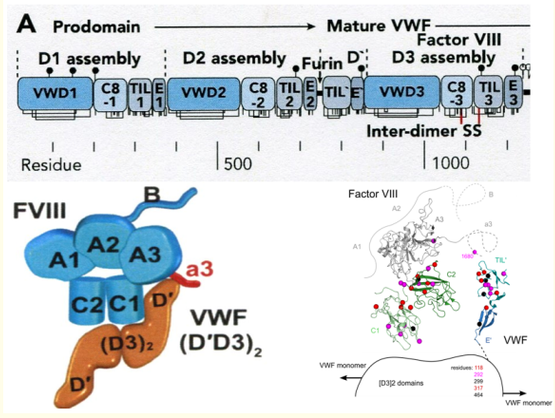


Figure 1: FFVIII is a heterodimer with a domain structure of A1-A2 and A3C1-C2. In the standard view and in accordance with cristal structures, they are illustrated with the polypeptide chain running clockwise from A1 to C2. After cleavage of proVWF into D1D2 (propeptide) and mature FVIII:C at the Furin cleavage site FVIII:C is bound to N-terminal TIL' and circulates in complex with VWF with a half life time of about 12 hours. Binding of FVIII:C to VWF is approximately by its dimeric D'D3 fragment as illustrated in the lower figures, with major contacts between D' and the C1 domain by interactions of VWF-TIL' with the A3 FVIII domain at the a3 acidi peptide as well as C2 domain. Thrombin cleavage of FVIII liberates the a3 peptide and the B domain, resulting in the dissociation of FVIII from VWF.

Figure 1A. Source Springer, Blood 2014;123:1412 [38].

Figure 1. Right below: Source Pritchard [39]

Modified and designed by Michiels

tions in 37 families were found in one family with T791M/T791M, in 3 families with R816W/R816W and in 33 families with R854Q/R854Q. R854Q was the first mutation combined with a second 2N mutation including C788Y, T791M, Y951C, R816W, and C858F in 14 of 35 families with VWD 2N and other combinations in 21 families (Tables 1, 2 and 3) [9]. Homozygosity for the C1060R in the D3 domain or double heterozygous C1060R with R854Q or non-sense (null) mutation is associated with severe VWD 2N (Tables 1-3) [9].

Homozygous (n = 38)		Compound heterozygous (n = 35)		
Mutation	Number of patients	Mutation on the first allele	Second gene defect	Number of patients
		C788Y	R854Q	1
T791M	1	T791M	Not identified	2
			R854Q	2
			Del C exon 18	1
		Y795C	R854Q	1
R816W	3	R816W	Not identified	1
			R854Q	2
R854Q	33	R854Q	Not identified	14
			Stop codon	2
			C788Y	1
			T791M	2
			Y795C	1
			R816W	2
			C858F	1
			C1060R	2
			Exon 25 splicing	2
		C858F	R854Q	1
		D879N	Stop codon	1
C1060R	1	C1060R	R854Q	2

Table 1: The von Willebrand factor (VWF) gene defects in 73 unrelated French type 2N von Willebrand Disease (VWD) patients are caused by either homozygous or double heterozygous missense mutations in the D' FVIII binding domain or are double heterozygous for a D' missense mutation and a second mutation (non-sense or secretion defect) inside (Del C exon 18) or outside the D' domain. Homozygosity for the C1060R in the D3 domain or double heterozygous C1060R with R854Q or non-sense (null) mutation is associated with severe VWD 2N (Source Mazurier et al 2001[9]).

The clinical manifestations of VWD 2N depend primarily the FVIII;C level ranging from around 0.05 U/dL to 0.20 U/dL and present with relatively moderate to mild hemorrhagic manifestations comparable with moderate to mild hemophilia A and normal VWF function.

Mutation	**FVIII:C**	**VWF:Ag**	**VWF:RCo**	**VIII:C/Ag**	**RCo/Ag**	**VWF MM**	**FVIII:BD**	**VWD type**
	U/ml	U/mL	U/mL	ratio	ratio		U/mL	
R816W/R816W [19]								
1	0.07	1.18	1.10	0.06	0.93	N	<0.10	2N severe
2	0.05	1.08	0.80	0.05	0.74	N	<0.10	2N Severe
3	0.04	1.04	1.00	0.04	0.96	N	<0.10	2N severe
R854Q/R854Q [19]								
1	0.38	1.05	nt	0.36		N	0.30	2N moderate
2	0.22	0.83	0.79	0.25	0.95	N	0.30	2N moderate
3	0.23	1.10	1.10	0.21	1.00	N	0.30	2N moderate
4	0.36	1.15	1.00	0.31	0.87	N	0.30	2N moderate
R816W/? [21]	0.14	0.49	0.52	0.29	1.06	N		2N moderate
R854Q/WT[21]	0.28	0.37	0.33	0.75	0.85	N		mild 1
M771I/WT[21]	0.20	0.38	0.59	0.53	1.55	N		mild 1
Family[27]								2N severe
C788T/null (family 1)	0.04	0.23	0.20	0.17	0.86	Abnormal	0.15	
C788T/R854W (1)	0.26	0.65	0.64	0.30	0.90	N	0.30	2N moderate
C788T/R854W (2)	0.30	1.00	0.90	0.30	0.90	N	0.30	2N moderate

Family[2Allen]								Severe 1
C788R/C788R	0.15	0.18	0.10	0.83	0.55	Abnormal		
C788R/WT	0.47	0.36	0.52	1.31	1.44	N		mild 1
C788R/WT	0.69	0.76	0.78	0.91	1.03	N		Normal
Family[28]								
R854Q/R760C	0.39	0.51	0.45	0.76	0.88	Abnormal	de-creased	2N mild
R760C/WT	0.48	0.49	0.35	0.98	0.71	Abnormal	low	mild 1
R854W/WT	0.98	1.06	0.77	0.92	0.72	N	0.50	Normal
Family[26]								
Y795C/R1566X (null)	0.10	0.91	0.51	0.11	0.57	Abnormal	0.10	2N severe
Y795C/WT						Abnormal	0.50	
R1566X/WT	1.04							Normal
Family[29]								
R854Q/R763G	0.17	0.76	0.71	0.22	0.93	Abnormal	0.20	2N moderate
R763G/WT	nt	N	nt			Abnormal	0.40	2N mild
R763G/WT	0.16	0.18	nt	0.89		Abnormal	0.40	2N mild
R763G/WT	0.37	0.33	nt	1.12		Abnormal	0.40	2N mild
Family[31]								
Y795C/R854Q	0.11	0.78	0.94	0.14	1.20	Abnormal	0.10	2N severe
C804F/R854Q	0.20	0.53	0.51	0.38	0.86	Normal	0.10	2N
R854W/WT	0.75	0.94	0.97	0.80	1.03	Normal	0.50	Normal

Table 2: Recessive von Willebrand Disease 2N mainly derived from French VWD studies: variable phenotypes of severe or moderate FVIII binding defect (FVIIIBD) and normal (VWD type 1) versus abnormal (VWD type 2) VWF multimers.

Conclusion: Causative mutations in the D' FVIII binding site domain of the VWF gene are R816W, R854Q, C788T, Y795C, and C804F when homozygous or double heterozygous for D'D' or D'-null, but not C788R and R763G.

Source	**FVIII:C**	**VWF:Ag**	**VWF:Rco**	**VIII:C/Ag**	**RCo/Ag**	**FVIII:BD**	**VIII:BD/**	**Mutation**	**VWD type**
Gender	U/mL	U/mL	U/mL	ratio	ratio	U/mL	VWF:Ag		
Casonato [23]									
I-1	0.24	0.40	0.48	0.6	0.48	0.13	0.31	R854Q/null	2N moderate
I-2	0.34	0.48	0.49	0.7	1.02	0.13	0.26	R854Q/null	2N moderate
I-3 carrier	1.46	0.95	0.65	1.5	0.68	0.96	1.0	WT/2680delC	Normal
II-1 carrier	0.55	0.33	0.38	1.6	1.15	0.43	1.3	WT/2680delC	Type 1 SD
II-2 carrier	0.76	0.33	0.22	2.3	0.67	0.36	1.0	WT/2680delC	Type 1 SD
II-3 carrier	1.16	0.86	0.49	1.3	0.57	0.78	0.9	WT/2680delC	Mild 1
Casonato [22]									
1 F	0.26	**0.69**		0.37		0.31	0.44	R854QR854Q	2N moderate
2 M	0.24	**0.80**		0.34		0.15	0.20	R854QR854Q	2N moderate
Casonato [22]									
1 F 2N	0.12	0.28	0.27	0.43	0.96	nt		R854QP812R	2N
2 M 2N	0.23	0.49	0.51	0.39	1.04	0.16	0.32	R854QP812R	2N
3 F 2N	0.22	0.61	0.44	0.36	0.72	0.10	0.16	R854QP812R	2N
4 M carrier	0.71	0.80	0.81	0.89	1.00	0.45	0.56	R854Q.W/WT	Normal
5 F carrier	0.58	0.58	0.81	1.0	1.14			R854Q.W/WT	Normal
6 M carrier	0.62	0.71	0.52	0.87	1.14			R854Q.W/WT	Normal

Table 3: Laboratory features of VWD type 2N due to D' domain mutations R854Q/null (2680delC = null mutation in exon 18), homozygous R854Q/R854Q and double heterozygous R854W/P812R and comparison of 2N carriers with normal VWF values vs null mutation carriers with features of VWD type 1SD in the Italian VWD studies of Casonato., *et al* [22].

Conclusion: R854Q/null and R854Q/P812R (null) have more severe VWD 2N as compared to homozygous R854Q/R854Q 2N VWD (Table 1). R854Q/WT carriers have normal VWF values, whereas WT/2680delC null carriers have features of mild VWD type 1 secretion defect (1SD).

The clinical manifestations of VWD 2N depend primarily on the FVIII:C level ranging from a few percentages but never less than 0.01 U/dL. Most VWD 2N patients have FVIIIC: levels from around 0.05 U/dL to 0.20 U/dL. VWD 2N patients present with relatively moderate to mild hemorrhagic manifestations comparable with moderate to mild hemophilia A and normal VWF function. The spontaneous hemorrhages are often not serious and usually featured by epistaxis, ecchymosis, muscular hematomas after a blow, but hemarthrosis and gastrointestinal bleedings are very rare. The bleeding type is usually mild and mainly post-traumatic, or related to surgery like adenoidectomy, tonsillectomy, and tooth extraction. Postpartum bleeding was frequently noted in young and adult women.

Structure and function of the FVIII binding region in the D' domain of VWF

The major FVIII binding region on VWF is composed of three distinct and conserved domains: TIL', E' and VWD3 (Figure 2) [10]. In electron microscopy (EM) structures, the trypsin-inhibitor like (TIL') E' domains form a protrusion from the D3 domain, thereby presenting the TIL'E' domains to the physiological binding partner FVIII. The VWF-FVIII binding site (FVIII:BS) consists of two distinct and conserved domains TIL' (residues 766-827) and E' (residues 829-863) that can be produced in bacterial cells, to allow for structural and dynamical investigations by NMR spectroscopy (Figures 2 and 3). The VWD3 (residues (867-1031) domain belongs to the D3 multimerization domain (Figures 1 and 2) [11]. Based on the high concentration of type 2N mutations, the TIL' and E' domains, previously known as D', are essential for FVIII binding by VWF.

The TIL'E' domains are located immediately after the Furin cleavage site (763aa) and form independently folding domains separating D' into two distinct domains TIL' and E' (Figure 3), which contain a majority of the VWD 2N mutations. All cysteine residues of these domains form disulfide bridges (Figure 3). The TIL' and E' domains tumble as a single entity with only limited inter-domain motion [11]. TIL' is formed of short beta sheets beta1:beta2 (residues 772-775;806-809) and beta3:beta4 (residues 814-817;820-823). The beta1-to-beta2 loop encompass an eight residues insertion between the second and third conserved TIL' cysteine residues. The second antiparallel beta sheet, formed of strands beta3 and beta4, is connected by a reversed turn forming a small hairpin structure. TIL' and E' are connected at 827-829. The greater proportion of E' is formed of a triple-stranded antiparallel beta-sheet formed of strands beta3 (residues 839-844), beta4 (residues 847-852) and beta5 (residues 855-858). The N-terminus of E' contains a short double strand beta1:beta2 sheet (residues 829-831:834-836). The relative positions of these two

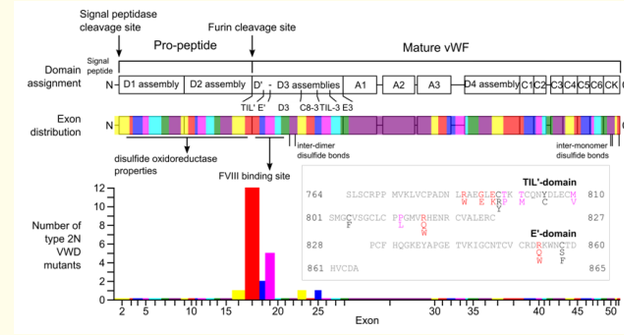


Figure 2: Type and distribution of von Willebrand factor (VWF) gene mutations in recessive von Willebrand disease (VWD) type 2N (VWD 2N) is featured by homozygous or double heterozygous mutations in the FVIII binding D' domain or D3 domain or the combination of at least one mutation in the FVIII binding D'domain or D3 domain plus one nonsense of missence mutation in any other domain in the VWF gene.

Source: Pritchard [39]

Blood 2015;126(8)939-942 [13].

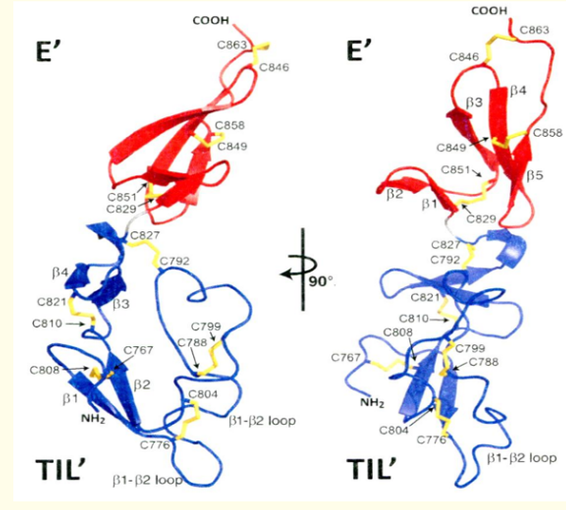


Figure 3: High-resolution structure of VWF TIL'E' domains. Ribbon diagram of the lowest-energy solution structure of TIL'(D' residues 766-827) and E' (D' residues 829-863) of the FVIII binding domain on VWF [8]. Disulfide bonds are colored in yellow. TIL' domain (bottom) is shown in blue, and the E' domain (top) is shown in red. The view of the structure shown to the right is a 900 rotation of the left view [9]. Beta strands are numbered according to the order in which they occur in the primary sequence of the individual domains. Cysteine linkages are associated with their residue numbers.

Source: (Reprinted with permission from Blood 2014;123: 4143-4151) [11].

Courtesy of Prof. Hansen

beta sheets are restrained by the E' Cys829-Cys851 disulfide bond. The dynamic characteristics of TIL'E' is of great importance for elucidating the mechanism by which FVIII is stabilized in plasma [11]. There is a distinguishable mobility differential between TIL' and E' domains: The E' domain is rigid whereas several residues in the beta1-to-beta2 loop of the TIL' domain show higher degree of conformational flexibility over a broad range of time scales. The TIL' beta1-to-beta2 loop is significantly more flexible than the remainder of TIL'E', which are connected by the C827-C829 bond. The TIL' beta1 to beta2 loop is strikingly dynamic on at least two times scales and the remainder of TIL'E' provides a rigid scaffold supporting this conformational dynamic entity [11].

Most non-cysteine VWD 2N missense mutations are distributed in the dynamic TIL' beta1-to-beta2 loop (Figure 4, green spheres) [11]. VWD 2N cysteine mutations (C788R/Y; Y795C and C804F in TIL'; C858C/F in E') are associated with aberrant multimerization, poor secretion and reduced FVIII binding (Figure 4, black spheres). Three non-cysteine VWD 2N mutations (T791M, R816W and R854Q) and R854Q account for the majority of VWD 2N patients [11]. The non-cysteine VWD 2N mutations T791M, R816W, T789P, M800V, R816Q and H817Q are clustered around a region of positive charge density on TIL'. The VWF residues R782, G785 and E787 are on the opposite of the structure (Figure 3B) yet in close proximity to the beta1-to-beta2 loop [21]. This strongly suggest that the severe VWD 2N non-cysteine mutations E787K, R782W and G785E lead to deficiencies in FVIII binding through a perturbation of the beta1-to-beta2 loop. Most non-cysteine VWD 2N mutations are found in the vicinity of the flexible regions of TIL' and all but one of the 2N mutations in TIL'E' result in the loss of positive charge. This is in agreement with the fact that high ionic charge and low pH result in a much lower affinity of the VWF:FVIII complex [11].

Two papers in Blood in 2015 by Chiu., *et al*. and Yee., *et al*. present complementary findings on the FVIII-VWF interaction with identification of the interaction between D'D3 and FVIII (Figure 1) [12,13]. The FVIII acidic a3 domain is critical to the high affinity binding to VWF (Figure 1). The positively charged and dynamic region on TIL'E' is very likely the major binding site to the negatively charged FVIII a3 domain. FVIII mutations VIII C1 domain [12-14]. and C2 domain [15]. impairing VWF binding to FVIII were identified as a one of the causes of mild/moderate hemophilia A. Mutations in the C_2 FVIII domain with low FVIII:C was due to retention of FVIII in the endoplasmic reticulum (secretion defect). Several mutations in the C1 domain resulting in mild/moderate hemophilia A have reduce binding to VWF [12,13]. A series of FVIII mutations ILe2098Ser, Ser2119Tyr, Asn2129Ser, Arg2159His and Pro-2153Gln clustered in the C1 domain are associated with reduced FVIII binding to VWF (Figure 4). Scat chard plot analysis of r-FVIIII mutations located in the FVIII light chain confirmed that the 3 evaluated mutations ILe2098Ser, Ser2119Tyr and Arg2150His (Figure 4) had indeed a reduced affinity for VWF with an impaired FVIII binding for VWF. The critical role or mechanism by which C1 influences the affinity of FVIII to VWF is in line with the observation that a monoclonal antibody recognizing the C1 domain, LE2E9, completely inhibited FVIII binding to VWF. Decreased binding to VWF due to mutations in the C1 domain of the FVIIII gene now appear to be a common cause of mild/moderate hemophilia A[12-15].

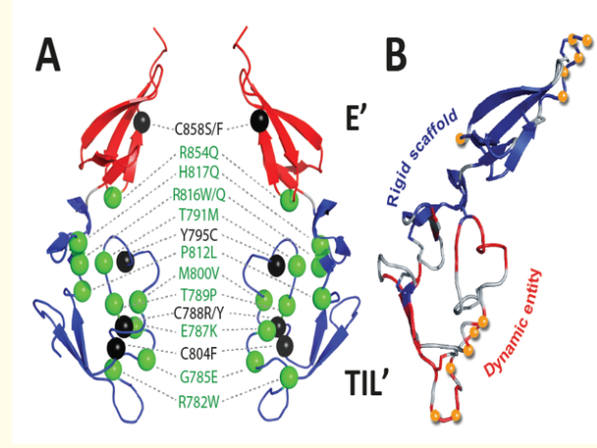


Figure 4: (A) Localization of type 2N VWD mutations in the D' domain (763-869aa, exon 18) [9]. The D' FVIII binding domain on VWF consists of two domains: trypsin-inhibitor-like (TIL'; residues 766-827; blue) and E' (residues 829-863; red). The non-cysteine mutations in TIL'E' domain (green spheres) are associated with 2N VWD [21]. Three non-cysteine type 2N mutations are associated with a severe 2N phenotype: E787K, T791M and R816W [9]. Type 2N mutations that involve a cysteine; C788R/Y, Y795C, and C804F (black spheres) in the TIL' domain, and C858S/F (black sphere) in the E' domain are associated with aberrant multimerization, poor secretion, and FVIII binding defect [9]. (B) Regions that are flexible (rigid) on the millisecond time-scale are colored red (blue). Yellow spheres depict residues that are mobile on the pico-nanosecond time-scale. Thus, the E' domain is overall rigid, whereas several residues in thebeta1 to beta2 loop of the TIL' domain show higher degree of conformational flexibility. TIL' and E' domains tumble as a single entity with only limited inter-domain motion.

Source: (Reprinted with permission from Blood 2014;123: 4143-4151) [11].

Courtesy of Prof. Hansen

Classical VWD 2 N Factor VIII Binding site Defect: FVIII:BD

Classical recessive VWD 2N is mainly due to the homozygous non-cysteine or double heterozygous noncysteine/null mutations R854Q and R816W in the D' Domain of the VWF gene which impair the binding of FVIII capacity of VWF (FVIII binding defect: FVIII-BD) but do not impair the function and multimeric structure of VWF [16-18]. All recessive VWD 2N non-cysteine mutations result in a clinical and laboratory phenotype indistinguishable from mild/moderate hemophilia. The accelerated clearance of FVIII:C and normal clearance of VWF after DDVAP in VWD 2N R854Q/R854Q vs R816W/R816W result in mild vs severe FVIII:C deficiency of 22 to 38% vs 5 to 7% with a clinical bleeding phenotype indistinguishable from mild vs moderate hemophilia A (Table 1) [19]. Measurement of FVIII coagulant activity (FVIII:C), FVIII antigen, VWF antigen (VWF:Ag) and VWF ristocetin cofactor activity, (VWF:RCo) after one intravenous dose of DDAVP (0.3 or 0.4 µg/kg) is mandatory to assess the severity of VWD 2N. A FVIII:C/VWF/Ag ratio below 0.50 and a normal VWF:RCo/VWF:Ag ratio appeared to be diagnostic for VWD 2N in patients with noncystein Arg91Gln (R854Q) or Arg53Trp (R816W) amino acid substitution[19]. In noncystein mutated VWD 2N patients, whatever their mutation, the DDAVP infusion resulted in a 2.3. ± 0.7 -fold increase of VWF and a variable rise (9.5 ± 7.7 times) of FVIII, whereas the VWF capacity to bind FVIII was not improved [19]. The FVIII:C response to DDAVP was more transient than VWF response to DDAVP, and FVIII:C half disappearance time was shortened to approximately 3 hours, whereas the VWF half life time is normal [19]. VWD 2N FVIII binding defects (FVIII:BD) due to the homozygous noncysteine mutation R854W/R854W is typically featured by normal VWF:Ag levels around 1.0 U/ml, a low FVIII:C/VWF:Ag ratio of about 0.30, Factor VIII binding defect (FVIII:BD) of about 0.30 U/mL and normal VWF multimers (Table 2, Figure 4) [9,16-21]. VWD 2N non-cysteine homozygous mutations E787K/E787K, T791M/T971M as well as the double heterozygous noncystein/null mutation R854Q/null (Arg91Gln/delC 2680 table 2) are severe with less than 10% FVIII binding to VWF (Tables 1 and 2) [22]. Heterozygous VWD 2N carriers R711I/WT, R816W/WT, R854W/WT and R854Q/WT have FVIII binding to VWF in the lower normal range with normal VWF levels and multimers are therefore usually diagnosed as VWD type 1 (Tables 1 and 3).

The laboratory characteristics of low FVIII:C/VWF:Ag ratios (0.31and 0.27) and FVIII Binding site Defect (FVIII:BD) of 12.7% in the study of Casonato., *et al*. was diagnostic for VWD 2N caused by double heterozygosity of R854Q/C842X (2N/null mutation) (Arg-91Gln = R854Q) [23]. Laboratory data of 4 heterozygous C842X/WT cases were consistent with VWD type 1 in 2 (II-1 and II-2) and normal in 2 (I-3 and II-3, Figure 3). The single cysteine deletion in exon 18 (2680delC) as the most common null mutation in the Swedish VWD type III patients [24,25]. was involved in double heterozygous VWD 2N (table 3). Casonato., *et al*. estimated that the most common R854Q mutation in VWD 2N occurred 10.000 to 40.000 years ago [22]. The R854Q mutation is limited to the Caucasian population suggesting that the R854Q mutational event occurred in the human population that had moved from Africa to Europe. As compared to homozygous R854Q/R854Q VWD 2N (Table 3 Casonato [22]. cases 1 to 3), double heterozygous R854Q combined with the P812R null mutation in the D' domain is associated with severe FVIII:BSD of less than 15% (Table 3 Casonato, cases I-1and I-2) that decreased FVIII:C/VWF:Ag ratios and low values in the FVIII:BD assay (Table 3). Three heterozygous R854Q/WT 2N carriers in the study of Casonato were asymptomatic with normal values for FVIII:C and VWF parameters (Table 3).

Abnormal VWF multimers in cysteine mutations in and outside the D' domain

The cysteine Y795C/null VWD 2N mutation in the D' domain of VWF is featured by a severe VWD 2N with very low FVIII:C due to FVIII:BD associated with abnormal smeary ultralarge VWF multimers (Table 2, (Figure 5) [26]. The FVIII:BD in asymptomatic heterozygous Y795C/WT carriers is about 50% showing the persistence of smeary ultralarge VWF multimers consistent with VWD 1sm [26]. Expression studies recombinant r795Y showed a similar pattern of a lack of triplet structure, a smeary pattern and the presence of supranormal multimers as in plasma pl795C. The multimeric pattern of recombinant non-cysteine r787K/rWT VWF was normal l [26].

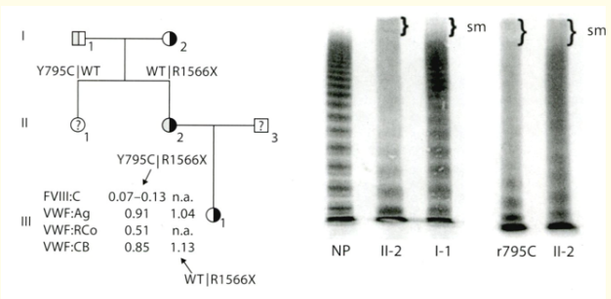


Figure 5: Segregation of heterozygous Y795C/WT and R1566X = null mutation into compound heterozygous Y795C/null mutation in a family with severe VWF;FVIIIB defect in rY795C and rE787K mutation (Table 1) [27]. The affected Y795C/null, and Y785C/WT and recombinant r795C show abnormal VWF multimers with a smeary pattern and the presence of ultralarge multimers.

Source: Blood 2000;95:3130-3145 [27].

Hilbert., *et al*. reported a VWD 2N case double heterozygous for Y795C/R854Q in a 37 year-old woman with a lifelong history of menorrhagia, normal bleeding time, normal VWF levels but dramatically reduced FVIII;C levels and reduced FVIII:C/VWF:Ag ratio (Table 1) associated with a smeary VWF multimeric pattern compared to control similar as has been described in VWD 2N Y795C/null double heterozygous VWD 2N and in asymptomatic carriers of Y795C/WT (Figure 6) [30]. Hilbert reported another VWD 2N case double heterozygous for C804F/R854Q in a 59-year-old woman who presented with a life-long history of mild to moderate hemorrhagic tendency featued by frequent epistaxis, gingivorrhagia and bleeding after tonsilectomy, with normal bleeding time, subnormal VWF levels (0.50 U/L bloodgroup A), but low FVIII:C levels (0,20 U/L) and decreased FVIII:C/VWF:Ag ratio 0.38 and 0.39 on repeated testing [30]. The FVIII-BD of VWF in all four recombinant (r) mutants rC795, rC854, rF804 and combinations of it were very low (Figure 6). Expression studies showed a typical smeary VWF multimeric pattern in rC795 and rC795/rC854 and in asymptomatic carriers of C795 (Figure 6) [30]. In contrast, the expression studies of C804F are featured by a severe secretion multimerization defect. The abnormal C804F VWF multimers in the double heterozygous C804F/R854W mutated VWD 2N is corrected to normal by the R854Q allele [30].

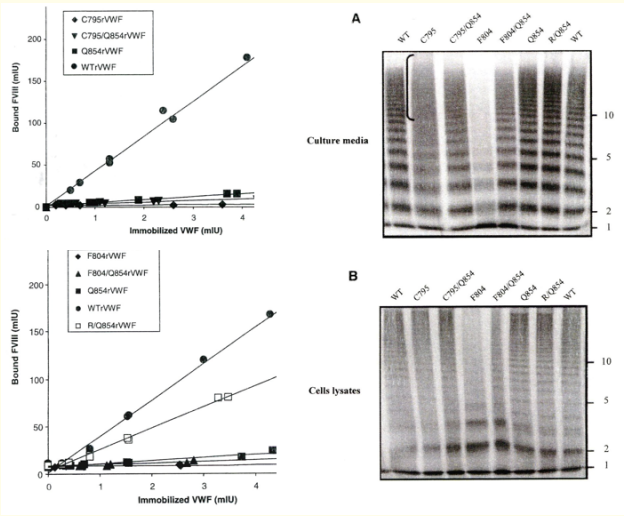


Figure 6: Upper left. Measurement of FVIII binding to VWF in VWD 2N patient A (Y795C/R854W), patient B (C804F/R854W), patient heterozygous for R816W and normal individual. VWF multimeric analysis in VWD 2N patient A Y795C/R854W with smeary VWF multimers and patient B C804F/R854W with normal VWF MM (upper right).

Source: [31].

Lower left. FVIII binding capacity of wild type (WT) VWF or recombinant (r) VWF expressed after transfection in COS-7 cells of rVWF mutant Y795C (A) or C804F (B). Lower right, multimeric profiles of WT or mutated rVWF expressed after transfection in COS-7 cells in cultured media (A) or cell lysates (B).

Source: Blood 1998;92(12):4663-4670 [31].

The cysteine mutations inside the D' domain C788R/Y, C788T and C804F in TiL', and C858S/F in E' and outside the D' domain C760C in D2, R763G Furin cleavage site only produce VWD 2N when combined with the R854Q mutation and are associated with aberrant multimerization due to a mixture of mature VWF and proVWF resulting in reduced FVIII:C/VWF:Ag ratio below 0.50 due to FVIII:BD (Table 2). Jorieux., *et al*. studied 3 patients from 2 unrelated families (P1 and P2, family 1 and P3, family 2 with a combined FVIII-BD and multimerization defect of VWF (Table 2, (Figure 7) [27]. P1 was double heterozygous for C788T/null allele and showed a hybrid picture of FVIII:BD (FVIII:C 0.04, VWF:Ag 0.23, combined with severe type 1, loss of large and intermediate VWF multimers (Table 2 and Figure 7) with prolonged bleeding time, and low levels of VWF parameters (around 0.20 U/mL due to a secretion multimerization defects (Figure 6 left, table 1). P2 was double heterozygous for C788T/R854Q (P2) presented with VWD 2N with normal bleeding time and normal VWF multimeric pattern (Figure 7 left, Table 2) indicating that the R854W allele corrected the VWF multimeric pattern [27]. Expression studies of recombinant VWF (rVWF) mutation showed a secretion and multimerization defect of rC788T and normal secretion and multimers for rR854Q. (Figure 7 middle) indicating that R854Q is determinative in causing VWD 2N. Case P3 from the second family was double heterozygous for C857F/R854Q with normal VWF multimers. Expression studies of recombinant VWF (rVWF) mutation showed a secretion and multimerization defect of rC858F and normal secretion and multimers for rR854Q. (Figure 6 left). Heterozygous expression of rVWFC858F with wild type or R854Q as well as C858F/WT showed a multimeric pattern with the relative decrease of large VWF multimers in 1.5% SDS agarose resolution gels (Figure 6) [27]. The conclusion from these two examples of double

heterozygous cysteine-noncysteine VWD 2N is that the non-cysteine R854Q determined and corrected the defective multimerization pattern of the cysteine mutations C788T (Figure 6 middle) and C858F (Figure 6 right).

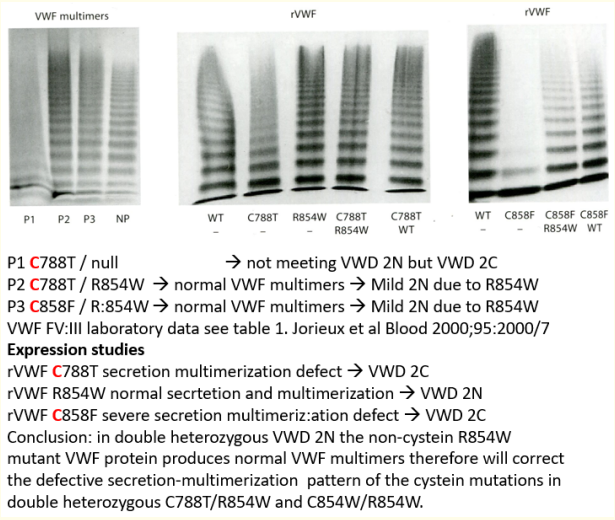
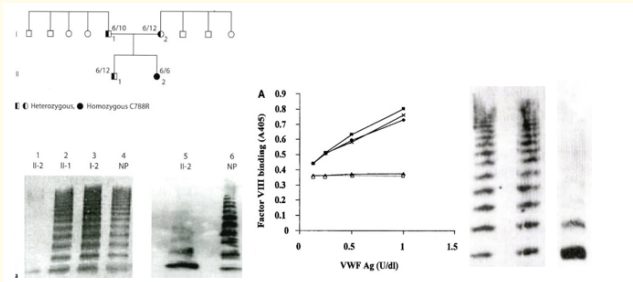


Figure 7: VWF multimers of VWD 2/N caused by C788T/null mutation (P1) and normal VWF multimers (VWD 1/Normandy) in double heterozygous C788T/C854W (P2) or C858F/C854W (P3) (Table 1) [26]. Expression studies of recombinant VWF show a multimerization defect in C788T and C858F, normal multimers in R854W, and normal or near normal VWF multimers in carriers of C788T, and C858F respectively.

Source: Thromb Haemostas 2004;92:36-41 [26].

Homozygous C788R/C788R mutation in the proband of a consanguineous family induced a severe recessive type 1 (Table 2) with low FVIII:C and VWF, decreased VWF:RCo/VWF:Ag ratio of 0.55, prolonged bleeding time and decrease of large VF multimers with lack triplets consistent with impaired secretion and multimerization defect similar as seen in VWD 2 with the absence of intermediate and large VWF multimers and a pronounced VWF D-dimer band (Figure 8). A FVIII:C/VWF:Ag ratio of 0.83 excluded the diagnosis of VWD 2N. The C788R/WT heterozygous father and brother were diagnosed as mild VWD Low VWF with normal VWF multimers because the normal WT VWF corrected the defective multimerization pattern of the cysteine mutations C788T (Table related to figure 8).



Mutation	FVIII:C %	VWF:Ag %	VWF:RCo %	FVIII:C/ VWF:Ag	VWF:RCo/ VWF:Ag
C788R/C788R	**15**	**18**	**10**	**0.83**	**0.55**
C788R father	47	36	52	1.3	1.4
C788R mother	69	76	78	0.90	1.0
C788R brother	44	57	84	0.77	1.5

Figure 8: Family pedigree. Left. C788R cysteine mutation in the VWF D' domain showing a family with recessive VWD severe type 2C in homozygous C788R/C788R (II-2) and mild type VWD type 2C in heterozygous C788R/WT and normal VWF multimeric pattern (II-1 brother, I-2 father).

Source: Brit J Haematol 2004;127:184-189 [30].

Upper left multimeric analysis. Left lane 1, absence of multimers in low resolution and lane 5 multimerization defect with presence of 4 VWF, and normal VWF multimers in C788C/WT heterozygotes (Lane 2 father (I-2) and (Lane 3, brother II-1).

Upper in between. Binding of FVIII to recombinant VWF (rVWF) wild type (black quadrant) rvWF Thr791M VWD 2N mutation (open quadrant) and rVWF C788R (black triangle). Factor VIII binding to rVWF C788R, and mutant/wildtype VWF hybrids secreted by COS-7 cells. The cells were transfected with the appropriate plasmid DNA, then placed in serum-free medium 24 hours posttransfection. The ability of each rVWF to bind factor VIII was 40% in m, rVWF C788R as compared to normal values in rVWF wild-type/C788R and WT/WT [30]. We conclude that defective multimerization of VWF is associated with decreased FVIII binding to mutant VWF.

Source: Brit J Haematol 2004;127:184-189 [30].

Upper right. Multimer analysis of rvWF C788R, and mutant/wild-type vWF hybrids secreted by COS-7 cells. The cells were transfected with the appropriate plasmid DNA, then placed in serum-free medium 24 hours posttransfection. 2 mU of each rvWF, as determined by ELISA, was analyzed on a nonreducing 2% SDS agarose gel: lane 1 wild-type rVWF and lane 2, rvWF C788R/wild-type hybrid showing normal VWF multimeric pattern and lane 3, rVWF C788R showing a secretion multimerization defect.

VWD 2N compound heterozygous for the noncystein R854Q in the D'-domain and R760C in the D2 domain is characterized by a mixture of normal VWF of R854Q and persistent VWF propeptide (pp) of mutant R760C VWF protein both in the proband R854Q/R760W and in the heterozygous father R760W/WT (Figure 9) [28]. Sodium dodecyl sulfate-polyacryl gel electrophoresis (SDS-PAGE) revealed 2 forms of circulating plasma VWF: one mature VWF band (225 kDa) from R854Q or WT and the other pro-VWF from R760C (Figure 9). The degree of FVIII binding defect (FVIII-BD) on VWF is markedly decreased in R854Q/R760W (11.9%) as compared to heterozygous R760W/WT (22.2%, father) and R854Q/WT (45.6%, mother) carriers (Table 2) [28]. The R760C cysteine mutant with persistent VWFpp induced a severe secretion defect and a FVIIIBD. Expression studies showed secretion of normal VWF multimers for rWT/VWF, a decrease of all multimers in rWT/R760C VWF and much more pronounced decreased in rR760C VWF mutant indicating that the R760W mutant induced a multimerization defect (Figure 9). Heterozygosity of R760C/WT in the D2 domain is associated with mild type 1 VWD and normal VWF multimers whereas compound heterozygosity of R854Q/R760C result in a FVIII-BD and abnormal banding and smeary VWF multimers (Figure 9, Table 2) [28]. Expression studies of the mutant rR760C mutation (D2 domain) showed decreased VWF multimerization and secretion with the absence of large VWF multimers and the persistence of pro-VWF (Figure 9) [28].

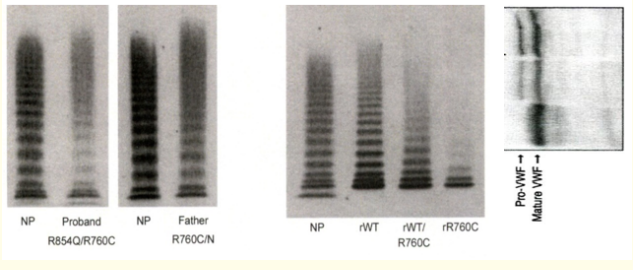


Figure 9: VWF multimeric pattern related to laboratory features in table 1 in VWD1/Normandy due to double heterozygous non-cysteine mutation R854Q in the D' domain and cysteine R760C mutation in the D2 domain three amino acids before the Furin cleavage site 763/764[28]. Recombinant (r) mutant rR760C mutation show the absence of intermediate and large VWF multimers and the presence of pro-VWF (right) indicating that the VWFpp is not cleaved off with the consequence of a severe secretion and multimerization defect of the rR760C mutant VWF (Table 1).
Source: Blood 2003;101:151-156 [28].

A unique family of VWD due to compound heterozygosity for the D' mutation R854Q and the R763G at the Furin cleavage site between D2 and D' domains has been described [29]. The double heterozygous proband R763G/R854Q and 3 heterozygous R763G/WT children showed a smeary pattern of VWF multimers with the presence of ultralarge VWF multimers (table 2, figure 10) [29]. The proband (I-1) had a lifelong history of severe epistaxis and bleeding after knee surgery and appendectomy. Three adult children (II-1, II-2 and II-3) had spontaneous hemorrhagic manifestations during childhood and were diagnosed as VWD based on reduced levels of VWF:Ag and abnormal VWF multimers [29]. Expression studies of rVWF showed secretion of normal VWF multimers for the rR854Q mutation (figure 9), but slower (s) moving bands of VWF for rR763G as compared to WT (figure 8). The heterozygous R763G/WT displayed a combination of mild VWD 2N with FVIII-BD of 30% and an abnormal smeary multimeric pattern due to slower (s) migration of the various multimers (table 2, figure 9) [29]. The hybrid rVWF R763G/R854Q displayed an abnormal multimeric pattern with broad bands (smeary pattern) and the presence of ultralarge VWF multimers (pro-VWF) also seen in the symptomatic carriers of the R763G mutation (Figure 10). The smeary pattern and presence of ultralarge VWF multimers is caused by the mixture of circulating non cleaved proVWF and normal mature VWF in carriers of the R763G mutation at the Furin cleavage site of VWF. The R763G mutant with persistence of VWFpp induces an abnormal banding of VWF multimers on top of FVIII binding defect (FVIII-BD) to VWF of about 30% (Figure 10). From this section we can conclude that the cysteine mutations inside the D' domain C788R/Y, C788T, C804F and C858S/F when combined with R854Q produce a typical VWD 2N with normal VWF multimers. In contrast the combination of R854Q with the cysteine mutations C760C in D2 and R763G Furin cleavage site produce a hybrid VWD 2N/2A phenotype (FVIII binding defect of VWF) associated with aberrant multimerization (smeary pattern) caused by a mixture of mature and proVWF with reduced FVIII:C/VWF:Ag ratio.

D3 domain mutations with FVIII-VWF binding and multimerization defects

The laboratory features in a symptomatic case of recessive double heterozygous VWD D879N in exon 20 and Arg1659stop in exon 28 (null allele) are shown in table 4. FVIII:C was decreased or equal to subnomal levels of VWF:Ag with FVIII:C/VWF:Ag ratios ranging from 0.33 to 1.05 at 4 different time point during a few years follow-up (Table 4) [31]. The VWF:Ag was decreased or bordeline and always higher than VWF:RCo with VWF:RCo/VWF:Ag ratios ranging from 0.18 to 0.45 consistent with type 2 VWD (Table 4). Plasma VWF binding to ristocetine activated plate-

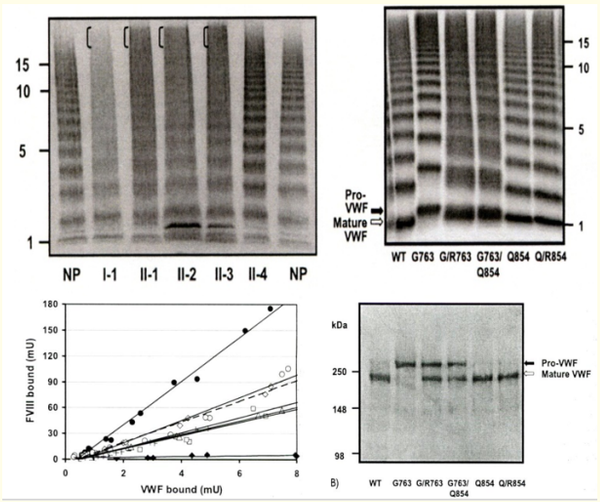


Figure 10: VWF multimers in a Family with VWD in table showing a smeary pattern and ultralarge multimers in the proband double heterozygous for R854Q/R763G with a life-long bleeding tendency and in 3 heterozygous carriers of R763G (II-1, II-2, II-3) with symptomatic VWD in early childhood, but normal VWF multimers in heterozygous R854Q (II-4) [29]. VWF multimers of recombinant VWF disclosing abnormal moving bands of mutant rR763G VWF multimers of Pro-VWF, smeary VWF multimers in heterozygous rwt/R763G and rR763G/Q854, and normal VWF multimers of mature VWF in rQ854 VWF and rwt VWF

Source: Thromb Haemostas 2006;96:290-294 [29].

Lower Left: Normal FVIII binding to VWF of normal in pool of normal plasma (NP, closed circle = 100%) in R816W mutant VWF (closed triangle = <5%) in a mixture NP and R816W (open circle = 50%) and in heterozygous carriers of R763G (open triangles and quadrangle = <30%).

Lower Right: presence in circulating plasma of pro-von Willebrand factor (Pro-VWF) in heterozygous carriers of R763G indicating that VWFpp is not cleaved off and absence of VWFpp in WT VWF and R854W VWF with the presence of mature VWF in normal WT VWF plasma and in heterozygous carriers of R/Q854 in the family with Q854W/R763G mutated VWD 2N (Table 1).

Source: Thromb Haemostas 2006;96:290-294 [29].

lets (RIPA) was decreased. High resolution agarose gel electrophoresis (2.5%) showed loss of large multimers and a loss of satelite band consistent with VWD type IIE (2E) (Table 4, Figure 11). The binding ability of FVIII to plasma VWF and to rVWFD879N were markedly decreased in the proband with the D879N/null mutation as compared to normal FVIII binding in normal VWF and rVWF/WT. Multimeric analysis of mutant VWFD879N/null in low resolution (1.5%) gel electrophoresis showed some loss of large VWF multimers and significant loss of large VWF multimers in plasma and platelets respectively as compared to controls (Figure 11 left). Multimeric analysis of rVWF-D879N revealed a typical type 2 E pattern of multimerization defect with significant loss of large VWF multimers and absence of a triplet structure of each VWF band as compared to wild type rVWF and heterozygous D879N/WT VWF (Figure 11) [31].

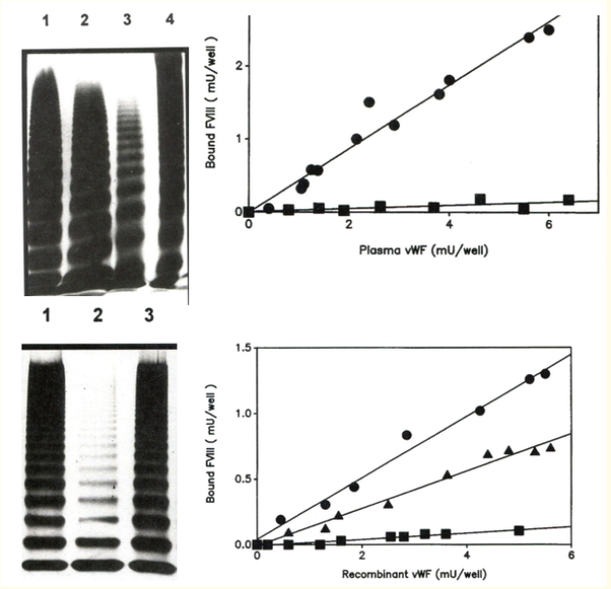


Figure 11: D897N/null 2N VWD. VWF multimeric analysis of VWD 2N D879N/null mutation of plasma and platelet lysate after electrophoresis in SDS 1.5% agarose gel and visualization with alkaline phosphatase conjugated anti VWF polyclonal antibodies: Lane 1 normal VWF MM pattern of normal plasma; Lane 2 some loss of large VWF MM in D897N mutated patient's plasma in direct comparison to normal VWF MM: Lane 3 loss of large VWF MM and no increase of small VWF MM in D897N mutated patient's platelet lysate: Lane 4 normal pattern of normal recombinant rVWF; Lane 4 lack of large and smaller bands in D879N mutated patient's rVWF; Lane 6 normal VWF MM pattern in hybrid normal rVWF and D897N mutated rVWF. The D879N/null mutation is featured by some loss of large VWF multimers in plasma (lane 2) as compared to wild type control plasma (lane 1), and significant loss of large VWF multimers in platelets (lane 3) as compared to control platelets (lane 4)[30]. FVIII binding to VWF is markedly decreased in mutant D879N/null plasma as compared to normal WT plasma.

Source: Brit J Haematol 2004;127:184-189 [30].

Mutation	Date	FVIII:C	VWF:Ag	VWF:RCo	VIII:C/Ag	RCo/Ag	VWF MM	FVIII:BD	VVWD
Proband		U/ml	U/mL	U/mL	ratio	ratio		U/mL	
D879N/null	1984	0.17	0.22	0.10	0.77	0.45	2E	<0.05	2N/2E
D879N/null	1985	0.23	0.55	0.10	0.42	0.18	2E	<0.05	2N/2E
D879N/null	1992	0.42	0.40	0.13	1.05	0.33	2E	<0.05	2N/2E
D879N/null	1995	0.16	0.48	0.25	0.33	0.52	2E	<0.05	2N/2E
	Sister	0.54	0.36	0.48	1.5	1.3	Normal	0.50	Normal
Normal values		0.50-1.5-	0.50-1.50	0.50-1.50					

Table 4: Repeated FVIII:C, VWF parameters and FVIII;BD in a case of VWD 2N/E due to the D879N/null mutation in the D3 domain of the VWF gene [31].

Conclusion: The combination of D879N mutation in the D3 domain and a nonsense null mutation is associated with pronounced 2N VWD and a type 2E VWF multimeric patterns in plasma as well as in expression studies (Figure 11)[31].

Hilbert., *et al*. identified two type 2N VWD missense mutations Q1053 and C1060R, in exon 24 of the D3 domain of the VWF gene in seven unrelated families. These missence mutations could be identified as Q1053/WT, C1060R/R854, C1060/C1060R, C1060/Y357X, C1060R/deletion and C1060R/R934Q [32]. The biological, blood group, platelets and FVIII and VWF laboratory findings related to ISTH defined VWD due to mutation in the D3 domain are shown in table 5 and Figure 12. The first patient heterozygous for Q1053/WT the bleeding tendency could not be explained by the vWF gene defect. Further molecular analysis revealed heterozygous C/T transition in the FVIII gene promotor at position -217 ahead of exon 1 in this patient and her father. Thus patient A is likely to be a hemophilia carrier who is also heterozygous for a type 2N VWD mutation showing a normal VWF multimeric pattern in SDS 1.5% agarose gel electrophoresis (Figure 12) [32].

The C1060R substitution was identified in VWD 2N cases of homozygous C1060R/C1060R or double heterozygous C1060R combined with R854Q in two and with a null allele (stop codon and deletion) in another two unrelated cases (Table 5) [32]. The combination C1060R/R854Q in two cases is associated with FVIII:C levels of 0.10 to 0.12 U/mL, normal VWF:Ag levels above 0.60 U/mL, and abnormal VWF multimers in plasma (Figure 12), whereas the two C1060R/null mutants had very low FVIII:C levels below 0.05 U/mL, decreased VWF:Ag levels of 0.43 and 0.38 respectively, and loss of large VWF multimers, both in plasma (Figure 12 and expression studies [34]. A similar observation of normal VWF:Ag levels associated with moderate FVIII:BD is seen in homozygous R854Q/R854Q and more pronounced FVIII:BD and decreased VWF:Ag levels in double heterozygous R854Q/null mutated VWD 2N patients with normal VWF multimers (Table 5) [22,23].

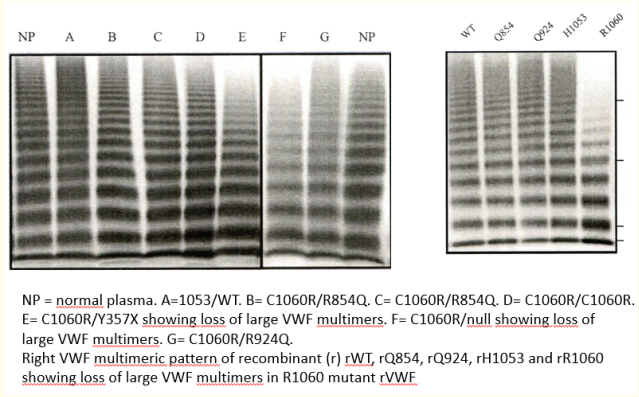


Figure 12: Left. Normal multimerization pattern in heterozygous mutant Q1053H/WT (A) and in the double heterozygous mutant, C1060R/R854Q (B and C), minor loss of large VWF multimers in C1060R/C1060R (D) and significant loss of large VWF multimers (E) in C1060R/Y357X as compared to normal plasma (NP left) [32]. Significant loss of large VWF multimers in C1060R/deletion (F) and minor loss of large VWF multimers in C1060R/R924Q (G) as compared to normal plasma (NP right). Right. Normal VWF multimeric pattern in wild type (WT) recombinant VWF (rVWF) and mutant rQ854, rQ924 and rH1053 VWF, but significant loss of large VWF multimers in rC1060R VWF.

Source: Brit J Haematol 2003;120:627-632. [32].

Case	FVIII:C	VWF:Ag	VWF:Rco	VIII:C/Ag	RCo/Ag	FVIII:BD	Mutation D3 domain	VWD type
Gender	U/mL	U/mL	U/mL	ratio	ratio	U/mL		
A 1 F	0.28	0.72	0.70	0.39	0.96	0.40	Q1053H/WT	Mild 1/2N
B 2 M	0.13	0.66	0.52	0.20	0.79	<0.05	C1060R/R854Q	2N severe
C 3 F	0.10	0.82	0.83	0.12	1.01	<0.05	C1060R/R854Q	2N severe
D 4 M	0.09	1.30	1.04	0.07	0.80	<0.05	C1060R/C1060R	2N severe
E 5 F	0.05	0.43	0.50	0.12	1.16	<0.05	C1060R/Y357X-null	2N / 2 E
F 6 M	0.03	0.38	0.25	0.08	0.66	<0.05	C1060R/deletion-null	2N / 2E
G 7 M	0.40	0.59	0.51	0.68	0.86	0.40	C1060R/R924Q	mild 1/2E
N values	0.50-1.50	50-1.50	50-1.50			0.50-1.50	WT/WT	Normal

Table 5: Laboratory findings in VWD 2N due to homozygous or double heterozygous C1060R mutations in the D3 domain of von Willebrand factor (Figure 12)[32]

Conclusion: the combination of homozygous C1060R/C1060R and double heterozygous C1060R/null is associated with the most severe variant of VWD 2N as compared to pronounced 2N VWD in double heterozygous C1036R/R854Q, whereas heterozygous C1060R/WT mutation is associated with mild VWD type 1 with some lack of large VWF due to a multimerization defect, which is also seen in VWD 2N double heterozygous for C1060R/null (Figure 11)[32]. C1060R combined with C1060R, null allele or R854Q are not reported in the Brno, Italien, Portugese and Spanish cohorts of VWD patients.

Hilbert identified a new VWF gene defect in a 24-year-old French male patient with a history of postsurgical bleedings, normal bleeding times, normal VWF antigen levels (VWF:Ag 67 and 91 IU dL and VWF:RCo 72 and 90 IU dL), normal VWF multimeric profile but low FVIII:C between 8–23 IU dL (one-stage chronometric assay) [33]. Capacity of plasma VWF to bind FVIII (FVIII:BD) was zero (Fig. 1) consistent with the diagnosis of VWD 2N after 5 years of misdiagnosis of mild hemophilia A after a bleeding episode following appendectomy. After intranasal spray of desmopressin VWF:Ag increased from 91 to 216 IU dL (x 2.37), while FVIII:C increased from 20 to 116 IU dL (x 5.8) and subsequently decreased with a short half-life of 3 hours. Sequencing of exons 18–27 of the patient's VWF gene revealed the presence of two molecular abnormalities in the heterozygous state: the 2561G to A transition in exon 20 inducing the R854Q mutation and the not yet reported 3223G to A transition in exon 25, inducing substitution of lysine for glutamic acid 1078 (E1078K). The patient's mother and sister who displayed subnormal FVIII:C levels (55 IU dL)1), normal multimeric profile and moderately decreased VWF:FVIIIB (Figure 1) harbored, respectively, only the R854Q or E1078K abnormality in the heterozygous state.

Experiences on VWD 2N from three European VWF Research Centers

In the extended Brno-Antwerp prospective collaborative VWF-VWD study of 320 collected VWD patients, the P812R missense mutation in the D' domain was rather frequent in the Brno VWD population (Smejkal., *et al*. manuscript in preparation, Table 6). Recessive cases with homozygous P812R/P812R presented with recessive type 3 or severe type 1 VWD with very low FVIII:C and VWF:Ag levels in two probands. The R854W mutation either double heterozygous with P812Rfs or homozygous R854W/R854W appeared to be the cause of recessive VWD 2N in all 11 cases in the extended cohort of the Brno VWD study (Table 6). Bleeding manifestation as measured by the Vincenza Bleeding Score (VBS) in VWD 2N patient in the Brno cohort are usually mild except in one. VWD 2N in the Brno study is featured by a decreased FIIIV:C binding to VWF associated with decreased FVIII:C/VWF:Ag ratio below 0.50 in all except one, which is associated with a typical VWD type 1 phenotype with normal VWF:GPIbM/VWF:Ag ratios. Double heterozygous VWD 2N mutated R854Q/P812R (null) have lower FVIII;C values as compared to homozygous R854Q/R854Q 2N VWD type 2N, similar as observed in the Italian Casonato study in table 3.

VWD	FVIII:C	VWF:Ag	GPIbM	FVIII:C/	GPIbM/	PFA-Epi	VBS	FVIII:B	Mutation defect
Type	%	%	%	Ag ratio	Ag ratio	seconds	score	%	D'Domain
2N	12	28	28	0.43	1.00	290		15	R854/P812rfs
2N	23	49	51	0.37	1.04	nt		16	R854/P812Rfs
2N	22	61	61	0.36	1.00	nt		10	R854/P812rfs
2N	33	48	62	0.69	1.29	146	2	8	R854/P812rfs
2N	13	29	22	0.45	0.76	240	4	<20	R854/P812rfs
2N	28	82	85	0.34	1.03	103	5	14	R854W/R854W
2N	18	75	75	0.24	1.00	219	19	12	R854W/R854W
2N	30	73	57	0.41	0.78	174	6	10	R854W/R854W
2N	30	86	90	0.35	1.04	98	0	10	R854W/R854W
2N	26	60	53	0.49	0.88	298	2	14	R854W/789Alat/Tyr
2N	22	69	73	0.30	1.05	163	10	11	not yet tested

Table 6: Laboratory finding in 11 cases with VWD 2N in the extended Brno cohort of VWD patients collected by Smejkal, Blatny and Penka

Conclusion: The R854W mutation either double heterozygous with P812Rfs or homozygous R854W/R854W is the cause of recessive VWD 2N in all 11 cases featured by decreased FVIII:C/VWF:Ag ratio below 0.50 except in one and a VWD type 1 VWF:GPIbM/VWF:Ag ratios in the Brno cohort of VWD patients . Double heterozygous VWD 2N mutated R854Q/P812R (null) have lower FVIII;C values as compared to homozygous R854Q/R854Q 2N VWD type 2N, similar as observed in the Italian Casonato study in table 3.

As part of the one center cohort of 92 Portugese VWD patients from 60 unrelated families comprising the entire spectrum of VWD patients study, Fidalgo., *et al*. from Portugal studied 12 VWD 2N probands with VWD 2N, who presented with 6 different variants of recessive homozygous or double heterozygous VWD 2N (Table 7) [34]. Seven different mutation combinations were found, which were located in the D'D' region as homozygous Arg854Gln/Arg-854Gln in seven, in the D'D' region as homozygous Arg816Trp/Arg816Trp in one, in the D'D3 region as double heterozygous arg854Gln/Asp879Glu in one, in the D'C1 region as double heterozygous Arg854Trp/Pro2297Leu in one, in the D'D1 region as Arg816/Arg34null and in the D'D3 region as His817Gln/Pro-1162Leu (Table 7). All VWD 2N patients had one R854Q or R816W mutation in the D' domain as the determinative cause of VWD 2N combined with a second D' mutation domain in eight cases and combined with a second mutation in the D1 (Arg34null), D3 (Asp-879Glu), D3 (Pro1162Leu) or C1 (Pro2297Leu) in four cases. The D' missense mutation homozygous for Arg816Trp or in combination with a null allele in two produced the lowest FVIII:C level of 7 to 11% reflecting a severe FVIII binding site defect whereas all VWF levels in homozygous Arg816Trp/Arg816Trp are completely normal and two times higher as compared to the Arg816Trp/null combination. The FVIII:C levels range from 18 to 33% and the VWF parameters were between 45 to 100% in the VWD 2N patients with the D' missense mutation homozygous for Arg854Gln in seven or in combination with a functional mutation defect located in the D3, D3 or C1 domain45 to 100%, which is completely in line with findings in the literature (Table 7) [34].

N Probands	FVIII:C	VWF:Ag	VWF:Rco	VIII:C/Ag	RCo/Ag	FVIII:BD	Mutation (Domain)	VWD type
Gender	U/mL	U/mL	U/mL	ratio	ratio	%		
2 M/5 F	0.28	0.75	0.71	0.37	0.95	0-2%	R854Q/R854Q (D'D')	2N
(range)	0.19-0.33	0.45-0.88	0.50-0.93					
1 F	0.18	0.46	0.50	0.39	1.09	.	R854Q/Pro2297Leu (D'C1)	2N
1 M	0.38	0.66	0.68	0.58	1.03	6%	R854Q/Asp879Glu (D'D3)	2N/2E
1 M	0.32	1.20	1.00	0.27	0.83	.	H817G/Pro1162Leu (D'D3)	2N/2E
1 F	0.07	1.00	1.00	0.07	1.00	.	R816W/R816W (D'D')	2N
1 F	0.11	0.52	0.47	0.20	0.94	0.5%	R816W/Arg34X (D'null)	2N

Table 7: Laboratory and molecular features of 7 probands with homozygous R854Q and 5 probands with double heterozygous mutations in the cohort of 92 VWD patients from 60 unrelated families in the Porteguese study [34]

Conclusion: The Portugese study confirms that R816W and R854Q are causative for 2N VWD (in blue) and discovered two new combinations of R854Q/Pro2297Leu missense mutation in the D'and C1 domain and the novel combination H817G and Pro1162Leu missense mutations in the D' and D3 are associated with VWD 2N and VWD 2N/E[34].

Among 28 Spanish 2N VWD patients 12 had a severe 2N phenotype of whom nine homozygous and three double heterozygous mutations for a FVIII binding site defect (FVIII:BD) (Table 8) [35,36]. The remaining 16 patients were heterozygous carriers of VWD 2N mutants and they presented with a mild 2N carrier state phenotype (Table 8) [36]. A compound phenotype 2N and 2A was found due to double heterozygosity of p.Asn879Asp and type 3 carrier status similar as described [30]. A single VWD 2N patient with a hybrid phenotype was compound heterozygous for the p. Cys-2773Ser in the CK domain and p.His817Gln in the D' domain (Table 8) [36]. Finally, Battle., *et al*. described three patients from two different families with a single mutation R763G at the Furin cleavage site associated with a VWD phenotype 2sm with decreased VIII:C/VWF:Ag ratios suspicious for moderate FVIII-BD defect mimicking VWD type 2N at the laboratory level (Table 9) [36]. Cases homozygous for R763G/R763G in the Furin cleavage site are predicted to produce non-cleaved proVWF associated a severe recessive secretion defect VWD type 1 but has not been reported in the literature. Heterozygous mutation R763G/WT in the Furin cleavage site produce a mild hybrid VWD phenotype 2N (FVIII binding defect of VWF) and type 1sm associated with aberrant multimerization (smeary pattern) caused by a mixture of mature and proVWF with reduced FVIII:C/VWF:Ag ratio (Table 9, Figure 10)

Proband	FVIII:C	VWF:Ag	VWF:Rco	VIII:C/Ag	RCo/Ag	FVIII:BD	Mutation (Domains)	VWD type
Gender	U/mL	U/mL	U/mL	ratio	ratio	U/ml		
1 F	0.05	0.59	0.86	0.08	1.46	0.00	R816W/R816W (D'D')	2N severe
1 F	0.05	1.74	1.71	0.03	0.99	0.00	R816W/R816W (D'D')	2N severe
1 F	0.05	0.51	0.49	0.10	0.96	0.00	R816W/R816W (D'D')	2N severe
1 F	0.08	1.14	0.93	0.07	0.82	0.00	R816W/R816W (D'D')	2N severe
1 M	0.10	1.02	1.10	0.10	1.08	0.10	R854Q/R854Q (D'D')	2N
1 M	0.12	0.42	0.40	0.29	0.95	0.02	R854Q/Arg324X (D'null)	2N
1 M	0.12	0.27	0.20	0.44	0.74	.	R854Q/Arg324X (D'null)	2N
1 F	0.14	0.33	0.34	0.42	1.03	0.05	R854Q/Gln895His(D'D3)	2N
1 F	0.16	0.32	0.32	0.50	1.00	0.06	R854Q/Gln895His(D'D3)	2N
1 M	0.24	0.44	0.56	0.55	1.27	0.10	R854Q/P2558X (D'null)	2N
Carriers								
1 F	1.24	0.74	0.95	1.68	1.28	0.56	R816W/WT	Carrier 2N
1 F	0.23	0.26	0.08	0.88	0.31	.	R816W/A1315C (D'A1)	2M smeary
1 F	1.10	1.28	1.49	0.87	1.16	0.52	R854Q/WT	Carrier 2N
1 F	1.14	0.67	0.71	1.70	1.06	0.49	R854Q/WT	Carrier 2N
1 F	0.46	0.62	0.61	0.74	0.98	0.65	R854Q/WT	Carrier 2N
1 M	0.54	0.34	0.34	1.59	1.00	0.57	R854Q/3144C>T (D'D3) plus 546 G>A D1	Carrier 2N
1 M	0.46	0.52	0.82	0.88	1.58	0.65	R854Q/Pro2063Ser (D'D4)	Carrier 2N
1 F	0.71	0.73	1.01	0.97	1.38	0.66	R854Q/3144C<T (D'D3)	Carrier 2N
1 M	0.57	0.75	0.60	0.76	0.80	0.44	R854Q/Intronic 11	Carrier 2N
1M	1.11	0.65	0.80	1.71	1.23	0.62	R854Q/ Intronic 19	Carrier 2N
1 F	1.08	0.82	0.85	1.31	1.02	0.63	R854Q/ Intronic 26	Carrier 2N

Table 8: Analysis of the genotype-phenotype correlation in 10 probands with VWD 2N and 11 carriers of 2N in the Spanish cohort of 480 VWD patients using next generation sequencing [36]

Conclusions. The Spanish study confirms previous findings that homozygous R816W and R854Q or combined with a null allele are causative for VWD 2N. A novel combination of R854Q/Gln895His missense mutations in the D'D3 domains produced a typical VWD 2N in 2 cases [36]. The double heterozygous R816W/Arg1315Cys mutation in the D'A1 domains results in VWD type 2M. The Pro2063Ser secretion defect (1SD) mutation in the D4 domain seems to me less pronounced as compared to Pro2063Ser/WT in VWD type 1 patients heterozygous for R854Q. Intronic and some other minor mutations (blue) do not influence the R854Q carrier phenotype.

Mutation	F/M Age	FVIII:C	VWF:Ag	VWF:RCo	VWF:CB	VIII:C/Ag	RCo/Ag	CB/Ag	VWD MM
Domain	Years	U/mL	U/mL	U/mL	U/mL	ratio	ratio	ratio	Smeary
R763G/WT	F 33	0.18	0.32	0.28	0.19	0.56	0.88	0.59	2sm 2N
R763G/WT	M 53	0.22	0.40	0.25	0.29	0.55	0.62	0.73	2sm 2N
R763G/WT	M 79	0.53	0.78	0.32	0.34	0.68	0.41	0.44	2sm 2N

Table 9: VWD type 2 smeary caused by heterozygous R763G/WT mutation in the Furin cleavage site diagnosed and decreased FVIII:C/VWF:Ag ratio not meeting the criteria for VWD 2N. The dominant R763G/WT mutation results in a mixture of circulating proVWF and mature VWF featured by a hybrid VWD phenotype of 2M/2N (Dominant VWD 2 Michiels-Normandy)

Discussion

Three non-cysteine VWD 2N mutations (T791M, R816W and R854Q) and R854Q in particular account for the majority of VWD 2N patients (Figure 13) [11]. The non-cysteine VWD 2N mutations T791M, R816W, T789P, M800V, R816Q and H817Q are clustered around a region of positive charge density on TIL'. The VWF residues R782, G785 and E787 are on the opposite of the structure in close proximity to the β1-to-β2 loop (Figure 3) [21]. Experimental RFVIII and RVWF binding studies show that the FVIII-VWF binding defect is mildly to moderately decreased for the Arg854Gln and Cys1060Arg mutation and severely decreased for the Thr791M, Arg816Trp and Arg763Gly in both supernatants (Figure 14) and in cell lysates (Figure 14, lower right). Such variabilities in the degree of FVIII:BD in VWD 2N are consistent with the severity of VWD type 2N disease mild to moderate in homozygous Arg854Gln and severe in homozygous Arg816Trp seen in Table 2.

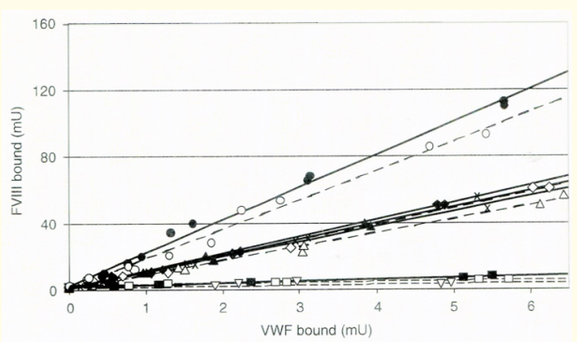


Figure 13: Binding of plasma and recombinant von Willebrand factor (rVWF) to factor FVIII in the study of Hilbert., *et al* 2004. Full symbols and straight lines are for plasma VWF. Empty symbols and dashed lines are for rVWF. Upper curves: FVIII to VWF-binding curves of rVWF and normal VWF [33]. Middle curves: FVIII binding to VWF curves of normal plasma and plasma of VWD2N with the heterozygous mutations R816W/WT, R854Q/WT and E1078K/WT. Lower curves: FVIII binding to VWF curves of double heterozygous K1078/Q854 and homozygous E1078K/E1078K mutations.

Source: [33].

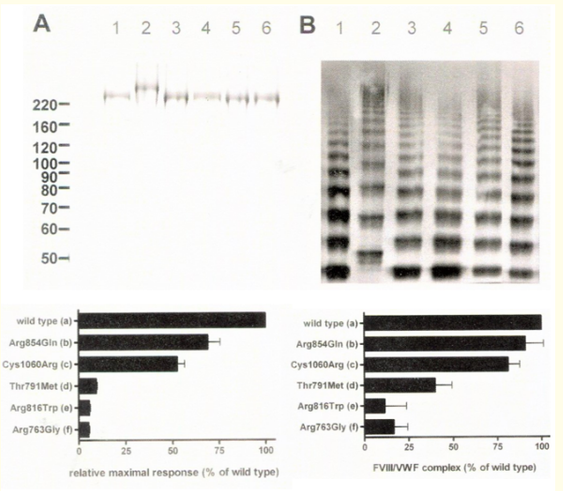


Figure 14: Purity and multimeric composition of recombinant von Willebrand factor (rVWF) type 2N von Willebrand disease (VWD). Mulitimeric composition was analyzed by 2.5% agarose gel electrophoresis followed by Western blot analysis using rabbit polyclonal antihuman VWF. Lanes correspond with wild type rVWF (lane 1), Arg763Gly Furin site mutation resulting in proVWF with abnormal VWF banding (lane 2), and three VWD 2N mutations Thr791Met (lane 3), Arg816Trp (lane 4) and Cys1060Arg (lane 6) showing mature VWF with normal VWF multimeric banding pattern indicating that FVIII-BD in VWD 2N mutants does not affect normal intracellular VWF multimerization, VWF storage in Weibel Palade bodies and secretion of VWF. Binding of recombinant FVIII (rFVIII) to wild type rVWF in control (lane a) and to mutant rVWF variants in VWD type 2N Arg854Gln (b), Cys1060Arg (c), Thr791Met (d) Arg816Trp € and Arg763Gly (f) expressed as relative maximal response percentage (%) of wild type rVWF (supernatant HEK293 cells, lower left) and as FVIII/VWF complex in percentage (%) of wild type RVWF (HEK293 cell lysates, lower right) in the rVWF expression study of Biggelaar., *et al* [5]. The FIIIV-VWF binding defect is mildly to moderately decreased for the Arg854Gln and Cys1060Arg mutation and severely decreased for the Thr791M, Arg816Trp and Arg-763Gly in both supernatants (lower left and in cell lysates (lower right) of rVWF expression studies. Such variabilities in the degree o FVIII:BD in VWD 2N are consistent with the severity of VWD type 2N disease mild to moderate in homozygous Arg854Gln and severe in homozygous Arg816Trp seen in Table 2.

Source: Plos One 2011;6(8):224163 [5].

Models of mutations in the N-terminal end of VWF TIL', E' and VWD3 domain with FVIII binding defect to VWF in patients with VWD 2N and 2E show that the FVIII C1 domain interacts with D' and the VWD3 platform (Figures 1 and 15). VWD 2N mutations cause defective binding to FVIII and map to D' and VWD3. The bipartite FVIII-binding site suggests that 2N mutations R854Q in the E' interface with TIL' and D879N in the Ca21-binding loop might indirectly inhibit FVIII binding by altering TIL'–E' and E'–D3 orientation (Figure 15) and thereby disturbing the orientation between the binding sites in TIL' and C8-3. The noncystein mutations P812L, H817Q, T791M,F787K, and M800V in the TIL'of the D' domain inhibit the FVIII binding by altering TIL'–E' and E'–D3 orientation in a similar way (Figure 15). The D879N mutation in the VWD3 Ca21-binding site disrupts the dual function of Asp-879 in coordinating Ca21 and hydrogen bonding to the backbone of the Ca21-binding loop (Dong., *et al*.) [37]. The D879N mutation causes both type 2E and 2N VW disease, and shows that the VWD3 Ca21-binding site is required both for efficient D3 multimerization and binding of FVIII to VWF. The specific type 2N Q1053H and E1078K mutations lie in C8-3 and decrease FVIII binding to VWF by 10-fold or more (Dong., *et al*.) [37]. VWD type 2E mutations result in the absence of long VWF multimers. The S979N mutation in C8-3 introduces a putative N-glycosylation site into its interface with VWD3 in close proximity to Cys-1099. Thus, S979N may disrupt proper intermodule orientation or structure around Cys-1099 required for D3 multimerization.

Non-cysteine VWD 2N mutations (T791M, R816W and R854Q) and R854Q in particular account for the majority of VWD 2N patients [11]. Noncystein mutations are associated with normal VWF levels, normal VWF multimers and normal platelet function (Figure 4 green spheres). VWD 2N cysteine mutations (C788R/Y; Y795C and C804F in TIL'; C858C/F in E') are associated with aberrant multimerization, poor secretion and reduced FVIII binding (Figure 4, black spheres). The 763 mutation in the Furin cleavage site is associated with the persistence of proVWF (Figures 10 and 14) which has a poor binding of FVIII to VWF (Figure 14). The hybrid rVWF R763G/R854Q displayed an abnormal multimeric pattern with broad bands (smeary pattern) and the presence of ultralarge VWF multimers (pro-VWF) also seen in the symptomatic carriers with the heterozygous R763G/WT mutation (Figure 10). The smeary pattern and presence of ultralarge VWF multimers is caused by the mixture of circulating non cleaved proVWF and normal mature VWF in carriers of the R763G mutation at the Furin cleavage site of VWF. The heterozygous R763G/WT mutant produces a mixture of circulating proVWF and mature VWF resulting in a smeary VWF multimeric pattern with a mild FVIII-BSD to VWF binding of about 40% to 60%(Dominant VWD 2M/2N, Table 9).

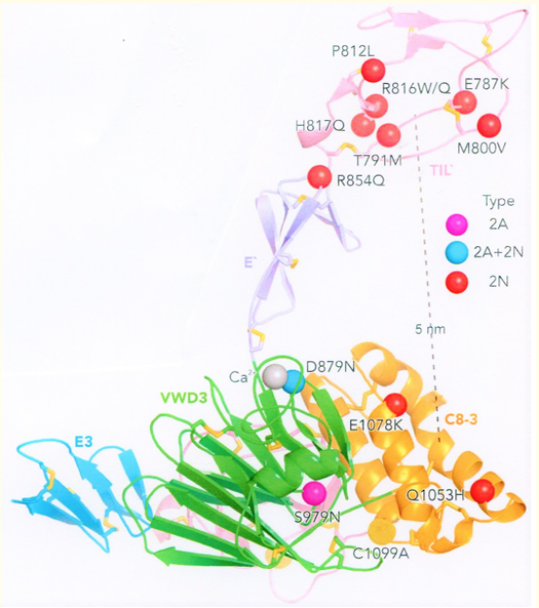


Figure 15: Model of mutations in the N-terminal end of VWF TIL', E' and VWD3 domain with FVIII binding defect to VWF in patients with VWD 2N and 2E (Dong., *et al*) [37]. (Reprinted with permission from Blood 2019;133(14):1523-1533).

Electron microscopy studies on FVIII–D'D3 binding complexes suggest that the FVIII C1 domain interacts with D' and appears to touch the VWD3 platform (Figure 1). TIL' approaches C8-3 more closely than any other VWD3 module. VWD 2N mutations cause defective binding to FVIII and map to D' and VWD3.The bipartite FVIII-binding site suggests that 2N mutations R854Q in the E' interface with TIL' and D879N in the Ca21-binding loop might indirectly inhibit FVIII binding by altering TIL'–E' and E'–D3 orientation, respectively, and disturbing the orientation between the binding sites in TIL' and C8-3. D879N in the VWD3 Ca21-binding site disrupts the dual function of Asp-879 in coordinating Ca21 and hydrogen bonding to the backbone of the Ca21-binding loop (Dong., *et al*)[37]. The D879N mutation causes both type 2E and 2N VWD disease, showing that the D3 Ca21-binding site is required both for efficient D3 dimerization and binding of FVIII. The specific type 2N Q1053H and E1078K mutations lie in C8-3 and decrease FVIII binding to rVWF by 10-fold and more than 10-fold. VWD type 2E mutations result in the absence of long VWF multimers. The S979N mutation in C8-3 introduces a putative N-glycosylation site into its interface with VWD3 in close proximity to Cys-1099. Thus, S979N may disrupt proper intermodule orientation or structure around Cys-1099 required for D3 multimerization.

Source: Blood 2019;133(14):1523-1533 [37].

Bibliography

1. Lenting PJ., *et al*. "The life cycle of coagulation factor VIII in view of its structure and function". *Blood* 92.11 (1998): 3983-3996.

2. Thompson AR. "Structure and function of factor VIIII gene and protein". *Sem Thromb Hemostas* 29.1 (2003): 11-22.

3. Leyte A., *et al*. "Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor". *Journal of Biological Chemistry* 266 (1991): 740-746.

4. Van den Biggelaar M., *et al*. "Requirements for cellular cotraffincking of factor VIII and von Willebrand factor to Weibel-Pallade bodies". *Journal of Thrombosis and Haemostasis* 5.11 (2007): 2235-2242.

5. Van den Biggelaar M., *et al*. "Storage of factor VIII variants with impaired von Willebrand factor binding in Weibel-Palade bodies in endothelial cells". *Plos One* 6.8 (2011): 224163.

6. Ebberink EHTM., *et al*. "Factor VIII/V C-domain swaps reveal discrete C-domain roles in factor VIII function and intracellular trafficking". *Haematologica* 102.4 (2017): 686-694.

7. Wang JW., *et al*. "Analysis of the storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from patients with von Willebrand disease". *Blood* 121.14 (2013): 2762-2772.

8. Jacquemin M., *et al*. "A novel cause of mild/moderate haemophilia A: mutations scattered in the factor VIII C1 domain reduce factor VIII binding to von Willebrand factor". *Blood* 96 (2000): 958-65.

9. Mazurier C., *et al*. "Type 2N von Willebrand disease". *Best Practice & Research Clinical Haematology* 14 (2001): 337-347.

10. Zhou Y-F., *et al*. "Sequence and structure relationships within von Willebrand factor". *Blood* 120.2 (2012): 449-458.

11. Shiltagh N., *et al*. "Solution structure of the major factor VIII binding region on von Willebrand factor". *Blood* 123.26 (2014): 4143-4151.

12. Chiu PL., *et al*. "Mapping the interaction between factor VIII and von Willebrand factor by electron microscopy and mass spectrometry". *Blood* 126.8 (2015): 935-938.

13. Yee A., *et al*. "Visualization of an N-terminal fragment of von Willebrand factor in complex with factor VIII". *Blood* 126.8 (2015): 939-942.

14. Krishnanwamy S. "FVIII-VWF dos-a-dos". *Blood* 126.8 (2015): 923-924.

15. Liu ML., *et al*. "Hemophilic factor VIII C1- and C2-domain missense mutations and their modeling to the 1.5-angstrom human C2-domain crystal structure". *Blood* 96 (2000): 979-987.

16. Nishino M., *et al*. "New variant of von Willebrand disease with defective binding to factor VIII". *Blood* 74 (1989): 1591-1599.

17. Mazurier C., *et al*. "A new von Willebrand factor (vWF) defect in a patient with factor VIII (FVIII) deficiency but with normal levels and multimeric patterns of both plasma and platelet vWF. Characterization of abnormal vWF/FVIII interaction". *Blood* 75.1 (1990): 20-26.

18. Tuley EA., *et al*. "Expression of von Willebrand factor "Normandy": an autosomal mutation that mimics hemophilia A". *Proceedings of the National Academy of Sciences of the United States of America*. 88 (1991): 6377-6381.

19. Mazurier C., *et al*. "Biological effect of desmopressin in eight patients with type 2N (Normandy) von Willebrand disease". *British Journal of Haematology* 88 (1994): 849-854.

20. Mazurier C and Meyer D. "Factor VIII binding assay of von Willebrand factor and the diagnosis of type 2N von Willebrand disease: results of an international survey on behalf of the subcommittee on von Willebrand factor of the Scientific and Standardization Committee of the ISTH". *Thrombosis and Haemostasis* 76 (1996): 270-274.

21. Goodeve A., *et al*. "Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD)". *Blood* 109 (2007): 112-121.

22. Casonato A., *et al*. "A common ancestor more that 10.000 years old for patients with R854Q-related type 2N von Willebrand's disease in Italy". *Haematologica* 98.1 (2013): 147-152.

23. Casonato A., *et al*. "Type 2N von Willebrand disease due to Arg-91Gln and a cysteine deletion in exon 18 of the von Willebrand gene". *British Journal of Haematology* 103 (1998): 39-41.

24. Zhang ZP., *et al*. "A single cytosine deletion in exon 18 of the von Willebrand factor gene is the most commonmutation inswedish VWD type 3". *Human Molecular Genetics* 9 (1992): 767-768.

25. Gadisseur A., *et al*. "Pseudohemophilia of Erik von Willebrand (VF) caused by one nucleotide deletion in exon 18 of the von Willebrand factor gene". *World Journal of Hematology* 2 (2013): 99-108.

26. Schneppenheim R., *et al*. "Recombinant expression of mutations causing von Willebrand disease type Normandy: characterization of a combined defect of factor VIII binding defect and multimerization". *Thrombosis and Haemostasis* 92 (2004): 36-41.

27. Jorieux S., *et al*. "Conformational changes in the D' domain of von Willebrand factor induced by CYS 25 and Cys 95 mutations lead to factor VIII binding defect and multimeric impairment". *Blood* 95 (2000): 3130-3145.

28. Casonato A., *et al*. "An Arg760Cys mutation in the consensus sequence of the von Willebrand factor propeptide cleavage site is responsible for a new von Willebrand disase variant". *Blood* 101 (2003): 151-156.

29. Hilbert L., *et al*. "Type 2N von Willebrand disease due to compound heterozygosity for R854Q and a novel R763G mutation at the cleavage site of von Willebrand factor propeptide". *Thrombosis and Haemostasis* 96 (2006): 290-294.

30. Hilbert L., *et al*. "Expression of two type 2N von Willebrand disease mutations identified in exon 18 of von Willebrand factor". *British Journal of Haematology* 127 (2004): 184-189.

31. Jorieux S., *et al*. "A novel mutation in the D3 domain of von Willebrands factor markedly decreases its ability to bind Factor VIII and affects its multimerization". *Blood* 92.12 (1998): 4663-4670.

32. Hilbert L., *et al*. "Two novel mutations Q1053H and C1060R, located in the D3 domain of von Willebrand factor are responsible for decreased FVIII-binding capacity". *British Journal of Haematology* 120 (2003): 627-632.

33. Hilbert L., *et al*. "First identification and expression of a type 2N von Willebrand disease (E1078K) located in exon 25 of von Willebrand gene". *Journal of Thrombosis and Haemostasis* 2 (2004): 2271-2273.

34. Fidalgo T., *et al*. "Genotype phenotype correlations Genotype phenotype correlations in a cohort of Portuguese patients comprising the entire spectrum of VWD types: impact of NGS". *Thrombosis and Haemostasis* 116 (2016): 17-31.

35. Battle J., *et al*. "Molecular and clinical profile of von Willebrand disease in Spain (PCM-EVW-ES): proposal for a new diagnostic paradigm". *Thrombosis and Haemostasis* 115 (2016): 40-50.

36. Borràs N., *et al*. "Molecular and clinical profile of 2017 von Willebrand disease in Spain (PCM-EVW-ES): comprehensive genetic analysis by next-generation sequencing of 480 patients". *Haematologica* (2017).

37. Dong X., *et al*. "The von Willebrand factor D'D3 assembly and stractural principles forFVIII binding and contamer biogenesis". *Blood* 133.14 (2019): 1523-1533.

38. Springer TA. "Von Willebrand factor, Jedi knight of the bloodstream". *Blood* 124 (2014): 1412-1425.

39. Pritchard Ruth Bronwen. "Developing integrated methods for probing flexible, hidden and metastable protein states: from side chains to global dynamics". Doctoral thesis (Ph.D), UCL (University College London) (2018).

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