

## Challenges in defining type 2M von Willebrand disease: results from a Canadian cohort study

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**To cite this article:** James PD, Notley C, Hegadorn C, Poon M-C, Walker I, Rapson D, Association of Hemophilia Clinic Directors of Canada, Lillicrap D. Challenges in defining type 2M von Willebrand disease: results from a Canadian cohort study. *J Thromb Haemost* 2007; 5: 1914–22.

**Summary.** *Background/methods:* In order to better characterize the genotype–phenotype correlation in type 2M von Willebrand disease (VWD), we sequenced the coding region for the mature subunit of the von Willebrand factor (*VWF*) gene (exons 18–52, including exon/intron boundaries) in 16 index cases originally submitted to the Canadian Type 1 VWD Study as type 1 VWD, but reclassified as type 2M VWD on the basis of phenotype (excessive mucocutaneous bleeding and von Willebrand factor: antigen (VWF:Ag) and/or von Willebrand factor: ristocetin cofactor (VWF:RCo) between 0.05 and 0.50 IU mL<sup>-1</sup> on at least two occasions and RCo/Ag ratio < 0.6 and no loss of high molecular weight multimers). Available family members (16 affected, 23 unaffected and six unknown) were sequenced for identified mutations. *Results:* We identified eight different missense mutations (R854Q, T1054M, R1315C, R1374C, R1374H, L1382P, S2179F, and T2647M) within these 16 families. We were significantly more likely to identify a *VWF* mutation in cases with RCo/Ag ratios < 0.50 ( $P < 0.05$ , chi-squared test). Importantly, every index case with an RCo/Ag ratio < 0.40 (4/4 index cases) had a mutation identified within the A1 domain, in contrast to 1/12 cases with an RCo/Ag ratio > 0.40. Difficulties with the standardization of the VWF:RCo may be responsible for the heterogeneity in cases with RCo/Ag ratios between 0.40 and 0.60. *Conclusions:* The genotype–phenotype correlation for cases with RCo/Ag ratios < 0.40 is clear. On the basis of our results, the phenotypic definition of type 2M VWD may need to be more stringent, and should be the subject of an international standardization initiative.

**Keywords:** mutations, type 2M VWD, Von Willebrand disease.

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Received 7 May 2007, accepted 14 June 2007

### Introduction

von Willebrand disease (VWD) is a common, inherited bleeding disorder caused by deficiency or dysfunction of von Willebrand factor (VWF). VWF circulates in the plasma as a multimeric glycoprotein, and plays key roles in hemostasis; it mediates platelet adhesion and aggregation at sites of vascular injury, and also binds and stabilizes factor VIII (FVIII) in the circulation [1].

The incidence of VWD varies widely between publications [2,3]. There are three main subtypes, classically characterized by excessive mucocutaneous bleeding, a positive family history, and abnormal VWF laboratory studies. Type 1 VWD is a partial deficiency of qualitatively normal VWF, type 2 VWD is caused by functionally abnormal VWF, and type 3 VWD represents a virtual absence of the VWF protein. Type 2 VWD is further divided into four subtypes: type 2N is characterized by abnormal binding of VWF to FVIII, type 2A by defective VWF-dependent platelet adhesion because of decreased high molecular weight (HMW) VWF multimers, type 2B by pathologically increased VWF–platelet interactions leading to the depletion of HMW VWF multimers, and type 2M by decreased VWF–platelet interactions not caused by the loss of HMW multimers [4]. The *VWF* gene was cloned and characterized by four groups simultaneously in 1985 [5–8], allowing for an improved understanding of the molecular basis of VWD.

Type 2M VWD, which is the focus of this report, is classically considered to be caused by decreased VWF–platelet interactions. Mutations within the A1 domain of the *VWF* gene, the region that encodes the binding site for platelet glycoprotein (GP)1b $\alpha$  [9–13] have been reported as causing type 2M VWD. Additionally, a missense mutation in the A3 domain has been reported to reduce VWF binding to collagen, thereby reducing platelet adhesion and also possibly causing type 2M VWD [14]. Recent large studies focusing on the molecular genetic basis of type 1 VWD have demonstrated the lack of a clear distinction between some VWD subtypes, namely type 1, type 2M and type 2A [15–17]. This distinction is important because of the variability of response to treatment

such as desmopressin [18]. The overlap of these subtypes appears to occur not only at the phenotypic level, but also at the genotypic level, and raises important questions about how these subtypes are currently defined. In this article, we describe 16 type 2M families (defined on the basis of phenotype), and highlight the similarity of those with RCo/Ag ratios between 0.40 and 0.60 to our type 1 VWD population. We also highlight the clear genotype–phenotype correlations in those cases with RCo/Ag ratios < 0.40.

## Patients, materials and methods

### Patients

In total, 194 families were submitted for enrolment to the Canadian Type 1 VWD Study [15] from 13 academic health science centers across Canada. After review, the index cases of 16 families met the phenotypic criteria for type 2M VWD. These criteria include: excessive mucocutaneous bleeding (defined as the occurrence of one or more significant mucocutaneous bleeding symptoms by the tertiary care Inherited Bleeding Disorders Clinic who enrolled the patient) and VWF:Ag and/or VWF:RCo between 0.05 and 0.50 IU mL<sup>-1</sup> on at least two occasions and mean RCo/Ag ratio < 0.6 and no loss of HMW multimers. Although an RCo/Ag ratio < 0.7 is often used to define type 2M VWD [19,20], on the basis of previous experience with the VWF:Ag and VWF:RCo assays within our laboratory, we have used a more stringent RCo/Ag ratio of < 0.6 to define type 2M disease in this study. The absence of a positive family history was not an exclusion criterion. All participants in the study were informed of the experimental nature of the study and gave informed consent. The study was approved by the Research Ethics Board of Queen's University and at each of the source institutions. Whole blood samples were collected by phlebotomy in both 3.2% sodium citrate (at a ratio of 9:1 v/v) and EDTA tubes from the index case and available family members. A plasma sample from the index case was frozen, and repeat VWD phenotypic studies were performed in a central laboratory.

### Coagulation studies

Laboratory tests for VWF:Ag, VWF:RCo and FVIII coagulant activity (FVIII:C) were performed at the source clinic attended by the patient according to local methods. These tests were repeated on frozen plasma samples (drawn at a separate time) at a central laboratory, the Clinical Hemostasis Laboratory at Kingston General Hospital (KGH) (Canada), and all available baseline (non-desmopressin trial) laboratory results were averaged. VWF laboratory tests at KGH were performed according to published methods [21]. The method used for performing the VWF:RCo assay was platelet aggregometry in both the central and source laboratories. In many cases, from the referral clinics, the VWF:RCo results were not reported as an absolute value, but as < 0.10 IU mL<sup>-1</sup>, for example. In

order to analyze our data, however, we needed to assign a numeric value to the VWF:RCo (to calculate the mean VWF:RCo and RCo/Ag ratio for an individual). Therefore, for a case with a VWF:RCo < 0.10 IU mL<sup>-1</sup> we used 0.10 IU mL<sup>-1</sup> as the absolute value, and we recognize that we may have overestimated the true VWF:RCo level and the RCo/Ag ratio in some cases. FVIII assays were performed according to published methods [22], as were VWF multimer analyses [23]. The VWF multimers were reviewed by three independent observers, and rated for the presence/absence of HMW multimers and triplet structure. Desmopressin trials were performed according to local institutional practises.

### DNA sequencing

A blood sample was obtained from all of the index cases, and genomic DNA was isolated from leukocytes using a salt extraction method [24]. Given the fact that type 2M VWD represents a qualitative subtype, full-length sequencing of the entire *VWF* coding region was not performed. DNA encoding the mature subunit (exons 18–52, including an average of 68 bp of sequence at the splice acceptor site and 98 bp of sequence at the splice donor site around each exon) was amplified by polymerase chain reaction (PCR) for all index cases. Primer sequences are available upon request. PCR amplification and purification of the products were performed using previously published methods [15]. The DNA sequences were compared with consensus *VWF* DNA sequences, with the assistance of Vector NTI Suite software (InforMax, Bethesda, MD, USA). When a putative mutation was identified, another template was PCR-amplified from the index case, and the opposite DNA strand was sequenced to confirm the sequence variation. Also, all available family members were sequenced for the putative mutation to confirm familial transmission. All sequence alignments and every sequencing chromatogram was analyzed independently by two technologists.

### ABO blood group genotyping

The method used for ABO blood group genotyping has been previously published [15].

## Results

### Patients and families

In total, 16 families (61 individuals) are reported here: 32 affected subjects, 23 unaffected subjects, and six subjects whose status is unknown. The mean number of individuals per family was 3.8. Ten of the families were two-generation families, five were three-generation families, and one was a single-generation family. Twelve of the index cases were female, and four were male. The mean age for the index cases was 24 years (range: 1–73 years). Half of the index cases (8/16) recorded their ethnicity as Caucasian, three as French Canadian, and two as Asian. The ethnicity of three index cases was unknown.

### Phenotype

All of the index cases reported excessive mucocutaneous bleeding. The most frequently reported symptom was menorrhagia (among females > 12 years); this was reported by 70% (7/10). Half (8/16) of the index cases reported easy bruising, and half (8/16) reported epistaxis. Approximately 38% (6/16) reported significant postoperative bleeding, 31% (5/16) reported excessive bleeding from minor wounds, and 13% (2/16) reported postdental procedure bleeding.

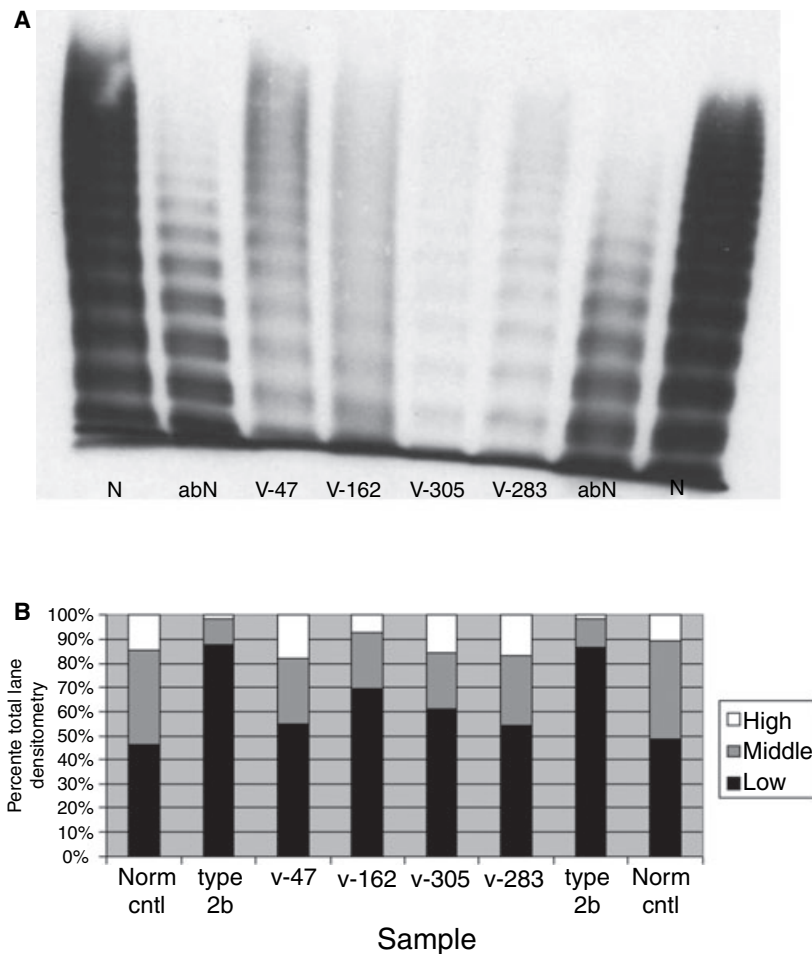
The mean VWF:Ag level for all index cases was 0.40 IU mL<sup>-1</sup> (range: 0.16–0.88 IU mL<sup>-1</sup>), the mean VWF:RCo level was 0.19 IU mL<sup>-1</sup> (range: 0.01–0.43 IU mL<sup>-1</sup>), and the mean FVIII level was 0.54 IU mL<sup>-1</sup> (0.20–1.04). The mean RCo/Ag ratio was 0.46 (range: 0.04–0.59). The correlation of source vs. central laboratory RCo/Ag ratios was excellent (correlation coefficient = 0.94). The multimers for 15 of the index cases were normal (no loss of HMW multimers; no abnormalities of triplet structure). One index case (V-162) showed a 'smearly' pattern [11] without obvious loss of HMW multimers (Fig. 1).

See Table 1 for phenotypic data on all index cases. None of the multimers showed an increase in HMW forms, as has been previously described in type 2M VWD (IC in the previous classification) [25]; however, the smearly pattern in V-162 is consistent with the concomitant loss of satellite bands seen in VWD type IC.

Desmopressin trial results are available for five of the index cases and three affected family members. The responses were generally poor. The mean increase in VWF:RCo in cases with baseline ratios > 0.40 was 0.29 IU mL<sup>-1</sup>, as compared to an increase of 0.14 IU mL<sup>-1</sup> in cases with baseline ratios < 0.40. This difference does not reach the level of statistical significance; however, this may be reflective of the small sample size. Detailed desmopressin results are shown in Tables 2 and 3.

### Sequencing results

We identified eight different putative missense mutations within seven of the 16 index cases (Tables 4). Six of these mutations have been previously reported (R1315C, R1374C,



**Fig. 1.** Multimer analysis. (A) Representative multimer analysis of four index cases for this study. V-47, V-305 and V-283 were all reported as normal, with no abnormalities of the triplet structure. V-162 was reported as no loss of high molecular weight (HMW) multimers, but smearly triplet pattern. The mutation identified in V-162 is T1054M, which lies within the D3 domain. (B) Distribution of the low, middle and HMW multimers: normal plasmas, 47.6% low ( $\pm 1.5\%$ ), 39.7% middle ( $\pm 1.3\%$ ) and 12.7% high ( $\pm 2.8\%$ ); type 2M von Willebrand disease plasmas, 60.2% low ( $\pm 7.2\%$ ), 25.2% middle ( $\pm 3.1\%$ ) and 14.5% high ( $\pm 4.7\%$ ). All values represent mean  $\pm$  SD.

**Table 1** Phenotypic data on all 16 index cases

ID	Age (years)	Gender	Mean VWF:Ag (IU mL <sup>-1</sup> )	Mean VWF:RCo (IU mL <sup>-1</sup> )	Mean FVIII (IU mL <sup>-1</sup> )	Mean RCo/Ag ratio	Multimers	ABO	Symptoms
V-013	9	F	0.21	0.12	0.43	0.57	Normal	A	b
V-047	36	F	0.37	0.20	0.94	0.54	Normal	O	abcd
V-052	40	F	0.45	0.25	0.63	0.56	Normal	A	acdef
V-128	25	F	0.68	0.38	0.33	0.56	Normal	O	bd
V-139	4	M	0.88	0.43	1.04	0.49	Normal	A	ce
V-145	19	F	0.58	0.31	0.83	0.53	Normal	A	d
V-162	4	F	0.54	0.29	0.49	0.54	Smeary	O	b
V-283	21	M	0.22	0.01	0.26	0.04	Normal	O	ae
V-305	1	M	0.25	0.14	0.33	0.56	Normal	A	a
V-347	9	F	0.64	0.38	0.97	0.59	Normal	O	b
V-402	14	F	0.18	0.10	0.2	0.56	Normal	O	ad
V-415	37	F	0.22	0.10	0.56	0.45	Normal	A	ae
V-421	73	M	0.32	0.10	0.31	0.31	Normal	A	be
V-431	52	F	0.16	0.06	0.36	0.38	Normal	A	abcdef
V-515	11	F	0.47	0.25	0.56	0.53	Normal	A	b
V-605	25	F	0.19	0.03	0.33	0.15	Normal	A	acd

Laboratory values are means of every baseline (non-DDAVP trial) result. Multimers were evaluated by three independent reviewers for the presence/absence of high molecular weight (HMW) forms and for triplet structure. All were normal for both, except for V-162. In this case, there was no obvious loss of HMW forms, but the triplet structure was 'smeary'. For the final column, listing mucocutaneous bleeding symptoms, a = bruising, b = epistaxis, c = excessive bleeding from wounds, d = menorrhagia, e = postoperative bleeding, and f = excessive bleeding following dental extraction/work. These mucocutaneous bleeding symptoms were deemed to be significant by the Inherited Bleeding Disorder Clinic who enrolled the patient.

VWF, von Willebrand factor.

**Table 2** Desmopressin responsiveness; VWF:Ag, VWF:RCo and FVIII levels

	Pre-VWF:Ag (IU mL <sup>-1</sup> )	Post-VWF:Ag (IU mL <sup>-1</sup> )		Pre-VWF:RCo (IU mL <sup>-1</sup> )	Post-VWF:RCo (IU mL <sup>-1</sup> )		PreFVIII (IU mL <sup>-1</sup> )	Post-FVIII (IU mL <sup>-1</sup> )		Mutation
		1 h	3 h		1 h	3 h		1 h	3 h	
V-402	0.22	0.51*	0.39	< 0.10	0.43*	0.23	0.33	1.09*	0.54	None
V-415	0.17	0.22	NA	0.14	< 0.10	NA	0.53	0.56	NA	T2647 M S2179 F
Daughter	0.20	0.74	0.39	0.14	0.73	0.26	0.28	1.06	0.66	S2179 F
V-421	0.39	0.30	0.53	< 0.10	0.19	0.20	0.72	0.44	0.73	R854Q R1315C
Daughter no. 1	0.31	0.65	0.55	< 0.10	0.32	0.25	0.68	1.02	0.80	R1315C
Daughter no. 2	0.21	0.55	0.34	< 0.10	< 0.10	0.10	0.75	0.56	0.59	R1315C
V-431	0.07	0.23	NA	< 0.05	0.25	NA	0.39	1.06	NA	R1374C
V-605	0.11	0.33	NA	Absent	< 0.10	NA	0.39	0.96	NA	L1382P

The route of administration was i.v. in all cases, except for V-431, where it was s.c. The dose was 0.3 µg kg<sup>-1</sup> (max. 20 µg). For V-402, the values with an asterisk were obtained at 30 min, rather than 1 h.

VWF, von Willebrand factor; NA, not available.

R1374H, R854Q, S2179F and T2647M), and two are new (T1054M and L1382P). Five index cases had one mutation identified, and two index cases had two putative mutations identified. Nine index cases had no mutations identified in the mature VWF subunit coding region; however, the RCo/Ag ratio was 0.49 or greater in every one of these nine cases. We were significantly more likely to identify a *VWF* mutation in cases with an RCo/Ag ratio < 0.50 ( $P < 0.05$ , chi-squared test), and every index case with a ratio < 0.40 had a mutation identified within the A1 domain (4/4 index cases with ratio < 0.40, as compared to 1/12 index cases with ratio > 0.40). Additionally, as has been described in type 1 VWD, we were more likely to identify a *VWF* mutation in cases with lower VWF:Ag levels. Nevertheless, in this population,

when the actual RCo/Ag ratio and VWF:Ag levels were used, rather than dichotomous variables, a low RCo/Ag ratio was more strongly associated with the identification of a *VWF* mutation ( $P = 0.016$ , Mann-Whitney *U*-test) than was a low VWF:Ag level ( $P = 0.071$ , Mann-Whitney *U*-test).

#### Cases with multiple mutations

Two putative missense mutations were identified in V-415: S2179F and T2647M. However, we believe that S2179F is the pathogenic mutation in this family, for the following reasons. The S2179F mutation cosegregates with affected status in this family, whereas T2647M does not. Additionally, the S2179F mutation has recently been shown to reduce VWF clearance

**Table 3** Desmopressin responsiveness; RCo/Ag ratios

	Baseline RCo/Ag ratio	Pre-RCo/Ag	1 h post-RCo/Ag	3 h post-RCo/Ag	Mutation
V-402	0.56	0.45	0.84	0.59	None
V-415	0.45	0.82	0.45	NA	T2647M S2179F
Daughter	NA	0.7	0.97	0.67	S2179F
V-421	0.31	0.26	0.63	0.38	R854Q R1315C
Daughter no. 1	NA	0.32	0.49	0.45	R1315C
Daughter no. 2	0.53	0.48	0.18	0.29	R1315C
V-431	0.38	0.71	1.07	NA	R1374C
V-605	0.15	0	0.30	NA	L1382P

The left-hand column shows the baseline ratio (mean of non-desmopressin trial results). The remainder of the RCo/Ag ratios presented here (pre-RCo/Ag, 1 h post-RCo/Ag, 3 h post-RCo/Ag) are calculated from a single result from each of the source laboratories (aliquots were not collected for retesting in the central laboratory).

**Table 4** Mutations and other sequence variation identified in index cases

ID	RCo/Ag ratio	Mutations identified	Other sequence variation identified
V-283	0.04	R1374H*	
V-605	0.15	L1382P*	
V-421	0.31	R854Q and R1315C*	c.6975 + 85_86delGT
V-431	0.38	R1374C*	
V-415	0.45	T2647M and S2179F	c.5842-111G > A
V-139	0.49	None	
V-515	0.53	None	c.6063 + 25 G > A
V-047	0.54	None	c.7056C > T, c.55-40C > T
V-145	0.53	None	
V-162	0.54	T1054M	c.6799-14T > C
V-052	0.56	None	c.6975 + 85_86delGT
V-305	0.56	R1315C*	
V-128	0.56	None	
V-402	0.56	None	c.8115 + 30T > G, c.6975 + 85_86delGT
V-013	0.57	None	
V-347	0.59	None	

The table is organized by ascending RCo/Ag ratio, in order to highlight the likelihood of identifying a mutation in cases with lower ratios. The mutations marked with an asterisk are all within the A1 domain, which encodes the von Willebrand factor–platelet GP1b $\alpha$  binding site.

to < 3 h [26] (which is consistent with the desmopressin trial results for the affected daughter of V-415, who only has S2179F). Interestingly, however, the mechanism of accelerated clearance is consistent with a type 1 VWD phenotype, not type 2M VWD. The RCo/Ag ratio in this index case is 0.45, and that in the affected daughter is 0.70, so this case might more appropriately be classified as type 1 VWD.

Two mutations were also identified in V-421: R854Q and R1315C. In the homozygous state, R854Q causes type 2N VWD [27], but it has often been reported in other VWD subtypes in the heterozygous state [15,16]. Although R1315C is listed in the ISTH VWF SSC database (<http://www.vwf.group.shef.ac.uk/index.html>) as a type 2M mutation, and lies within the platelet GP1b-binding region, it has been reported to affect multimerization [28]. This is not the case with our patient, in whom the RCo/Ag ratio is 0.31 and VWF multimers are normal.

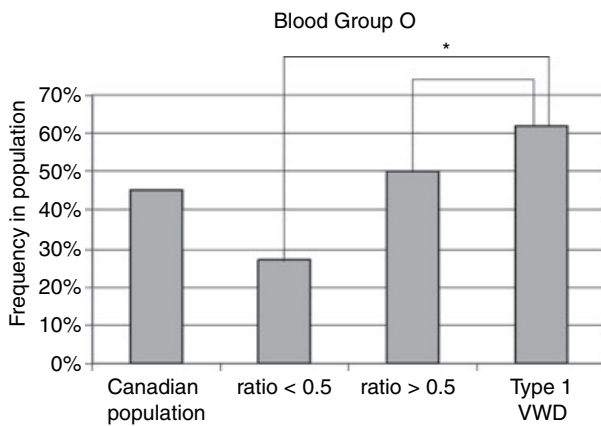
#### Low-VWF:Ag, mutation-negative cases

Two index cases merit further comment: V-13 and V-402. Neither case had a *VWF* mutation identified in the region

encoding the mature subunit, although both had VWF:Ag levels at the lower end of the spectrum (V-13 VWF:Ag = 0.21 IU mL<sup>-1</sup>, and V-402 VWF:Ag = 0.18 IU mL<sup>-1</sup>). It is possible that there are *VWF* mutations in these cases outside of the area that we sequenced for this report (i.e. in exons 1–17); however, exons 1–17 are not known to be involved with VWF–platelet or collagen interactions, except through the facilitation of multimer assembly. Both cases had RCo/Ag ratios > 0.50, and might be better classified as type 1 VWD cases. It is known that 30–37% of type 1 VWD index cases do not have *VWF* mutations identified, and despite the fact that *VWF* mutations were found more readily in cases with lower VWF:Ag levels, cases with low VWF:Ag levels (< 0.20 IU mL<sup>-1</sup>) in whom no *VWF* mutations were found have been described [15,16].

#### Family studies

Formal linkage and association studies have been performed on our overall study population, and the results from the type 1 VWD families are reported separately [29]. As reported in that publication, the LOD scores in most of our type 1 VWD



**Fig. 2.** Frequency of blood group O. The relative frequency of blood group O in four different groups. Canadian population refers to the general Canadian population, ratio < or > 0.5 refers to the type 2M von Willebrand disease (VWD) population stratified on the basis of RCo/Ag ratio, and type 1 VWD refers to the frequency of blood group O as reported in our overall type 1 VWD publication (James *et al. Blood* 2007; 109: 145–54.). There is no statistical difference between the frequency of blood group O in the type 1 VWD population and in the higher-ratio (> 0.5) group ( $P = 0.506$  Fisher's exact test, two-sided); however, there is a significant difference (\*) between the frequency of blood group O in the type 1 VWD population and in the lower-ratio (< 0.5) group ( $P = 0.035$ , Fisher's exact test, two-sided).

families cluster tightly around zero, as a result of the structure and relatively small family size in our study. This is consistent with the findings in most of our type 2M families; however, there is one family that deserves special mention. The LOD score for the family of V-047, which, in addition to the index case, includes the father, mother, sister and niece (daughter of the sister), is  $-1.85$ , suggesting non-linkage. There were no *VWF* mutations identified within this family, and the RCo/Ag ratio in the index case is 0.54.

In every family in which a *VWF* mutation was identified in the index case, there is complete cosegregation of affected status with the presence of the presumed pathogenic mutation. Conversely, there are no informative data available (linkage or association) on the cases in which a mutation was not identified in the region encoding the mature subunit.

#### ABO blood group

ABO results for each index case are shown in Table 1; six of the 16 index cases (38%) included in this report are blood group O. However, the relative frequency of blood group O in this population varies with the RCo/Ag ratio; cases with higher ratios are more likely to be blood group O. Half of the cases with ratios > 0.5 are blood group O (5/10), as compared to 17% (1/6) of index cases with ratios < 0.5 ( $P =$  non-significant, Fisher's exact test, two-sided) (Fig. 2). The frequency of blood group O in the Canadian type 1 VWD population is 62% [15], and there is a significant difference between this and the frequency of blood group O in the low-ratio group ( $P = 0.035$ , Fisher's exact test, two-sided). Given that type 2M VWD is a qualitative disorder, and that the effect

	A1/A3+	A1/A3–	Total
Ratio < 0.40	4	0	4
Ratio > 0.40	1	11	12
Total	5	11	16

Sensitivity = 80%, Specificity = 100%

	A1/A3+	A1/A3–	Total
Ratio < 0.50	4	2	6
Ratio > 0.50	1	9	10
Total	5	11	16

Sensitivity = 80%, Specificity = 81.8%

	A1/A3+	A1/A3–	Total
Ratio < 0.60	5	11	16
Ratio > 0.60	13	110	123
Total	18	121	139

Sensitivity = 27.8%, Specificity = 90.0%

**Fig. 3.**  $2 \times 2$  Tables for RCo/Ag ratio and A1/A3 domain von Willebrand factor (*VWF*) mutation identified (+) or not identified (–);  $2 \times 2$  tables for three different levels of RCo/Ag ratio. Sensitivity and specificity for the identification of an A1/A3 domain *VWF* mutation are shown immediately beneath each  $2 \times 2$  table for that level of RCo/Ag. The values for ratio > 0.60 are from our type 1 von Willebrand disease publication (James *et al. Blood* 2007; 109: 145–54.).

of blood group O on *VWF* is felt to be quantitative in nature, we would not expect to see an enrichment of blood group O in a true type 2M population [30]. The increased frequency of blood group O in the index cases with higher RCo/Ag ratios (> 0.50) supports the classification of these individuals as type 1 VWD as opposed to type 2M VWD.

#### Analysis of RCo/Ag ratio as a predictor for *VWF* mutations in the A1/A3 domain

We evaluated the sensitivity and specificity of RCo/Ag ratios of < 0.4, 0.5 and 0.6 for predicting whether or not a mutation in the A1 or A3 domain of *VWF* was identified. Detailed results are shown in Fig. 3.

#### Discussion

According to the recently updated classification of VWD published by the Subcommittee on *VWF* of the International Society of Thrombosis and Hemostasis, type 2M VWD includes 'qualitative variants with decreased *VWF*-dependent platelet adhesion without a selective deficiency of HMW *VWF* multimers. The assembly and secretion of large molecular weight multimers is approximately normal and the functional defect is caused by mutations that disrupt binding of *VWF* to

platelets or subendothelium. Most cases of VWD type 2M have been identified based upon a value for VWF:RCo that is disproportionately low compared with VWF:Ag and such patients usually have mutations within VWF A1 domain that impair binding to platelet Gp1b' [4]. In this article, we have shown that for the genotype–phenotype correlation to be as clear as intended in this classification, the ratio of VWF:RCo to VWF:Ag that distinguishes type 2M from type 1 must be quite low; in fact, lower than what has traditionally been felt to represent the cut-off.

In the Canadian VWD population, an RCo/Ag ratio  $< 0.40$  (in individuals with normal multimers) is strongly associated with mutations in the A1 domain of VWF. Cases with ratios of  $> 0.40$  or perhaps  $> 0.50$  might more accurately be classified as type 1 VWD, because of the heterogeneity within this group. VWF A1 domain mutations are not always found in this group, and additionally there is an enrichment of blood group O, as seen in a type 1 VWD population. Within the population reported here, the only family with evidence of VWF non-linkage (which has been reported in a subset of type 1 VWD families) [15,16] had an RCo/Ag ratio in the index case of 0.54. Additionally, we suspect that with more desmopressin trial information, we would have been able to show a correlation between low RCo/Ag ratios and poorer desmopressin responses.

Our analysis of the sensitivity and specificity of the RCo/Ag ratio for the prediction of mutations within the A1/A3 domains of VWF (Fig. 3) is another factor to consider in determining the appropriate RCo/Ag cut-off between types 1 and 2M VWD. On the basis of these results, an RCo/Ag ratio of  $< 0.50$  performs well as a screening test, with a reasonably high sensitivity and a lower specificity than for  $< 0.40$  or  $< 0.60$ . Therefore, we suggest that an RCo/Ag ratio of  $< 0.50$  be used to distinguish type 2M from type 1 VWD. Interestingly, there were 13/123 index cases with RCo/Ag ratios  $> 0.60$  with an A1 domain mutation identified. This is reflective of the genetic heterogeneity seen within a type 1 VWD population, and shows that in cases with higher ratios, an A1 domain mutation is not the sole determinant of the phenotype. It is possible that other genetic or environmental factors act as modifiers in these cases.

It is important to comment on the primary assay used to distinguish type 1 VWD from type 2M VWD in our study: the VWF:RCo. Although this assay has been felt by some to be the most useful for the diagnosis of VWD [19], we have found significant variability in the results of this test in Canada using the platelet aggregation method. This variability becomes even more problematic when the required discrepancy for type 2M VWD between the VWF:RCo and VWF:Ag is not extreme. Although we are suggesting a threshold of RCo/Ag ratio of  $< 0.50$  for type 2M VWD in Canada, it is important to recognize that this threshold will vary with the specific method and characteristics of that method in different institutions. Importantly, the cases included in this article came from nine different institutions in Canada, and therefore the mean VWF laboratory values reported here take into account the different

methodologies and variability between those nine institutions. We did not perform VWF:CB assays on our patients; this assay has previously been shown to be insensitive to mutations that impair VWF–platelet binding [31], but it is useful in settings where collagen binding is impaired. As none of our patients had A3 domain mutations, we do not feel that this assay would have added value to this article.

It is also important to consider the impact of multimer analysis on the distinction between type 2A and type 2M VWD in this study. The multimers in type 2M VWD are meant to be ‘approximately normal’ [4]; however, subtle abnormalities of triplet structure or the presence of increased HMW multimers are acceptable. It is critical to acknowledge, however, that multimer analysis is also technically challenging, and subtle distinctions such as those described here (i.e. V-162) may not always be appreciable.

In conclusion, type 2M VWD is often described in terms of the molecular pathogenesis underlying the condition, i.e., reduced platelet-dependent function of VWF (binding to either GPIb or collagen) without a deficit of HMW multimers. The genetic basis of this dysfunction is beginning to be characterized, and our understanding is likely to evolve as further knowledge is gained, and as further attempts to more carefully define type 2M VWD continue. In this article, we have described a cohort of phenotypically defined type 2M VWD cases, and have highlighted the clear genotype–phenotype correlations in cases with lower RCo/Ag ratios. We suggest that the threshold ratio for making the diagnosis of type 2M VWD needs further evaluation, preferably in the form of an international standardization initiative, and because of the heterogeneity seen in cases with higher ratios, propose that these might better be classified as type 1 VWD.

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### Acknowledgements

The authors thank the Canadian Association of Nurses in Hemophilia Care, C. Pruss and M. Bowman for their contributions to this project. This project was funded by a CIHR (Canadian Institutes for Health Research) Operating Grant (MOP-42467) and funds from CSL Behring. D. Lillicrap holds a Canada Research Chair in Molecular Hemostasis and is a Career Investigator of the Heart and Stroke Foundation of Ontario.

### Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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