Pathophysiology of von Willebrand factor in bleeding and thrombosis

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CHAPTER 6

VON WILLEBRAND FACTOR NEUTRALIZING AND NON-NEUTRALIZING ALLOANTIBODIES IN 213 SUBJECTS WITH TYPE 3 VON WILLEBRAND DISEASE ENROLLED IN 3WINTERS-IPS

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ABSTRACT

Background

Type 3 von Willebrand disease (VWD) is the most severe form of this disease owing to the almost complete deficiency of von Willebrand factor (VWF). Replacement therapy with plasma-derived products containing VWF or recombinant-VWF rarely cause the development of alloantibodies against VWF that may be accompanied by anaphylactic reactions.

Aim

To assess the prevalence of anti-VWF alloantibodies in type 3 VWD subjects enrolled in the 3WINTERS-IPS.

Methods

An indirect in-house ELISA has been used to test all alloantibodies against VWF. Neutralizing antibodies (inhibitors) have been tested with a Bethesda-based method using a VWF collagen binding assay (VWF:CB). Samples positive for anti-VWF antibodies were further tested with Bethesda-based methods using the semi-automated gain-of-function glycoprotein-Ib binding (VWF:GPIbM) and a VWF antigen (VWF:Ag) ELISA.

Results

18/213 (8.4%) subjects tested positive for anti-VWF antibodies and 13/213 (6%) had VWF:CB inhibitors. These 13 were among the 18 with anti-VWF antibodies. Of the five without VWF:CB inhibitors, three had non-neutralizing antibodies, one only inhibitor against VWF:GPIbM, and one could not be tested further. Ten of 13 subjects with VWF:CB inhibitors also had VWF:GPIbM inhibitors, 6 of which also had VWF:Ag inhibitors. Subjects with inhibitors were homozygous for VWF null-alleles (11/14), homozygous for a missense variant (1/14) or partially characterized (2/14).

Conclusions

Anti-VWF antibodies were found in 8.4% of type 3 VWD subjects, whereas neutralizing VWF inhibitors were found in 6%, mainly in subjects homozygous for *VWF* null-alleles. Because inhibitors may be directed towards different VWF epitopes, their detection is dependent on the assay used.

INTRODUCTION

von Willebrand disease (VWD) is an inherited bleeding disorder caused by quantitative (types 1 and 3) and qualitative (type 2) defects of the multimeric protein von Willebrand factor (VWF) [1-3]. Type 3 VWD is the rarest form of VWD with a prevalence that ranges from 0.1 to 5.3 per million inhabitants and increases in regions with a high rate of consanguinity [1,4,5]. It is characterized by an almost complete deficiency of VWF and by consequence also reduced factor VIII (FVIII) levels in plasma [1,3]. Type 3 VWD is inherited as an autosomal recessive trait. These patients are mainly homozygotes/compound heterozygotes for VWF null defects, for a missense and null defect or homozygous for missense variants. The type of genetic defects contributes to explain the different mechanisms responsible for the deficiency of VWF, such as reduced synthesis, impaired secretion, increased clearance or a combination of them [6].

Type 3 patients may have severe clinical manifestations which include mucocutaneous bleeding, menorrhagia, joint and gastrointestinal bleeding [7-9]. These symptoms impair quality of life [10] and require management based upon replacement therapy with VWF-containing products [9,10]. Replacement therapy may be delivered on-demand to stop bleeding episodes and prevent bleeding before a surgical procedure, or as secondary long-term (SLT) prophylaxis to prevent frequently recurring bleeding [10-13]. Upon treatment with VWF-containing concentrates type 3 VWD patients can develop alloantibodies that may neutralize VWF (also called inhibitors), make replacement therapy ineffective and expose patients to the risk of anaphylactic reactions [13,14]. In previous studies, the prevalence of alloantibodies has been estimated at 5-10% [13,14].

In this study, we aimed to assess the prevalence of alloantibodies against VWF in the frame of type 3 von Willebrand International Registries Inhibitor Prospective Study (3WINTERS-IPS), a multicentric retrospective and prospective study enrolling European and Iranian subjects with type 3 VWD. Due to current lack of consensus on which test should be performed to accurately evaluate the prevalence of this adverse effect of replacement therapy, we chose to evaluate the presence of non-neutralizing as well as neutralizing antibodies directed against VWF using several different assays.

MATERIALS AND METHODS

Study population

The 3WINTERS-IPS study includes 265 type 3 VWD subjects of European and Iranian ancestry enrolled at 22 centers. Inclusion criteria were a previous diagnosis of type 3 VWD obtained at the recruiting centers, available data on their bleeding history and administration of VWF-containing products and availability to follow-up. The study has been approved by the local ethical committees of all participating Centers and subjects gave written informed consent.

Confirmation of type 3 VWD diagnosis

At the time of enrollment, plasma samples and buffy coats were collected to confirm centrally the diagnosis. To this purpose, von Willebrand factor antigen (VWF:Ag) was measured using an ELISA based method, whereas FVIII:C was measured by a one-stage clotting assay using FVIII deficient plasma (Siemens, Erlangen, Germany) and the APTT reagent Triniclot (TCoag, Wicklow, Ireland). The VWF propeptide (VWFpp) was measured by an ELISA using antibodies from Sanquin (Amsterdam, the Netherlands) [15-16]. The molecular analysis of VWF was based on next-generation sequencing, PCR with Sanger sequencing and multiplex-ligation dependent probe amplification [17]. The subject bleeding history was collected at enrollment and the bleeding score (BS) calculated using a bleeding assessment tool [18] along with the information available about therapy. The results obtained were reported as medians and interquartile ranges (IQR) for continuous variables, whereas categorical data were reported as percentages.

Anti-VWF antibodies

The presence of all antibodies against VWF was determined in plasma samples obtained from subjects at the time of enrolment using an in-house indirect ELISA [19], that identifies all antibodies irrespective of the immunoglobulin subclass. Briefly, 96 wells ELISA plates (Nunc A/S, Roskilde, Denmark) were coated with 1 IU/mL of a recombinant VWF from Chinese hamster ovary cells (a generous gift of Shire/Takeda), previously heat inactivated for 30 minutes at 56°C to destroy the small FVIII traces within this product. The plates were then incubated at 2-8°C overnight, washed with phosphate-buffered saline (PBS)/albumin (1%) and blocked with a PBS/albumin (5%) solution for 30 minutes. In a first screening round subject plasma was used 1:50 diluted with PBS/albumin (5%), seeded into the plates and incubated for 60 minutes at 37°C. The plates were then washed and incubated with anti-human IgG, IgA, and IgM antibodies labeled with horseradish peroxidase (HRP). Binding was revealed through a colorimetric reaction by measuring absorbance at 492/620 nm. The assay cut-off was set at 2 times the optical density (O.D.) of normal pooled plasma from more than 30 healthy donors. A mixture of subject plasma with IgG or IgM antibodies has been used as positive control. Positive plasma sample was further diluted geometrically until it showed negative results (<2 times the O.D. of normal pooled plasma).

Neutralizing antibodies (inhibitors)

Plasma samples were evaluated for the presence of neutralizing antibodies with a Bethesda-based method using an in-house collagen type III ELISA [20]. A reference plasma consisting of a lyophilized pool plasma from healthy donors (Technoclone, Diapharma, Vienna, Austria) was resuspended following manufacturing instructions and used as normal pooled plasma (NPP) for mixing studies. Undiluted plasma samples and serial dilutions performed using the PBS/albumin (5%) dilution buffer were mixed 1:1 with the NPP. The anti-human

VWF rabbit antibody (A0082; Dako, Glostrup, Denmark) was pre-diluted from 1:40 to 1:640 using the dilution buffer, mixed 1:1 with NPP and used as a positive control for VWF inhibitors, whereas the NPP was mixed 1:1 with the dilution buffer and used as reference plasma. Then, all samples were incubated for 1 hour at 37°C and kept on ice until loaded into the plate. For each plate, a calibration curve was obtained by diluting NPP (from 1:5 to 1:320) with the dilution buffer. The normal and low-range controls (Haemochrom Diagnostica, Essen, Germany) were used as internal controls.

Microtiter plates (NUNC Roskilde, Denmark) were coated with collagen type III (Biozol Eching, Germany) overnight. After washing 3 times with PBS/albumin (0.1%), plates were incubated with the blocking solution PBS/albumin (2.5%) for 30 minutes at room temperature. Then, plates were washed 3 times and samples added. For each plate, the calibration curve and all controls were seeded in duplicate. The plates were incubated for 1 hour and 30 minutes at 37°C. After washing 3 times, the rabbit anti-human VWF conjugated with horseradish peroxidase (HRP; A0092, Dako) was added for 1 hour and 30 minutes at 37°C. Binding was revealed through a colorimetric reaction by measuring absorbance at 492/620 nm. Samples were considered positive for VWF inhibitors in presence of a titer ≥ 0.3 Bethesda unit (BU). A Bethesda unit (BU) was defined as the amount of the antibody which inactivates 50% of VWF after 1-hour incubation at 37°C.

Two additional versions of Bethesda-based methods have been used to detect VWF inhibitors in subjects positive for anti-VWF antibodies and with an available plasma sample. These samples were tested with a method using the gain-of-function mutant glycoprotein(GP)Ib binding assay ([VWF:GPIbM], INNOVANCE VWF Ac test kit; Siemens, Marburg, Germany). Undiluted plasma samples and serial dilutions were mixed 1:1 with the NPP. A 1:1 mixture of the NPP and dilution buffer was taken as a reference plasma, whereas serial mixtures of the NPP and anti-human VWF rabbit antibody (A0082; Dako) were used as a positive control for VWF inhibitors. Then, the assay was performed following manufacturing instructions. Briefly, gain-of function rGPIb molecules carrying mutations G233V and M239V have been added to all plasma mixtures and spontaneously bind VWF in the absence of ristocetin. Then, polystyrene beads coated with an anti-GPIb antibody are added to each mixture. The binding of rGPIb-VWF complexes causes the agglutination of polystyrene beads resulting in a decrease in light transmission which is directly proportional to the VWF-GPIb binding activity in plasma.

Differently from the other two Bethesda-based methods, the Bethesda-based method using VWF:Ag does not measure a residual VWF activity but the residual amount of VWF:Ag present in the NPP after incubation with plasma samples. For this assay, the microtiter plates (NUNC Roskilde, Denmark) were coated overnight with the anti-human VWF rabbit antibody (A0082; Dako). The mix 1:1 of the plasma samples with the NPP, the reference plasma, the calibration curve and positive controls were prepared and underwent the same steps already described for VWF:CB Bethesda-based assay.

RESULTS

Study population

A total of 265 subjects were enrolled in 3WINTERS-IPS. Of these, 52 were excluded from further study because DNA samples were not available or essential data were missing (Figure 1).

The remaining 213 subjects can be divided in three groups (Table 1). The first group includes 162 subjects having a confirmed diagnosis of type 3 VWD with plasma VWF:Ag≤ 3 IU/dL and identified VWF defects. Of them, 5 have only a partial genotyping because the second genetic defect was not identified. Fourteen of 162 subjects were reported to be on SLT prophylaxis at sampling time, but this information was missing for one subject. The second group included 9 subjects with VWF:Ag ≤3 IU/dL in whom genetic analyses failed to identify a VWF defect. Of them, one was on prophylaxis at sampling time. The third group included 42 fully characterized subjects with VWF:Ag >3 IU/dL and identified VWF, although in 6 subjects the second genetic defect was not identified. Of them, 27 were on treatment at sampling time.

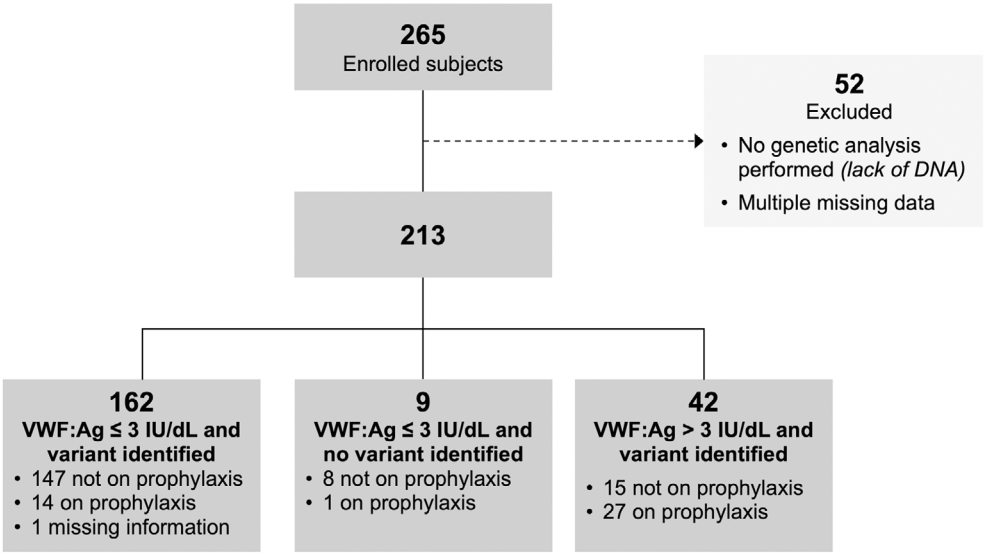

Figure 1. Flowchart of the study population. 265 subjects with a previous type 3 diagnosis were enrolled in 3WINTERS-IPS. Of them, 52 were excluded because of multiple missing data, whereas the remaining 213 subjects were tested for both non-neutralizing and neutralizing VWF antibodies using an indirect ELISA and a Bethesda-based method assay on von Willebrand factor collagen binding, respectively. These 213 subjects were further divided depending on the availability of biochemical and molecular information.

Table 1. Characteristics of enrolled subjects

	All subjects*, †	VWF:Ag ≤3 IU/dL, genetic variant identified*,‡	VWF:Ag ≤3 IU/dL, no genetic variant identified**	VWF:Ag >3 IU/dL, genetic variant identified††
Subjects, n (%)	213 (100)	162 (76)	9 (4)	42 (20)
Age at enrolment, (years)	28.0 (6.0-43.0)	27.0 (15.8-40.3)	18.0 (11.0-28.5)	42.5 (23.8-58.5)
Sex, n (%)				
Male	89 (42)	67 (41)	3 (33)	19 (45)
Female	124 (58)	95 (59)	6 (67)	23 (55)
BS, (score)				
all	15.0 (8.0-21.0)	14.5 (8.0-20.0)	9.0 (4.5-17.0)	18.0 (11.5-25.5)
treated	22.0 (13.5-27.5)	17.0 (7.0-27.5)	14	23.0 (16.5-28.0)
untreated	14.0 (7.3-19.0)	14.0 (8.0-19.0)	8.5 (4.3-17.5)	11.0 (6.0-18.0)
unclassified‡‡	3	3	-	-
Prophylaxis at sampling time, n (%)				
yes	42 (19.7)	14 (8.6)	1 (11)	27 (64)
no	170 (79.8)	147 (90.7)	8 (89)	15 (36)
unclassified‡‡	1 (0.5)	1 (0.6)	-	-
VWF:Ag, (IU/dL)				
all	0.5 (0.5-0.5)	0.5 (0.5-0.5)	1.7 (1.2-2.4)	8.6 (4.6-35.5)
treated	5.6 (2.4-32.0)	1.9 (1.3-2.4)	1.7	21.0 (6.3-42.0)
untreated	0.5 (0.5-0.5)	0.5 (0.5-0.5)	1.8 (1.2-2.4)	5.2 (4.1-6.3)
unclassified‡‡	0.5	0.5	-	-

Table 1 (continued). Characteristics of enrolled subjects

	All subjects*, †	VWF:Ag ≤3 IU/dL, genetic variant identified*,‡	VWF:Ag ≤3 IU/dL, no genetic variant identified**	VWF:Ag >3 IU/dL, genetic variant identified††
VWFpp, (IU/dL)				
all	1.9 (0.8-5.4)	1.6 (0.7-4.3)	2.9 (2.4-6.8)	5.1 (1.2-11.0)
treated	2.8 (0.9-8.5)	2.1 (0.6-6.0)	7.6	4.3 (1.0-9.6)
untreated	1.8 (0.8-5.1)	1.5 (0.7-4.2)	2.8 (2.3-4.4)	8.5 (3.4-15.9)
unclassified‡‡	0.7	0.7	-	-
FVIII:C, (IU/dL)				
all	2.6 (2.0-4.7)	2.4 (1.8-3.2)	2.0 (1.6-3.5)	22.8 (14.7-64.8)
treated	13.9 (5.5-63.2)	5.4 (4.1-8.1)	7.5	56.7 (13.9-79.3)
untreated	2.4 (1.8-3.2)	2.3 (1.8-2.9)	1.8 (1.6-2.7)	19.1 (14.9-33.4)
unclassified‡‡	1.3	1.3	-	-

Continuous variables were reported as median and interquartile range (IQR). Descriptive variables were reported as numbers with percentages. BS, bleeding score; VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide; FVIII:C, Factor VIII coagulant activity.

* Most of subjects had a VWF antigen below the limit of detection (0.8 IU/dL) and therefore in those subjects it was arbitrarily set as 0.5 IU/dL. † Missing values: FVIII:C, n=4; VWFpp, n=8; BS, n=11; incomplete genotyping, n=11. ‡ Missing values: FVIII:C, n=1; VWFpp, n=4; BS, n=6; incomplete genotyping, n=5. ** Missing values: FVIII:C, n=1; VWFpp, n=1. †† Missing values: FVIII:C, n=2; VWFpp, n=3; BS, n=5; incomplete genotyping, n=6. ‡‡One subject in the main group was unclassified because of missing information about treatment.

All anti-VWF antibodies

The 213 subjects have been tested for the presence of all antibodies against VWF at the time of their enrolment using an in-house ELISA that detects IgG, IgA and IgM antibodies (**Table 2** and **Figure 2**).

In total, 18 subjects tested positive (8.4%), 11 from Europe and 7 from Iran (61% vs. 39%). Their median age was 40.5 years (IQR: 24.5-44.3) and they were mainly females (13/18; 72%). These subjects have a median BS of 16, IQR: 7.0-25.0 (n=17; 1 missing data) similar to that of type 3 subjects who tested negative for anti-VWF alloantibodies who had a median BS of 15, IQR: 8.0-21.0 (n=185; 10 missing data). None of them was on SLT prophylaxis at sampling time, although all but one (missing information) reported to have received previous treatments. All but one of these subjects belonged to the first and main group consisting of subjects with a confirmed diagnosis (VWF:Ag ≤3 IU/dL and a complete molecular characterization of VWF). Most of them had unmeasurable VWF:Ag

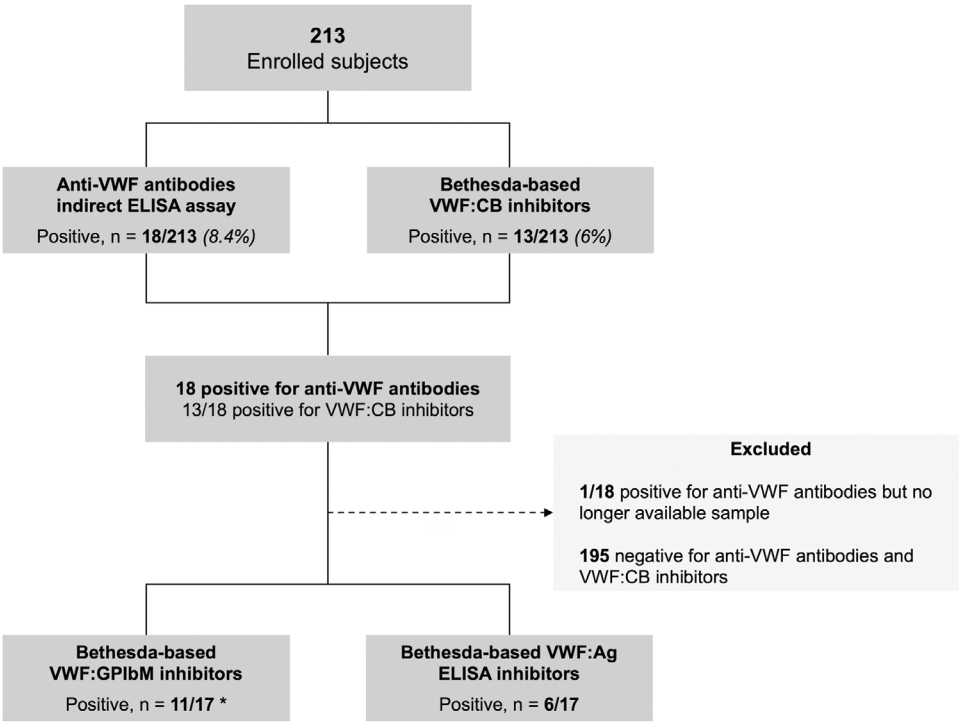

Figure 2. Sample workflow and related results. In total, 213 subjects have been screened for all antibodies against VWF (without distinguish between non-neutralizing and neutralizing antibodies) using an indirect ELISA assay. The same 213 subjects have been tested for VWF inhibitors with a Bethesda-based method able to measure the residual VWF collagen binding activity (VWF:CB). The Bethesda based method using VWF:GPIbM and the Bethesda based method using VWF:Ag ELISA were performed on the 17 subjects positive for anti-VWF antibodies and with an available plasma sample. * One subject had only VWF:GPIbM inhibitors.

as arbitrarily set at 0.5 IU/dL. The remaining subject was classified among those with an unconfirmed type 3 diagnosis due to VWF:Ag >3 IU/dL (5.2 IU/dL).

Neutralizing antibodies (inhibitors)

The 213 type 3 subjects were investigated in order to assess the presence of VWF neutralizing antibodies (inhibitors) with a Bethesda-based method using the VWF:CB

Table 2. Type 3 VWD subjects who developed non-neutralizing and neutralizing antibodies (inhibitors) against VWF

Subject ID (E/I)	Age[†]	Sex	BS	VWF:Ag *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C *(IU/dL)*	Anti-VWF (OD subject/ OD cut-off)[‡]	VWF:CB Inhibitor (BU)
32 (E)	55	F	27	0.5	43.1	2.2	8.4	5
37 (E)	42	F	32	0.5	1.2	1.1	7.84	1.8
81 (E)	41	F	8	0.5	0.2	2	11.28	15
82 (E)[§]	40	M	9	5.2	8.5	33.4	4.92	<0.3
96C (E)	44	F	16	1.5	11.4	5	7.96	0.4
99D (E)	43	M	17	0.5	1.4	3.4	9.34	10
101D (E)	45	F	28	0.5	0.3	2.4	6.08	0.3
102D (E)	42	M	20	0.5	1.3	3.6	8.0	1.3
106 (E)	29	F	6	0.5	0.8	2.9	8.53	3.8
113 (E)	20	F	20	2.2	14.1	1.7	5.16	<0.3
114 (E)	63	F	33	0.5	0.2	2.2	5.36	1
6 (I)	2	M	5	1.4	8.6	5.1	6.97	1.18
47 (I)	45	F	-	0.5	3.1	1.4	7.17	23
61 (I)	18	F	5	0.5	0.6	1.2	7.26	56
66 (I)	30	F	2	0.5	0.3	1.9	2.44	<0.3

ELISA (**Figure 2**). A sample was considered positive if the inhibitor titer was ≥0.3 BU. VWF:CB inhibitors were detected in 13 subjects, with a prevalence of 6%, and in them the titer ranged from 0.3 to 56 BU.

Seventeen out of 18 subjects who had tested positive for anti-VWF antibodies by indirect ELISA and with available plasma samples were further investigated to evaluate the presence of an inhibitory antibody directed against a different VWF epitope

VWF:GPIbM Inhibitor (BU)	VWF:Ag Inhibitor (BU)	VWF Gene Defect specification (HGVS description, allele 1/ HGVS description, allele 2) ††
2.8	0.3	NM_000552.3:c.4975C>T (p.Arg1659*)/ NM_000552.3:c.4975C>T (p.Arg1659*)
<0.3	<0.3	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)
5.9	0.7	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)
<0.3	<0.3	NM_000552.3:c.1534-3C>A (p.Leu512Profs*11)§§/ NM_000552.3:c.7085G>T (p.Cys2362Phe)
<0.3	<0.3	NM_000552.3:c.8155+1G>T (p.G2706_C2719delfs*25)/ NM_000552.3:c.8155+1G>T (p.G2706_C2719delfs*25) §§
3.8	2.0	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)
0.3	<0.3	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)
0.7	<0.3	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)
0.4	<0.3	NM_000552.3:c.6182delT (p.Phe2061Serfs*38)/ NM_000552.3:c.6182delT (p.Phe2061Serfs*38)
1	<0.3	NM_000552.3:c.6917delT (p.Leu2306Argfs*4)/NONE
<0.3	<0.3	NM_000552.3:c.7636A>T (p.Asn2546Tyr)/ NM_000552.3:c.7636A>T (p.Asn2546Tyr)
0.5	<0.3	NC_000012.11:g.(?_6058180)_(6105389_6120781)del (delEx35_Ex52)/ NC_000012.11:g.(?_6058180)_(6105389_6120781)del (delEx35_Ex52)
52	1.5	NM_000552.3:c.4036C>T (p.Gln1346*)/NONE
70	7.6	NM_000552.3:c.311_312delAG (p.Gln104Argfs*19)/ NM_000552.3:c.311_312delAG (p.Gln104Argfs*19)
n.d.	n.d.	NM_000552.3:c.4975C>T (p.Arg1659*)/ NM_000552.3:c.4975C>T (p.Arg1659*)

Table 2 (continued). Type 3 VWD subjects who developed non-neutralizing and neutralizing antibodies (inhibitors) against VWF

Subject ID (E/I)	Age†	Sex	BS	VWF:Ag *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C *(IU/dL)*	Anti-VWF (OD subject/ OD cut-off)‡	VWF:CB Inhibitor (BU)
87 (I)	9	F	10	0.5	4	3.3	5.84	13
94L (I)	32	F	23	0.5	3.6	2.6	2.96	<0.3
103M (I)	26	M	14	0.5	4.2	3.3	2.56	<0.3

E, European subject; I, Iranian subject. † Age at enrolment. Sex: F, female; M, male. BS, Bleeding score. VWF:Ag, von Willebrand factor antigen. VWFpp, von Willebrand factor propeptide. FVIII:C, Factor VIII coagulant activity. VWF:CB, von Willebrand factor collagen binding. VWF:GPIbM, the gain-of-function mutant GPIb binding was performed using INNOVANCE reagent. BU, Bethesda units. ‡ The presence of anti-VWF antibodies has been evaluated using an indirect ELISA assay. A sample was considered positive if the optical density (OD) was at least 2-times higher than that of normal

6

(**Figure 2**) using a VWF:GPIbM assay and VWF:Ag ELISA. Eleven subjects were positive for VWF:GPIbM inhibitors (11/17; 65%). Of them, 10 were also found to be positive for VWF:CB inhibitors (10/11; **Table 2**), whereas one was positive for VWF:GPIbM but not for VWF:CB inhibitors. The Bethesda-based method measuring residual amount of VWF:Ag by ELISA was positive for 6 of 17 subjects (35%) characterized by high inhibitor titers. In total, 14 subjects tested positive for VWF inhibitors and they all belong to the group of 18 subjects positive for anti-VWF antibodies. Six had VWF:CB, VWF:GPIbM plus VWF:Ag inhibitors, four had VWF:CB plus VWF:GPIbM inhibitors, three had only VWF:CB inhibitors and one had only VWF:GPIbM inhibitors. The three subjects who only tested positive with the indirect ELISA assay (3/17) were therefore diagnosed to have non-neutralizing antibodies.

Genetic variants identified in subjects positive for non-neutralizing and neutralizing VWF antibodies.

The variants identified in the 18 subjects positive for VWF inhibitors and/or all anti-VWF antibodies against VWF are listed in **Table 2**. The majority of the subjects were homozygous for a genetic defect (n=15; 83%), one was a compound heterozygote for a missense and a splice variant (6%) and two were incompletely characterized because the genetic defect on the second VWF allele was not identified (11%; one heterozygous for a small deletion and the other one heterozygous for a nonsense variant).

The 13 subjects with inhibitors using at least the Bethesda VWF:CB assay were more frequently Europeans than Iranians (9 vs. 4). Of them, 11 were homozygous for a null allele including a complete VWF gene deletion (n= 5), a large deletion involving exons 35-52 (n= 1), small deletions (n= 3), a splice mutation confirmed at mRNA level as

VWF:GPIbM Inhibitor (BU)	VWF:Ag Inhibitor (BU)	VWF Gene Defect specification (HGVS description, allele 1/ HGVS description, allele 2) ††
28	1.7	NM_000552.3:c.4309delG (p.Ala1437Profs*4)/ NM_000552.3:c.4309delG (p.Ala1437Profs*4)
<0.3	<0.3	NM_000552.3:c.2376C>G (p.Cys792Trp)/ NM_000552.3:c.2376C>G (p.Cys792Trp)
<0.3	<0.3	NM_000552.3:c.2376C>G (p.Cys792Trp)/ NM_000552.3:c.2376C>G (p.Cys792Trp)

pooled plasma. § This subject has an unconfirmed type 3 VWD diagnosis because of VWF:Ag >3 IU/dL. A sample was considered positive for neutralizing antibodies if the inhibitor titer was ≥0.3 BU. N.d., not determined. †† The large deletions are also reported using a simpler nomenclature. §§ This variant has been previously evaluated at mRNA level. Subjects 113 (E) and 47 (I) have an incomplete genotyping as the respective second genetic defect was not found. Subject 66 (I) was not tested for VWF:GPIbM and VWF:Ag inhibitors because of insufficient plasma sample.

responsible for alternative splicing (n= 1) and a nonsense variant (n= 1). One subject was homozygous for a missense variant. The remaining subject had an incomplete molecular characterization because only heterozygosity for a nonsense variant was identified, with an unknown defect for the second VWF allele. The subject who tested positive only for VWF:GPIbM inhibitors was heterozygous for a small deletion leading to a frameshift and a premature stop codon, but the second genetic defect was not identified.

Subject history on therapies and anaphylactic reactions

All the data herein reported refer to the retrospective phase of 3WINTERS-IPS and were collect-ed at subject enrollment. Neither the 13 subjects positive for VWF:CB inhibitors nor the only one with the VWF:GPIbM inhibitor were on SLT prophylaxis at sampling time, and all were al-ready known to be carriers of inhibitors at the time of the enrolment in 3WINTERS-IPS. In total, 13 subjects reported a previous exposure to replacement therapy, for one this information was missing (**Table 3**). Four subjects reported the previous use of recombinant activated FVII (rFVIIa) and three have been treated with a recombinant FVIII. In all cases, the administration of rFVIIa followed that of at least one product containing VWF. One subject reported to have only been treated with rFVIII, whereas another reported to have used the prothrombin complex after the previous administration of a concentrate containing VWF and rFVIIa. Six subjects, all Europeans, reported to have been treated with at least two different products. All Iranian subjects but one (unavailable information) reported to have been treated only with a plasma concentrate con-taining VWF. The three subjects who had non-neutralizing antibodies and the subject who has been only tested for VWF:CB inhibitor (insufficient plasma sample) reported previous treatments with a plasma-derived product containing VWF.

Types and titers of anti-VWF were also evaluated pertaining to a previous history of anaphylactic reactions (**Table 3**). The three subjects with non-neutralizing antibodies and the one with incomplete characterization did not report any anaphylactic reactions notwithstanding their exposure to plasma-derived VWF products. A history of anaphylactic reactions was reported in eight (8/18) subjects characterized by VWF:CB, VWF:GPIbM and VWF:Ag inhibitor assays. The titers of these inhibitors were variable from very low (0.3

Table 3. Previous treatment(s) and anaphylactic reactions reported at enrolment

Subject ID	Year of Birth	Type of replacement therapy (First year of exposure)	Non-neutralizing antibodies only
32 (E)	1959	plasma derived VWF-FVIII (1994)	-
37 (E)	1972	Recombinant FVIII (2014)	-
81 (E)	1971	plasma derived VWF-FVIII (1980); plasma derived VWF-FVIII (1986); plasma derived VWF-FVIII (1993); recombinant FVIII (1993)	-
82 (E) †	1972	plasma derived VWF-FVIII (2011)	+
96C (E)	1969	plasma derived VWF-FVIII (1991); plasma derived VWF-FVIII (2003)	-
99D (E)	1970	plasma derived VWF-FVIII(1991); activated recombinant FVII (1995)	-
101D (E)	1968	plasma derived VWF-FVIII (1994); activated recombinant FVII (2011)	-
102D (E)	1971	plasma derived VWF-FVIII (1977); recombinant FVIII (1997); activated recombinant FVII (1997); plasma derived VWF-FVIII (2001)	-
106 (E)	1984	plasma derived VWF-FVIII (2000)	-
113 (E) ‡	1993	plasma derived VWF-FVIII (1994); activated recombinant FVII (2006); activated prothrombin complex (2013)	-
114 (E)	1950	plasma derived VWF-FVIII (1994)	-
6 (I)	2011	plasma derived VWF-FVIII (2011)	-
47 (I)	1967	N.A.	-
61 (I)	1994	plasma derived VWF-FVIII (2008)	-
66 (I)	1983	plasma derived VWF-FVIII (2012)	-
87 (I)	2003	plasma derived VWF-FVIII (2008)	-
94L (I)	1980	plasma derived VWF-FVIII (2007)	+
103M (I)	1986	plasma derived VWF-FVIII (2008)	+

E, European subject; I, Iranian subject. † This subject has an unconfirmed type 3 VWD diagnosis because of VWF:Ag >3 IU/dL. BU, Bethesda units. ‡This subject tested positive for VWF inhibitors with a Bethesda based assay using VWF:GPIbM (1 BU). N.A., not applicable. N.d., not determined. All data were collected at enrolment (3WINTERS-IPS retrospective

BU) to high (56 BU). The behavior of the remaining six subjects (6/18) who at enrolment reported no history of anaphylaxis is un-clear. Three (3/6) European subjects reported no anaphylactic reaction and they had low titers of anti-VWF inhibitors. One of them reported to be only treated with recombinant FVIII, whereas the other one had been switched to recombinant FVIIa and then activated prothrombin complex concentrates by the attending physicians owing to previous experience of anaphylaxis episodes in

VWF:CB inhibitors (BU)	VWF:GPIbM Inhibitor (BU)	VWF:Ag Inhibitor (BU)	Anaphylactic reaction	VWF Gene Defect specification (Allele 1/Allele 2)
5	2.8	0.3	+	p.Arg1659*/p.Arg1659*
1.8	<0.3	<0.3	-	delEx1_Ex52/delEx1_Ex52
15	5.9	0.7	+	delEx1_Ex52/delEx1_Ex52
<0.3	<0.3	<0.3	-	p.Leu512Profs*11[§]/p.Cys2362Phe
0.4	<0.3	<0.3	-	p.G2706_C2719delfs*25/ p.G2706_C2719delfs*25[§]
10	3.8	2.0	+	delEx1_Ex52/delEx1_Ex52
0.3	0.3	<0.3	+	delEx1_Ex52/delEx1_Ex52
1.3	0.7	<0.3	+	delEx1_Ex52/delEx1_Ex52
3.8	0.4	<0.3	+	p.Phe2061Serfs*38/p.Phe2061Serfs*38
<0.3	1	n.d.	-	p.Leu2306Argfs*4/NONE
1	<0.3	<0.3	+	p.Asn2546Tyr)/p.Asn2546Tyr
1.18	0.5	<0.3	-	delEx35_Ex52/delEx35_Ex52
23	52	1.5	-	p.Gln1346*/NONE
56	70	7.6	+	p.Gln104Argfs*19/p.Gln104Argfs*19
<0.3	n.d.	n.d.	-	p.Arg1659*/p.Arg1659*
13	28	1.7	-	p.Ala1437Profs*4/p.Ala1437Profs*4
<0.3	<0.3	<0.3	-	p.Cys792Trp/p.Cys792Trp
<0.3	<0.3	<0.3	-	p.Cys792Trp/p.Cys792Trp

phase). Subjects 113 (E) and 47 (I) had an incomplete genotyping as the respective second genetic defect was not found.
[§] This variant has been previously evaluated at mRNA level. Subject 66 (I) has been only tested for anti-VWF antibodies and VWF:CB inhibitors because of insufficient sample.

other subjects followed at the same center; one received plasma-derived VWF for many years (until 2003) before enrolment and apparently did not receive any other treatment. Three Iranian (3/6) subjects who reported no anaphylactic reaction were characterized by variable titers of inhibitors with values> 10 BU in two of them: all these subjects had been exposed to plasma-derived VWF products but the data about exposure was missing in one case.

DISCUSSION

In this study, we evaluated the prevalence of all alloantibodies against VWF (both neutralizing and not-neutralizing) in the 3WINTERS-IPS cohort, the largest cohort of type 3 subjects so far investigated for this purpose. All antibodies against VWF were detected using an indirect ELISA assay, whereas the presence of neutralizing antibodies (inhibitors) was detected using a Bethesda method based on the measurement of residual VWF:CB in plasma. Overall, 18 of 213 type 3 subjects tested positive for VWF alloantibodies, thus with a prevalence of 8.4%. All but one of them (due to unavailable data) reported previous treatments with at least one product containing VWF. Three of 18 subjects tested positive for anti-VWF antibodies using the indirect ELISA, but they were negative for VWF inhibitors irrespective of the Bethesda-based method used. This led us to conclude that these subjects only had non-neutralizing antibodies which do not inhibit VWF function(s). Nevertheless, it was impossible to assess whether or not these antibodies were present before treatment with VWF containing products. Suiter et al. [21] previously reported the presence of high-titer non-neutralizing anti-VWF antibodies in 3 of 39 cases previously treated with cryoprecipitate or plasma-derived FVIII products containing VWF. Of them, one received no further infusion after positivity for VWF:CB inhibitor, whereas the remaining two showed a poor recovery of VWF:Ag, VWF ristocetin cofactor activity, VWF:CB and FVIII:C plasma levels after infusion of plasma-derived or recombinant VWF but without developing neutralizing antibodies [21]. Notwithstanding the still unsettled role of non-neutralizing anti-VWF alloantibodies, these data suggested that their presence may be associated with a decreased recovery and/or increased clearance following replacement therapy.

To date, there is no consensus on which functional method should be preferred to detect VWF inhibitors, because these methods are not standardized and their availability is confined to specialized laboratories [14]. In the present study, the detection of VWF inhibitors was performed by means of a Bethesda-based method using an in-house VWF:CB ELISA. Neutralizing antibodies were found in 13 of 213 subjects (prevalence 6%). Most of them (10 cases) also tested positive for neutralizing antibodies against VWF:GPIbM. An additional subject was positive for VWF:GPIbM inhibitors but not for VWF:CB inhibitors. In a subject, who tested negative for VWF: CB inhibitors, it was not possible to complete the VWF inhibitors characterization with the other Bethesda-based methods because the sample was no longer available. These results show that the use of

the VWF:CB method may be a valid choice for the identification of VWF inhibitors, but also that inhibitor assessment may be inconclusive when based on a single functional test. This is in line with previously reported data [22] which highlighted that the capacity to detect VWF inhibitors and thus their true prevalence is affected by the functional epitope recognized by the antibodies. Differently from the Bethesda-based method using VWF:CB or VWF:GPIbM, the method using the in-house VWF:Ag ELISA allows to measure the residual amount of VWF:Ag but not residual VWF activity. This assay was the least sensitive, because it was able to detect VWF:Ag antibodies only in the 6 samples with a VWF:CB inhibitor titer ≥5 BU (6/17; 35%), perhaps because only high-titer antibodies do precipitate VWF allowing their detection [23].

A link between the type of VWF defect and the development of VWF inhibitors was previously reported, with large or complete gene deletions being the most common defects followed by nonsense and missense variants [24-26]. This finding is largely confirmed in the present study, because the majority of subjects who developed inhibitors were homozygous for complete or large gene deletions or genetic defects resulting in null alleles, whereas only one subject was homozygous for a missense variant. However, not all the type 3 subjects enrolled in this study carrying partial gene deletions [17] developed inhibitors. This is in line with the findings by Mohl et al. [27], who described 5 homozygous carriers of a large deletion involving exons 1-3 who developed no inhibitor despite frequent replacement therapy, thus suggesting that other cofactors are involved [27]. In agreement with these data, a Hungarian subject enrolled in the present study who had the same genotype (c.delEx1-3/c.2435delC) did not develop a VWF inhibitor even though she has been treated with a product containing VWF. Thus, having a specific VWF defect does not automatically imply the development of VWF inhibitors even when subjects are related, suggesting partial penetrance [28]. Accordingly, VWF inhibitors have been detected in only 5 of 7 subjects carrying a complete gene deletion (6 in homozygosity and one in heterozygosity because the second genetic defect was not found). Of them, 4 homozygous subjects were siblings, but only three of them developed VWF inhibitors. Other peculiar cases have been highlighted in the present cohort. Four unrelated Iranian subjects were homozygous carriers for the p.Gln104Argfs*19 variant. Of them, 3 reported previous treatments but only one developed an inhibitor. Similarly, only one of 2 unrelated Italian subjects who were homozygous carriers for the p.Phe2061Serfs*38 developed an inhibitor. Lastly, among three unrelated Dutch subjects who were homozygous carriers for p.Asn2546Tyr, two reported the previous use of a concentrate containing VWF but only one of them developed an inhibitor. Taken together, these results indicate that risk cofactors other than the genotype are responsible for inhibitor development, as already established for haemophilia subjects [29].

Anaphylactic reactions after exposure to plasma-derived products containing VWF have been reported in type 3 VWD subjects since 1995 [30,31]. In the present study 8/18 had a history of anaphylactic reactions according to the clinical data collected

and reported by the attending physicians. All these subjects had measurable levels of neutralizing anti-VWF inhibitors but with different titers, perhaps depending on the time of the last exposure to plasma-derived VWF products. Three subjects with non-neutralizing antibodies and the one who tested positive for anti-VWF antibodies but was partially tested for inhibitors (VWF:CB <0.3 BU) showed no anaphylactic reactions even if previously exposed to plasma-derived VWF containing products. The interpretation of the behavior of the remaining 6 subjects who apparently did not develop anaphylaxis despite previous exposure to plasma-derived VWF concentrates is inconclusive and more detailed information about these cases will be collected in the prospective phase of the 3WINTER-IPS project.

This study stems from the 3WINTERS-IPS, an investigator-driven observational study designed to assess the clinical, laboratory and genetic background as well as the related therapeutic approaches in a very large cohort of type 3 VWD subjects. However, the sample size, albeit large considering the rarity of VWD type 3, still remains one of the study limitations. Since all these subjects have been already proven to carry VWF inhibitor at the time of enrollment, a second limitation of the study is that we could not evaluate whether or not the presence of non-neutralizing antibodies indicates the future development of inhibitors, or whether their detection is clinically useful to monitor subjects' response to treatment. Third, we were unable to obtain an accurate record of the time and circumstances related to subject exposure to plasma-derived and/or recombinant VWF products prior to inhibitors detection (e.g., exposure day and dosages), nor how the therapeutic approach changed afterwards. Lastly, the assays used to determine the presence of anti-VWF antibodies are not standardized, even though our choice to perform them centrally has perhaps contributed to reduce variability.

In conclusion, the presence of alloantibodies, that includes both non-neutralizing and neutralizing antibodies against VWF had a prevalence of 8.4% in our study population. Not all subjects who were antibody positive using the indirect ELISA assay had VWF inhibitors. However, all subjects with VWF inhibitors were detected using this assay, suggesting that it may represent a valid screening method. All subjects had previous treatments, but it was impossible to establish whether or not the non-neutralizing antibodies are the consequence of replacement therapy nor any exposure time relationship. The development of neutralizing antibodies assessed using a Bethesda-based method measuring residual VWF:CB has been found to be a rare event with a prevalence of 6%. Nevertheless, this diagnosis is related to the type of functional epitopes recognized by anti-VWF antibodies and is therefore influenced by the assay method used to detect them. The present results also suggest that at least one method to be chosen between VWF:CB and VWF:GPIbM should be performed to maximize the capacity to detect inhibitors, whereas the use of the method based upon the VWF:Ag ELISA should be discouraged due to the low sensitivity that allows to detect only high titers antibodies.

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