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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Received: 15 September 2022 | Revised: 23 December 2022 | Accepted: 3 January 2023

https://doi.org/10.1016/j.jtha.2023.01.001

ORIGINAL ARTICLE

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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Manuscript handled by: David Lillicrap

Final decision: David Lillicrap, 27 December 2022

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Funding information
The 3WINTERS-IPS project received unconditional research grants from several Pharmaceutical Companies: their annual grants were paid to the AB Bonomi Foundation sponsor of the study. According to the amounts of grants paid, we acknowledge the representatives of Baxter-ShireTakeda, CSL Behring, Grifols, LFB, Octapharma. This work was partially supported by the Hungarian National Research Development and Innovation Office (NFKI) grant OTKA-K19_131945 (I.B.). This work was partially supported by the Italian Ministry of Health – Bando Ricerca Corrente 2021 (F.P.).

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.

Objective: The objective of this study was to assess the prevalence of anti-VWF alloantibodies in subjects with type 3 VWD enrolled in the 3WINTERS-IPS.

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

Results: In total, 18 of the 213 (8.4%) subjects tested positive for anti-VWF antibodies and 13 of 213 (6%) had VWF:CB inhibitors. These 13 were among the 18 with anti-VWF antibodies. Of the 5 without VWF:CB inhibitors, 3 had non-neutralizing antibodies, 1 only inhibitor against VWF:GPIbM, and one could not be tested further. Ten of the 13 subjects with VWF:CB inhibitors also had VWF:GPIbM inhibitors, 6 of whom 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 subjects with type 3 VWD, whereas neutralizing VWF inhibitors were found in 6%, mainly in subjects homozygous for *VWF* null alleles. Because inhibitors may be directed toward different VWF epitopes, their detection is dependent on the assay used.

KEYWORDS
anaphylaxis, antibodies neutralizing, diagnosis, von Willebrand disease, von Willebrand factor

1 | 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 that include mucocutaneous bleeding, menorrhagia, and joint and gastrointestinal bleeding [7–9]. These symptoms impair the quality

of life [10] and require management based on the replacement therapy with VWF-containing products [9,10]. Replacement therapy may be delivered on demand to stop the bleeding episodes and prevent bleeding before a surgical procedure or as secondary long-term (SLT) prophylaxis to prevent frequently recurring bleeding [10–13]. On 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% to 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. Owing to the 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 and neutralizing antibodies directed against VWF by using several different assays.

Essentials

- Patients may develop anti-von Willebrand factor (VWF) antibodies and/or anaphylactic reaction because of replacement therapy.
- The development of VWF inhibitor is confirmed to be a rare event with a prevalence of 6%.
- Anaphylactic reactions have been reported for 8 of 18 subjects with anti-VWF antibodies.
- Detection of epitope-specific VWF inhibitors is determined by the type of test used.

2 | MATERIALS AND METHODS

2.1 | Study population

The 3WINTERS-IPS study includes 265 subjects with type 3 VWD of the European and Iranian ancestries 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.

2.2 | Confirmation of type 3 VWD diagnosis

At the time of enrolment, plasma samples and buffy coats were collected to confirm centrally the diagnosis. To this purpose, the von Willebrand factor antigen (VWF:Ag) was measured by using an enzyme-linked immunosorbent assay (ELISA)-based method, whereas FVIII:C was measured by using a 1-stage clotting assay using FVIII-deficient plasma (Siemens) and the APTT reagent Triniclot (TCoag). The VWF propeptide (VWFpp) was measured by using an ELISA by using antibodies from Sanquin [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 subjects' bleeding history was collected at enrolment, and the bleeding score (BS) was calculated by using a bleeding assessment tool [18] along with the information available about therapy. The results obtained were reported as medians and interquartile ranges (IQRs) for continuous variables, whereas categorical data were reported as percentages.

2.3 | Anti-VWF antibodies

The presence of all antibodies against VWF was determined in plasma samples obtained from subjects at the time of enrolment by using an in-house indirect ELISA [19], which identifies all antibodies irrespective of the immunoglobulin subclass. Briefly, 96-well ELISA plates (Nunc A/S) 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 the 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 antihuman IgG, IgA, and IgM antibodies labeled with horseradish peroxidase (HRP). Binding was revealed through a colorimetric reaction by measuring the absorbance at 492/620 nm. The assay cutoff was set at 2 times the optical density (OD) of the 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 OD of normal pooled plasma).

2.3 | Neutralizing antibodies (inhibitors)

Plasma samples were evaluated for the presence of neutralizing antibodies with a Bethesda-based method by using an in-house collagen type III ELISA [20]. A reference plasma consisting of a lyophilized pool plasma from healthy donors (Technoclone, Diapharma) was resuspended following the manufacturer's instructions and used as normal pooled plasma (NPP) for mixing studies. Undiluted plasma samples and serial dilutions performed by using the PBS/albumin (5%) dilution buffer were mixed 1:1 with the NPP. The anti-human VWF rabbit antibody (A0082; Dako) was prediluted from 1:40 to 1:640 by 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 the 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) were coated with collagen type III (Biozol Eching) 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, the plates were washed 3 times, and samples were 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 for 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 the absorbance at 492/620 nm. Samples were considered positive for VWF inhibitors in the presence of a titer ≥ 0.3 Bethesda unit (BU). A BU was defined as the amount of the antibody that 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 by using the gain-of-function mutant glycoprotein(GP)Ib binding assay ([VWF:GPIbM], INNOVANCE VWF Ac test kit; Siemens). 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 that is directly proportional to the VWF-GPIb binding activity in plasma.

Differently from the other 2 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) 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.

3 | RESULTS

3.1 | 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 3 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 the sampling time, but this information was missing for 1 subject. The second group included 9 subjects with VWF:Ag ≤3 IU/dL in whom genetic analyses failed to

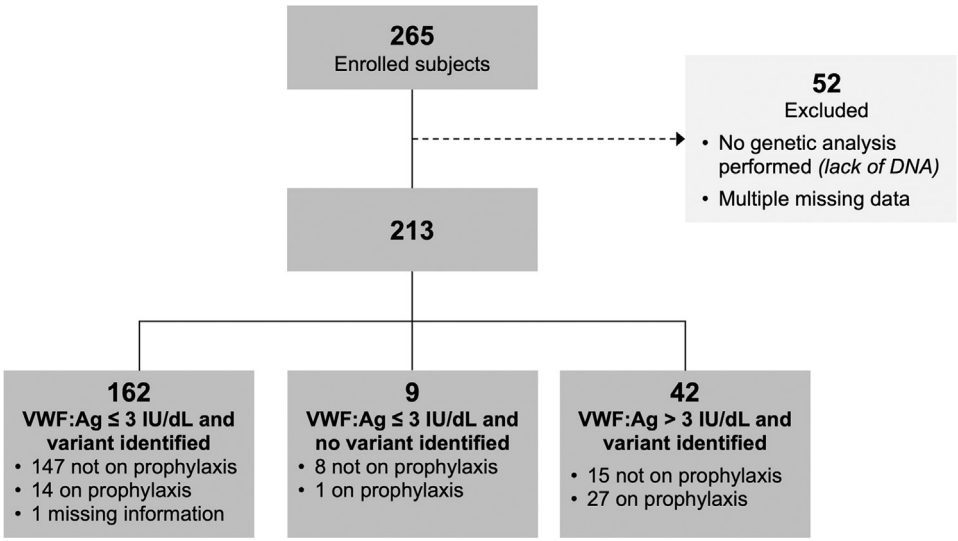

FIGURE 1 Flowchart of the study population. In total, 265 subjects with a previous type 3 diagnosis were enrolled in 3WINTERS-IPS. Of them, 52 were excluded because of multiple missing data, whereas the remaining 213 subjects were tested for both non-neutralizing and neutralizing VWF antibodies by using 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[a,b]	VWF:Ag ≤3 IU/dL, genetic variant identified[a,c]	VWF:Ag ≤3 IU/dL, no genetic variant identified[d]	VWF:Ag >3 IU/dL, genetic variant identified[e]
Subjects, *n* (%)	213 (100)	162 (76)	9 (4)	42 (20)
Age at enrolment (y)	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[f]	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[f]	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[f]	0.5	0.5	-	-
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[f]	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[f]	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.
[a] 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.
[b] Missing values: FVIII:C, *n* = 4; VWFpp, *n* = 8; BS, *n* = 11; and incomplete genotyping, *n* = 11.
[c] Missing values: FVIII:C, *n* = 1; VWFpp, *n* = 4; BS, *n* = 6; and incomplete genotyping, *n* = 5.
[d] Missing values: FVIII:C, *n* = 1 and VWFpp, *n* = 1.
[e] Missing values: FVIII:C, *n* = 2; VWFpp, *n* = 3; BS, *n* = 5; and incomplete genotyping, *n* = 6.
[f] One subject in the main group was unclassified because of missing information about treatment.

identify a *VWF* defect. Of them, 1 was on prophylaxis at the 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 the sampling time.

3.2 | All anti-VWF antibodies

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

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

Subject ID (E/I)	Age[a]	Sex	BS	VWF:Ag *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C *(IU/dL)*	Anti-VWF (OD subject/ OD cutoff)[b]	VWF:CB Inhibitor (BU)	VWF:GPIbM Inhibitor (BU)	VWF:Ag Inhibitor (BU)	VWF Gene Defect specification (HGVS description, allele 1/HGVS description, allele 2)[d]
32 (E)	55	F	27	0.5	43.1	2.2	8.4	5	2.8	0.3	NM_000552.3:c.4975C>T (p.Arg1659*)/ NM_000552.3:c.4975C>T (p.Arg1659*)
37 (E)	42	F	32	0.5	1.2	1.1	7.84	1.8	<0.3	<0.3	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180) _(6233842_?)del (delEx1_Ex52)
81 (E)	41	F	8	0.5	0.2	2	11.28	15	5.9	0.7	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180) _(6233842_?)del (delEx1_Ex52)
82 (E)[c]	40	M	9	5.2	8.5	33.4	4.92	<0.3	<0.3	<0.3	NM_000552.3:c.1534-3C>A (p.Leu512Profs*11)[e]/ NM_000552.3:c.7085G>T (p.Cys2362Phe)
96C (E)	44	F	16	1.5	11.4	5	7.96	0.4	<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)[e]
99D (E)	43	M	17	0.5	1.4	3.4	9.34	10	3.8	2.0	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180) _(6233842_?)del (delEx1_Ex52)
101D (E)	45	F	28	0.5	0.3	2.4	6.08	0.3	0.3	<0.3	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180) _(6233842_?)del (delEx1_Ex52)
102D (E)	42	M	20	0.5	1.3	3.6	8.0	1.3	0.7	<0.3	NC_000012.11:g.(?_6058180)_(6233842_?)del (delEx1_Ex52)/ NC_000012.11:g.(?_6058180) _(6233842_?)del (delEx1_Ex52)
106 (E)	29	F	6	0.5	0.8	2.9	8.53	3.8	0.4	<0.3	NM_000552.3:c.6182delT (p.Phe2061Serfs*38)/ NM_000552.3:c.6182delT (p.Phe2061Serfs*38)
113 (E)	20	F	20	2.2	14.1	1.7	5.16	<0.3	1	<0.3	NM_000552.3:c.6917delT (p.Leu2306Argfs*4)/NONE
114 (E)	63	F	33	0.5	0.2	2.2	5.36	1	<0.3	<0.3	NM_000552.3:c.7636A>T (p.Asn2546Tyr)/ NM_000552.3:c.7636A>T (p.Asn2546Tyr)
6 (I)	2	M	5	1.4	8.6	5.1	6.97	1.18	0.5	<0.3	NC_000012.11:g.(?_6058180)_(6105389_6120781)del (delEx35_Ex52)/ NC_000012.11:g.(?_6058180) _(6105389_6120781)del (delEx35_Ex52)
47 (I)	45	F	-	0.5	3.1	1.4	7.17	23	52	1.5	NM_000552.3:c.4036C>T (p.Gln1346*)/NONE
61 (I)	18	F	5	0.5	0.6	1.2	7.26	56	70	7.6	NM_000552.3:c.311_312delAG (p.Gln104Argfs*19)/ NM_000552.3:c.311_312delAG (p.Gln104Argfs*19)

TABLE 2 (Continued)

Subject ID (E/I)	Age[a]	Sex	BS	VWF:Ag *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C *(IU/dL)*	Anti-VWF (OD subject/ OD cutoff)[b]	VWF:CB Inhibitor (BU)	VWF:GPIbM Inhibitor (BU)	VWF:Ag Inhibitor (BU)	VWF Gene Defect specification (HGVS description, allele 1/HGVS description, allele 2)[d]
66 (I)	30	F	2	0.5	0.3	1.9	2.44	<0.3	n.d.	n.d.	NM_000552.3:c.4975C>T (p.Arg1659*)/ NM_000552.3:c.4975C>T (p.Arg1659*)
87 (I)	9	F	10	0.5	4	3.3	5.84	13	28	1.7	NM_000552.3:c.4309delG (p.Ala1437Profs*4)/ NM_000552.3:c.4309delG (p.Ala1437Profs*4)
94L (I)	32	F	23	0.5	3.6	2.6	2.96	<0.3	<0.3	<0.3	NM_000552.3:c.2376C>G (p.Cys792Trp)/ NM_000552.3:c.2376C>G (p.Cys792Trp)
103M (I)	26	M	14	0.5	4.2	3.3	2.56	<0.3	<0.3	<0.3	NM_000552.3:c.2376C>G (p.Cys792Trp)/ NM_000552.3:c.2376C>G (p.Cys792Trp)

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.

A sample was considered positive for neutralizing antibodies if the inhibitor titer was ≥0.3 BU.

BS, bleeding score; BU, Bethesda units; E, European subject; F, female; M, male; FVIII:C, factor VIII coagulant activity; I, Iranian subject; n.d., not determined; VWF:Ag, von Willebrand factor antigen; VWF:CB, von Willebrand factor collagen binding; VWF:GPIbM, the gain-of-function mutant GPIb binding was performed using INNOVANCE reagent; VWFpp, von Willebrand factor propeptide.

[a] Age at enrolment.

[b] 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 pooled plasma.

[c] This subject has an unconfirmed type 3 VWD diagnosis because of VWF:Ag >3 IU/dL.

[d] The large deletions are also reported using a simpler nomenclature.

[e] This variant has been previously evaluated at mRNA level.

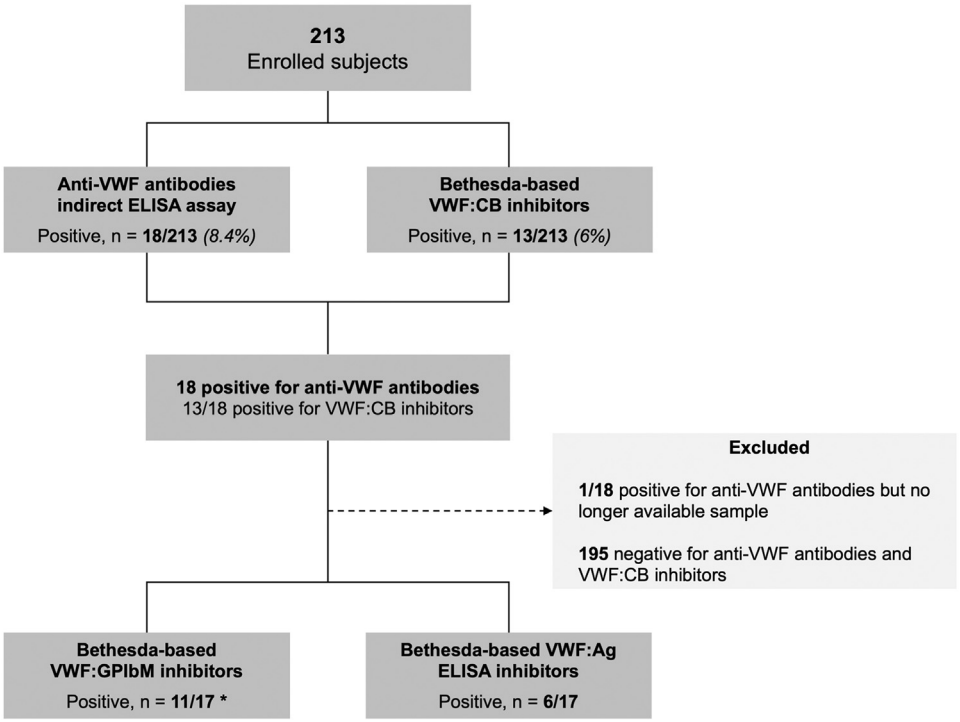

FIGURE 2 Sample workflow and related results. In total, 213 subjects have been screened for all antibodies against VWF (without distinguish between non-neutralizing and neutralizing antibodies) by using an indirect ELISA assay. The same 213 subjects have been tested for VWF inhibitors with a Bethesda-based method able to measure the residual VWF collagen binding activity (VWF:CB).The Bethesda-based method using VWF:GPIbM and the Bethesda-based method by 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.

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 women (13/18; 72%). These subjects have a median BS of 16, IQR: 7.0-25.0 (n = 17; data missing for 1 subject) similar to that of subjects with type 3 VWD who tested negative for anti-VWF alloantibodies who had a median BS of 15, IQR: 8.0- 21.0 (n = 185; data missing for 10 subjects). None of them was on SLT prophylaxis at the sampling time, although all but 1 (missing information) reported to have received previous treatments. All but 1 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 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).

3.3 | Neutralizing antibodies (inhibitors)

The 213 subjects with type 3 VWD were investigated to assess the presence of VWF neutralizing antibodies (inhibitors) with a Bethesda-based method by using the VWF:CB 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 of the 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 (Figure 2) by 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 1 was positive for VWF:GPIbM but not for VWF:CB inhibitors. The Bethesda-based method measuring the 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, 4 had VWF:CB plus VWF:GPIbM inhibitors, 3 had only VWF:CB inhibitors, and 1 had only VWF:GPIbM inhibitors. The 3 subjects who only tested positive with the indirect ELISA assay (3/17) were therefore diagnosed to have non-neutralizing antibodies.

3.4 | 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. Most subjects were homozygous for a genetic defect (n = 15; 83%), 1 was a compound heterozygote for a missense and a splice variant (6%) and 2 were incompletely characterized because the genetic defect on the second *VWF* allele was not identified (11%; 1

heterozygous for a small deletion and the other 1 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 responsible for alternative splicing (n = 1), and a nonsense variant (n = 1). One subject was homozygous for a missense variant. The remaining subjects 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.

3.5 | Subjects' history on therapies and anaphylactic reactions

All the data herein reported refer to the retrospective phase of 3WINTERS-IPS and were collected at the time of subject enrolment. Neither the 13 subjects positive for VWF:CB inhibitors nor the only 1 with the VWF:GPIbM inhibitor was on SLT prophylaxis at the sampling time, and all were already 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 1 this information was missing (Table 3). Four subjects reported the previous use of recombinant activated FVII (rFVIIa) and 3 have been treated with a recombinant FVIII. In all cases, the administration of rFVIIa followed that of at least 1 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 2 different products. All Iranian subjects but 1 (unavailable information) reported to have been treated only with a plasma concentrate containing VWF. The 3 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 3 subjects with non-neutralizing antibodies and the 1 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 8 subjects (8/18) characterized by VWF:CB, VWF:GPIbM, and VWF:Ag inhibitor assays. The titers of these inhibitors were variable from very low (0.3 BU) to high (56 BU). The behavior of the remaining 6 subjects (6/18) who at enrolment reported no history of anaphylaxis is unclear. 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 1 had been switched to recombinant FVIIa and then activated prothrombin complex concentrates by the attending physicians owing to the previous experience of anaphylaxis episodes in other subjects followed at the same center; 1 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 2 of them: all these subjects had been exposed to plasma-derived VWF products, but the data about exposure were missing in 1 case.

4 | 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 subjects with type 3 VWD enrolled so far investigated for this purpose. All antibodies against VWF were detected by using an indirect ELISA assay, whereas the presence of neutralizing antibodies (inhibitors) was detected by using a Bethesda method based on the measurement of residual VWF:CB in plasma. Overall, 18 of 213 subjects with type 3 VWD tested positive for VWF alloantibodies, thus with a prevalence of 8.4%. All but 1 of them (because of unavailable data) reported previous treatments with at least 1 product containing VWF. Three of 18 subjects tested positive for anti-VWF antibodies by 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 that do not inhibit VWF function(s). Nevertheless, it was impossible to assess whether 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, 1 received no further infusion after positivity for VWF:CB inhibitor, whereas the remaining 2 showed a poor recovery of VWF:Ag, VWF ristocetin cofactor activity, and 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 by 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 not only that the use of the VWF:CB

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	VWF:CB inhibitors (BU)	VWF:GPIbM inhibitor (BU)	VWF:Ag inhibitor (BU)	Anaphylactic reaction	VWF Gene Defect specification (Allele 1/Allele 2)
32 (E)	1959	plasma derived VWF-FVIII (1994)	-	5	2.8	0.3	+	p.Arg1659*/p.Arg1659*
37 (E)	1972	Recombinant FVIII (2014)	-	1.8	<0.3	<0.3	-	delEx1_Ex52/delEx1_Ex52
81 (E)	1971	plasma derived VWF-FVIII (1980); plasma derived VWF-FVIII (1986); plasma derived VWF-FVIII (1993); recombinant FVIII (1993)	-	15	5.9	0.7	+	delEx1_Ex52/delEx1_Ex52
82 (E)[a]	1972	plasma derived VWF-FVIII (2011)	+	<0.3	<0.3	<0.3	-	p.Leu512Profs*11[c]/ p.Cys2362Phe
96C (E)	1969	plasma derived VWF-FVIII (1991); plasma derived VWF-FVIII (2003)	-	0.4	<0.3	<0.3	-	p.G2706_C2719delfs*25/ p.G2706 _C2719delfs*25[c]
99D (E)	1970	plasma derived VWF-FVIII (1991); activated recombinant FVII (1995)	-	10	3.8	2.0	+	delEx1_Ex52/delEx1_Ex52
101D (E)	1968	plasma derived VWF-FVIII (1994); activated recombinant FVII (2011)	-	0.3	0.3	<0.3	+	delEx1_Ex52/delEx1_Ex52
102D (E)	1971	plasma derived VWF-FVIII (1977); recombinant FVIII (1997); activated recombinant FVII (1997); plasma derived VWF-FVIII (2001)	-	1.3	0.7	<0.3	+	delEx1_Ex52/delEx1_Ex52
106 (E)	1984	plasma derived VWF-FVIII (2000)	-	3.8	0.4	<0.3	+	p.Phe2061Serfs*38/ p.Phe2061Serfs*38
113 (E)[b]	1993	plasma derived VWF-FVIII (1994); activated recombinant FVII (2006); activated prothrombin complex (2013)	-	<0.3	1	n.d.	-	p.Leu2306Argfs*4/NONE
114 (E)	1950	plasma derived VWF-FVIII (1994)	-	1	<0.3	<0.3	+	p.Asn2546Tyr)/p.Asn2546Tyr
6 (I)	2011	plasma derived VWF-FVIII (2011)	-	1.18	0.5	<0.3	-	delEx35_Ex52/delEx35_Ex52
47 (I)	1967	N.A.	-	23	52	1.5	-	p.Gln1346*/NONE
61 (I)	1994	plasma derived VWF-FVIII (2008)	-	56	70	7.6	+	p.Gln104Argfs*19/ p.Gln104Argfs*19
66 (I)	1983	plasma derived VWF-FVIII (2012)	-	<0.3	n.d.	n.d.	-	p.Arg1659*/p.Arg1659*
87 (I)	2003	plasma derived VWF-FVIII (2008)	-	13	28	1.7	-	p.Ala1437Profs*4/ p.Ala1437Profs*4
94L (I)	1980	plasma derived VWF-FVIII (2007)	+	<0.3	<0.3	<0.3	-	p.Cys792Trp/p.Cys792Trp
103M (I)	1986	plasma derived VWF-FVIII (2008)	+	<0.3	<0.3	<0.3	-	p.Cys792Trp/p.Cys792Trp

All data were collected at enrolment (3WINTERS-IPS retrospective phase). Subjects 113 (E) and 47 (I) had an incomplete genotyping as the respective second genetic defect was not found. Subject 66 (I) has been only tested for anti-VWF antibodies and VWF:CB inhibitors because of insufficient sample.

BU, Bethesda units; E, European subject; I, Iranian subject; N.A., not applicable; n.d., not determined.

[a] This subject has an unconfirmed type 3 VWD diagnosis because of VWF:Ag >3 IU/dL.

[b] This subject tested positive for VWF inhibitors with a Bethesda-based assay using VWF:GPIbM (1 BU).

[c] This variant has been previously evaluated at the mRNA level.

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 by 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 most subjects who developed inhibitors were homozygous for complete or large gene deletions or genetic defects resulting in null alleles, whereas only 1 subject was homozygous for a missense variant. However, not all the subjects with type 3 VWD 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, although 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 1 in heterozygosity because the second genetic defect was not found). Of them, 4 homozygous subjects were siblings, but only 3 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 1 developed an inhibitor. Similarly, only 1 of 2 unrelated Italian subjects who were homozygous carriers for the p.Phe2061Serfs*38 developed an inhibitor. Finally, among 3 unrelated Dutch subjects who were homozygous carriers for p.Asn2546Tyr, 2 reported the previous use of a concentrate containing VWF but only 1 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 hemophilia 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 1 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 and the related therapeutic approaches in a very large cohort of subjects with type 3 VWD. However, the sample size, albeit large considering the rarity of VWD type 3, still remains 1 of the study limitations. Because all these subjects have been already proven to carry VWF inhibitor at the time of enrolment, a second limitation of the study is that we could not evaluate whether 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 before the inhibitors' detection (eg, exposure day and dosages) nor how the therapeutic approach changed afterward. Finally, the assays used to determine the presence of anti-VWF antibodies are not standardized, although our choice to perform them centrally has perhaps contributed to reduce variability.

In conclusion, the presence of alloantibodies, which 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 by using the indirect ELISA assay had VWF inhibitors. However, all subjects with VWF inhibitors were detected by using this assay, suggesting that it may represent a valid screening method. All subjects had previous treatments, but it was impossible to establish whether the non-neutralizing antibodies are the consequence of replacement therapy nor any exposure time relationship. The development of neutralizing antibodies assessed by 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 1 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 on the VWF:Ag ELISA should be discouraged because of the low sensitivity that allows to detect only high titers antibodies.

ACKNOWLEDGMENTS

We would like to thank the Members and current Chair of the Sub-Committee on VWF of the Scientific Standardization Committee (SSC) of the ISTH who promoted the endorsement of 3WINTERS-IPS

project among the scientific activities of this Sub-Committee. We also would like to thank Javier Battle, Erik Berntorp, Cosimo Ettore, Charles R.M. Hay, Hamid Hoorfar, Maria Gabriella Mazzucconi, Massimo Morfini, Rafael Parra Lòpez, and Omidreza Zekavat for subjects' enrolment and collections of clinical data and Anne Goodeve for her work on genotyping.

We wish to thank Paolo De Simoni, Luca Maravigna, and Elisabetta Musazzi of the CRO Sintesi Research for the study coordination. We acknowledge L. F. Ghilardini for the illustration work. The Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico is member of the European Reference Network (ERN) EuroBloodNet.

AUTHOR CONTRIBUTIONS

U.B. I.P., F.P., A.B.F., and J.E. conceived, designed, and supervised the study; L.B., U.B., J.G., R.S., and J.E. performed and/or supervised laboratory measurements; M.T.P. and U.B. performed and/or supervised data analysis; M.T.P.,U.B., L.B., J.E, I.P., P.M.M., A.B.F., and F.P., wrote and revised the manuscript; M.A., E.B., Z.B., M.R.B., O.B.H., I.B., G.C., P.E., J.G., M.K., B.K., R.L., F.W.G.L., M.F.L.F., R.M., J.O., C.S., R.S., A.T., G.T., A.T., M.T., H.Y., E.Z., J.E., and F.P. enrolled subjects and collected subjects' data; M.A. supervised the laboratory samples collected at the Iranian centers, and all authors contributed to the editing of the manuscript and approved the final version.

DECLARATION OF COMPETING INTEREST STATEMENT

J.E. received research support from CSL Behring outside the scope of this project and fees for educational activities from Roche and Celgene, which fees go to the institution. J.G. in the last 3 years received fees for lectures or consultancy from LFB outside the scope of this work. F.L. received unrestricted research grants from CSL Behring, Takeda, SOBI, and uniQure. He is a consultant for CSL Behring, Takeda, BioMarin, and uniQure, of which the fees go to the University. He has been a DSMB member of a study sponsored by Roche. P.M.M. is member of the scientific board for the Bayer Awards. Speaker fee from Bayer and Kedrion, Roche for lectures at educational symposia. C.S., in the last 3 years, has received honoraria for consulting or speaker bureau from Bayer, CSL Behring, Takeda, Novonordisk, Sobi, Novartis, Pfizer, Amgen, and Roche. A.T. received research support or honoraria for lectures or consultancy from CSL Behring, Octapharma, and Takeda outside of this work. F.P. reported participation at advisory board of Sanofi, Sobi, Takeda, Roche, and BioMarin. A.B.F. reported advisory board participation of CSL Behring, Grifols, Takeda, Octapharma, LFB, and Kedrion. J.O. has received research funding from Bayer, Biotest, CSL Behring, Octapharma, Pfizer, Swedish Orphan Biovitrum, and Takeda; consultancy, speakers bureau, honoraria, scientific advisory board, and travel expenses from Bayer, Biogen Idec, BioMarin, Biotest, Chugai Pharmaceutical Co., Ltd., CSL Behring, Freeline, Grifols, Novo Nordisk, Octapharma, Pfizer, F. Hoffmann-La Roche Ltd., Sanofi, Spark Therapeutics, Swedish Orphan Biovitrum, and Takeda. GC reported advisory board participation/speaker fee of CSL Behring, Grifols, Takeda, LFB, and Kedrion. The remaining authors declare no competing financial interests.

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