Pathophysiology of von Willebrand factor in bleeding and thrombosis

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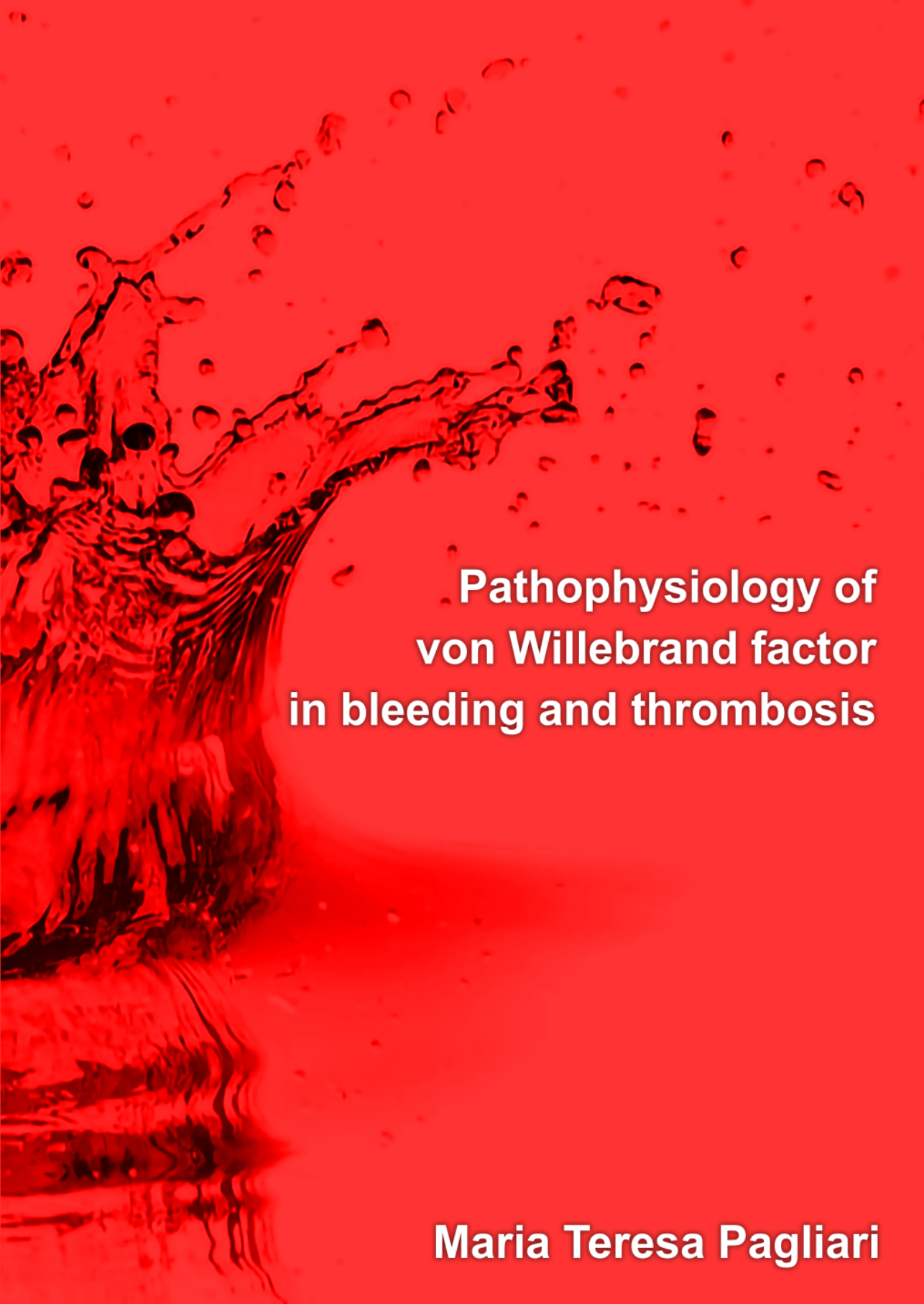

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Maria Teresa Pagliari

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PATHOPHYSIOLOGY OF VON WILLEBRAND FACTOR IN BLEEDING AND THROMBOSIS

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CHAPTER 1

INTRODUCTION AND OUTLINE OF THE THESIS

VON WILLEBRAND FACTOR SYNTHESIS

von Willebrand factor (VWF) is a multimeric glycoprotein principally synthesized in the endothelial cells and in a lower percentage in megakaryocytes [11]. VWF is synthesized as a pre-pro-polypeptide of 2813 amino acids (aa) that includes a signal peptide (22 aa), a propeptide (741 aa), and the VWF (2050 aa), which undergoes several post-translational modifications before it reaches its mature form [2-5]. The first series of modifications occur in the endoplasmic reticulum (ER) where the signal peptide is cleaved and VWF is glycosylated at N-linked glycosylation sites. Then, the subunits dimerize through the formation of disulfide bonds at the carboxyl-terminal extremity [6, 7]. Once in the Golgi, further glycosylation by adding O-linked glycans occurs along with sulfation.

At this stage, the VWF propeptide (VWFpp) is cleaved by furin. The VWFpp remains non-covalently bounded to VWF exerting an important regulatory role in driving the multimerization process which occurs through the formation of disulfide bonds involving the N-terminal extremity of dimers [6, 8].

The VWFpp also regulates the storage of mature VWF within the Weibel Palade Bodies (WPBs) in endothelial cells or the package into alpha-granules in platelets [9]. The mature VWF can be secreted in absence of stimuli (basal secretion [9-11]) or it can be released after endothelial or platelet activation (regulate secretion [12]).

VON WILLEBRAND DISEASE

von Willebrand disease is an inherited bleeding disorder caused by the presence of a mutation in *VWF* [13] and it can be distinguished in quantitative or qualitative defects. Quantitative defects include a partial deficiency of VWF defined as type 1 VWD, whereas the virtual absence of VWF is referred to as type 3 VWD. These two forms differ for the severity of the quantitative reduction and for the inheritance pattern which is autosomal dominant in the former and recessive for the latter.

The qualitative defects, mainly inherited with a dominant pattern, are generally recognized as type 2 VWD and can be further divided into four subgroups. Briefly, type 2A includes those variants characterized by an altered multimeric pattern with lacking of the most hemostatically active high molecular weights multimers (MWM) and in most severe forms even intermediate-MWM are missing. Type 2B includes those variants with increased VWF capacity to interact with glycoprotein Ib (GPIb) complex exposed on platelets surface, thus resulting in a faster consumption of MWM. Type 2M includes those variants responsible for an altered VWF function with reduced platelet or collagen binding activity although in presence of a normal multimeric pattern. Lastly, type 2N is caused by variants affecting VWF capacity to bind Factor (F) VIII [13, 14].

AVAILABLE TREATMENTS FOR VWD

Bleeding manifestations are due to either quantitative or qualitative VWF defects and in a second stage to the reduced FVIII levels. Therefore, the therapeutical approach focuses

on the normalization of VWF activity levels [15, 16]. The most common treatment consists of the infusion of the desmopressin (1-deamino-8-d-arginine vasopressin, DDAVP) which stimulate the transient release of both VWF and FVIII [17, 18]. The therapeutic effect of DDAVP should be tested by a trial administration to evaluate patient-individual responsiveness. Generally, it is administrated to type 1 patients with exception e.g., for those with an increased VWF clearance or when a prolonged treatment is required. Even a few type 2 patients may benefit from this treatment, such as in case of minor bleeding [19], although it is contraindicated in 2B as the rise in circulating mutant VWF with increased capacity to bind platelets may cause thrombocytopenia. Self-evident, DDAVP is not effective in type 3 patients because of the complete lack of VWF [20].

For these exceptions and in case of major and prolonged bleeding, regardless of VWD classification, the use of concentrate containing VWF is recommended. Depending on their origin, we can distinguish between plasma-derived (pd) VWF and recombinant (r) VWF. Pd-VWF can differ by the source, the purification process, the method used for viral inactivation and the variable amount of FVIII in the concentrate. The rVWF is produced in Chinese hamster ovary cells. Due to the method of production and the lack of ADAMTS13 cleavage activity, the rVWF shows ultra-large (UL) VWF multimers with a longer half-life than pd VWF products and it does not contain FVIII [21-23].

In some cases, patients may be treated with other additional drugs like tranexamic acid, a fibrinolysis inhibitor that can be administrated either alone or in combination with both DDAVP and VWF concentrate to treat minor mucocutaneous bleeding or in case of dental surgeries [24-26].

VWF BETWEEN BLEEDING AND THROMBOSIS

The most known function of VWF consists in the maintenance of the primary hemostasis exerted through the recruitment of platelets and by mediating their adhesion at the site of vascular damage [1, 27]. Indeed, the VWF can recognize and bind the different types of collagen exposed after the damage through the A3 (collagens I and III [28, 29]) and A1 (collagen IV and VI) domains [30, 31]. After anchoring to the vessel, the VWF is stretched by the shear which allows the exposure of A1 domains and as a consequence their interaction with the GPIb complex on platelets. Nevertheless, the VWF also plays a role in the secondary hemostasis circulating in a complex with FVIII stabilizing and protecting it from the proteolytic degradation [1].

The multimeric structure of VWF is strongly correlated with its hemostatic activity, in which the UL multimers can spontaneously bind to platelets [32]. The regulation of the pro-hemostatic function of VWF and thereby the prevention of spontaneous thrombosis is exerted by the disintegrin and metalloprotease with thrombospondin Type 1 repeats 13 (ADAMTS13) that is primarily synthesized in the hepatic stellate cells [33]. In physiological conditions, ADAMTS13 binds to the VWF A2 domain cleaving the Y1605-M1606 bond and resulting in smaller and less functional VWF molecules [34].

Severe deficiency of ADAMTS13 may cause thrombotic thrombocytopenic purpura (TTP), a life-threatening disorder characterized by the formation of thrombi rich in platelets and VWF [35]. On the contrary, the presence of mutations localized within the A2 domain may be responsible for an increased susceptibility of VWF to proteolysis by ADAMTS13 as described in patients with type 2A VWD with loss of VWF high- MWM [36].

The perturbation of the equilibrium between VWF and ADAMTS13 plasma levels has been investigated in the onset of different thrombotic disorders. The increase of plasma VWF and FVIII levels have been independently reported as associated with the risk of venous thromboembolism (VTE) [37-40]. Similarly, a reduction of ADAMTS13 levels has also been investigated in the onset of different thrombotic disorders including myocardial infarction, coronary artery disease, and venous thromboembolisms [41-43]. Nevertheless, the complete mechanism which links the increase of VWF and FVIII levels and a decrease of ADAMTS13 still remains to be clarified.

OUTLINE OF THE THESIS

This thesis focuses on the role of VWF in bleeding and thrombosis. In the first part of the thesis, the role of VWF variants and the development of VWD are considered. The second part focuses on type 3 VWD, the most severe form of this disease caused by the complete lack of VWF. Finally, the third part considers the VWF as a risk factor in the onset of deep vein thrombosis either alone or in combination with changes in ADAMTS13.

Part I: Genetics of von Willebrand disease

Chapter 2 describes the approach used to perform a differential diagnosis between type 2A and 2B VWD. For this purpose, the platelet-dependent VWF activity (VWF:GPIbM) over the VWF ristocetin cofactor activity (VWF:RCo) ratio has been used as an alternative to the ristocetin-induced platelet aggregation (RIPA).

In **Chapter 3**, a group of five patients carrying the type 1 variant p.Arg1379Cys has been characterized. One was diagnosed as affected by type 1 VWD, whereas the four who also carried the polymorphism p.Ala1377Val *in cis* with p.Arg1379Cys had a type 2M phenotype. The *in vitro* expression study evaluating the capacity of the wild-type, mutants, and hybrid recombinant (r) VWF to bind recombinant GPIb in presence of an increasing concentration of ristocetin showed that their synergistic effect is the cause of type 2M phenotype.

The genotypic characterization of two unrelated Italian VWD patients has been described in **Chapter 4**. The *novel* variant p.Thr274Pro found at heterozygous state has been expressed *in vitro* and rVWF localization has been further evaluated using immunofluorescence. This contributes to explain that the reduction of VWF levels measured in patients' plasma was caused by a combination of reduced synthesis, impaired secretion and multimerization.

Part II: Type 3 von Willebrand disease: results from the 3WINTERS-IPS

Chapter 5 focuses on the role of VWFpp in the determination of the pathomechanism underlying type 3 VWD in the largest cohort of type 3 VWD patients so far collected, the 3WINTERS-IPS. In this study, we showed that the VWFpp over VWF antigen (VWF:Ag) ratio may discriminate between homozygous/compound heterozygous carriers for a *VWF* missense variant and homozygous/compound heterozygous carriers of *VWF* null alleles. The use of FVIII over VWF:Ag ratio, however, failed to discriminate missense from null defects.

In **Chapter 6**, the development of alloantibodies against VWF in the 3WINTERS-IPS cohort has been investigated. We confirmed that the development of neutralizing antibodies represents a rare side effect of the replacement therapy with VWF concentrates and it is mainly found in type 3 patients homozygous for null defects. Because VWF inhibitors may recognize different epitopes on VWF, the prevalence estimation is strongly affected by the type of assay used.

Part III: Role of von Willebrand Factor in deep vein thrombosis

In the last two chapters, we have investigated the role of ADAMTS13, VWF, and FVIII as risk factors for deep vein thrombosis (DVT). In **Chapter 7** we demonstrated that a slight decrease in ADAMTS13 activity levels or an individually increase in VWF and FVIII levels are associated with an increased DVT risk. Moreover, we showed how the combination of slightly reduced plasma ADAMTS13 activity levels combined with high VWF levels is responsible for a marked increase in DVT risk in our study population. Subsequently, in **Chapter 8** we investigated the genetic component of DVT, focusing on *ADAMTS13*, *VWF* and *F8*. In this work, we applied a targeted next-generation sequencing approach to evaluate the association of common, low-frequency, and rare variants located in the coding region of these 3 genes with the onset of DVT. Finally, **Chapter 9** will include a general discussion and conclusions about the topics described in this thesis focusing on the future perspectives including a new cellular model to study and characterize VWF.

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PART I

GENETICS OF VON WILLEBRAND DISEASE

CHAPTER 2

DIFFERENTIAL DIAGNOSIS BETWEEN TYPE 2A AND 2B VON WILLEBRAND DISEASE IN A CHILD WITH A PREVIOUSLY UNDESCRIBED *DE NOVO* MUTATION

Pagliari MT, Baronciani L, Stufano F, Colpani P, Siboni SM, Peyvandi F.

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LETTER TO THE EDITOR

Type 2 von Willebrand disease (VWD) is due to qualitative defects of von Willebrand factor (VWF) and can be further divided into four subgroups: (i) type 2A includes variants with decreased VWF-dependent platelet adhesion and the deficiency of high molecular weight multimers (HMWM); (ii) type 2B includes variants with increased affinity of VWF for platelet glycoprotein Ibα (GPIbα) and a variable deficiency of HMWM; (iii) type 2M includes variants with decreased VWF-dependent platelet adhesion without selective deficiency of HMWM and (iv) type 2N includes variants with markedly decreased binding affinity for factor (F)VIII [1]. Patients with the most common form of type 2A [2] and type 2B [3] VWD may have similar FVIII and VWF levels (FVIII coagulant activity [FVIII:C], VWF antigen [VWF:Ag], VWF ristocetin cofactor activity [VWF:RCo], VWF collagen binding [VWF:CB]) and also the multimeric pattern may be quite similar. On the contrary, ristocetin induced platelets agglutination (RIPA) is remarkably different in type 2A and 2B VWD patients, being impaired in the former and heightened in the latter. RIPA is the most commonly used method to discriminate type 2B from other VWD types, although it suffers of limitations such as the need of a fresh blood sample and the requirement of an adequate patient platelet count [4]. Furthermore, we prefer not to perform RIPA in paediatric patients, due to the relatively large amount of required blood. There are other assays which are able to discriminate type 2B VWD using smaller amount of blood such as: (i) the measurement of ristocetin-induced binding of VWF to platelets by flow cytofluorimetry, performed using autologous platelets [5] and (ii) the whole blood ristocetin-activated platelet impedance aggregometry [6], although these assays are not currently available in our laboratory. Molecular analysis is another valid alternative to RIPA assay even if it might be occasionally inconclusive as in the case herein discussed.

Flood *et al*., [7] developed a ristocetin independent ELISA method using a recombinant gain-of-function GPIbα [7] to evaluate the platelet-dependent VWF activity (VWF:GPIbM) [8] and claimed to be able to discriminate type 2B from the other type 2 VWD patients using this assay [7]. Recently, we proposed a modified version of the Flood's ELISA as an alternative to RIPA to differentiate type 2B from the other VWD types, showing that the VWF:GPIbM/VWF:RCo ratio distinguishes most type 2B VWD patients from non-type 2B VWD. Indeed, type 2B VWD patients showed much higher VWF:GPIbM/VWF:RCo ratios than healthy subjects and other VWD patients. Furthermore, among type 2B patients, the increase in this ratio is proportional to the loss of HMWM [9].

With this background, we describe the practical use of the VWF:GPIbM/VWF:RCo ratio as an alternative to RIPA to perform the differential diagnosis between type 2A and 2B VWD in a paediatric patient with a previously undescribed *de novo* mutation. All subjects were aware of the study and gave their informed consent, according to the Declaration of Helsinki. The bleeding history of the proband, collected using the International Society On Thrombosis And Haemostasis-Bleeding Assessment Tool (ISTH-BAT) [10] and obtaining a score of 5, was characterized by easy bruising, prolonged bleeding after minor wounds

and recurrent epistaxis that in one instance required cauterization. The last event of epistaxis was followed by hemorrhagic shock which required blood transfusion and replacement therapy with a FVIII-VWF plasma-derived concentrate in association with tranexamic acid. The biochemical analysis of the 2 years old girl showed a platelet count of 323.000/mm^3 and FVIII:C of 56 IU/dL, but reduced VWF levels (VWF:Ag 33 IU/dL, VWF:RCo 6 IU/dL, VWF:CB 3 IU/dL) [11]. Multimeric analysis, performed by electrophoresis using 1.2% HGT agarose gel in presence of 0.1% SDS [12], showed the lack of high/intermediate MWM in the proband's plasma (**Figure 1A**).

The proband's parents had normal FVIII and VWF levels (FVIII:C 156 IU/dL, VWF:Ag 128 IU/dL and VWF:RCo 115 IU/dL for the mother and FVIII:C 130 IU/dL, VWF:Ag 119 IU/dL and VWF:RCo 110 IU/dL for the father).

The lack of high/intermediate MWM and the markedly reduced VWF:CB (3 IU/dL; normal range 45-174 IU/dL [11]) excluded a diagnosis of type 2M, but led to a possible diagnosis of type 2A or 2B VWD. The normal platelet count of the proband (323.000/mm^3) suggested a type 2A VWD diagnosis, because most type 2B patients with a severe loss of high/intermediate MWM are thrombocytopenic.

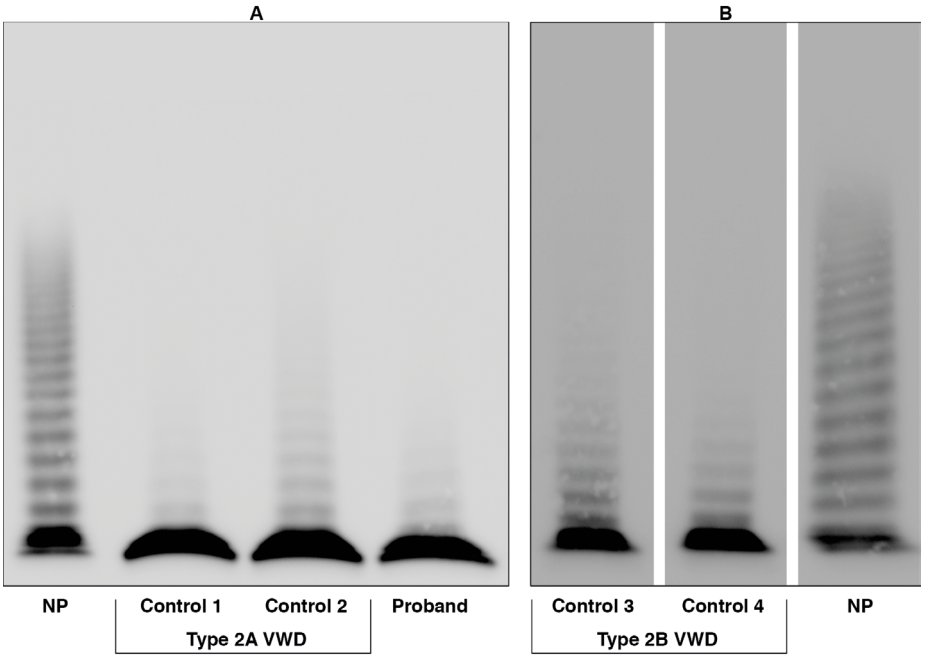

Figure 1. Multimeric analysis of proband and type 2A and 2B VWD patients used as controls. Multimeric structure of plasma von Willebrand factor (VWF) visualized in a non-reducing agarose gel (1.2% HGT agarose/0.1% SDS). The plasma VWF of the proband and those of type 2A (**A**) and 2B VWD (**B**) patients used for comparison showed a comparable multimeric pattern with the lack of high and intermediate molecular weight multimers. Lanes from the same gel are delimited by a black line.

Sanger sequencing of *VWF* exon 28 was performed for the proband in order to investigate the presence of a type 2 VWD mutation in both the A1 and the A2 domains [13] and a previously undescribed heterozygous in-frame deletion (c.[4606_4611delCACGTC];[=], p.[H1536_V1537del];[=]) was identified in addition to the already reported polymorphisms c.4141A>G (p.Thr1381Ala) and c.4641T>C (p.Thr1457Thr), both in homozygosis (reference sequence NM_000552.4). Then, the candidate mutation (p.H1536_V1537del) found in the proband was also searched in both parents without success. Short tandem repeats (STR)I and II were analysed by PCR in the proband and her parents using labelled oligonucleotides as previously described [14] to confirm the *de novo* origin of the mutation. The proband showed the same alleles (STRI-II 101-162 bp and STRI-II 121-170 bp) and also identified in her mother (STRI-II 101-162 bp; STRI-II 105-158 bp) or father (STRI-II 121-170 bp; STRI-II 125-182 bp).

Therefore, we could conclude that p.H1536_V1537del is a *de novo* mutation, which arose either in the proband herself at the early stages of embryogenesis or in the germ line cells of one of her parents. *De novo* mutations are rarely reported for VWD and their frequency is not precisely estimated [15], although Shen *et al.* [16] have recently described 4 *de novo* mutations in 15 families affected by type 2 VWD . Based on the STRI-II analysis, the proband has inherited the *VWF* alleles from her unaffected parents. Therefore, the *de novo* mutation identified is likely to be the disease-causing mutation, as also supported by the *in silico* evaluation performed using PROVEAN (-6.5; cut-off of -2.5) [17], SIFT-indel (0.894; max= 1) [18] and MutationTaster (damaging mutation) [19].

In this case, molecular analysis did not help us to distinguish between type 2A or type 2B VWD, because this mutation has never been reported before. Then, we carried out the VWF:GPIbM assay. The plasma samples of two type 2A and two type 2B VWD patients, characterized by the lack of the high/intermediate MWM and by plasma VWF levels similar to those of the proband, were used as controls (**Figure 1 A,B**). Each plasma sample was determined twice at two different dilutions.

The proband had a VWF:GPIbM/VWF:RCo ratio of 1, similar to those of the type 2A VWD controls but lower than those of type 2B VWD (**Table 1**).

This allowed us to exclude a type 2B gain-of-function effect for this mutation, thus obtaining the type 2A VWD diagnosis. A limitation of this approach is that the VWF:GPIbM/VWF:RCo ratio is inadequate to evaluate a possible type 2A patient with a VWF:RCo< 6 (IU/dL). In this case, the comparisons between VWF:GPIbM and VWF:RCo versus the corresponding VWF:Ag values have to be considered. Indeed, in type 2A VWD patients, the discrepancy between VWF activity and VWF:Ag is present when the activity is measured with both VWF:GPIbM and VWF:RCo assays, whereas in type 2B VWD patients this discrepancy is absent when the VWF:GPIbM assay is performed (**Table 1**).

In conclusion, this is a rare case of a previously undescribed *de novo VWF* mutation causing type 2 VWD. Both the platelet count and the localization of the p.H1536_V1537del mutation in the A2 domain suggested the type 2A VWD phenotype, although a firm diagnosis was obtained only by evaluating the VWF:GPIbM/VWF:RCo ratio.

Table 1. Biochemical and molecular data of proband and type 2A and 2B VWD patients used as controls

Patient	Type	Amino acid substitution	VWF:Ag *(IU/dL)*	VWF:RCo *(IU/dL)*	VWF:GPIbM *(IU/dL)*	VWF:GPIbM/ VWF:RCo
Proband	2A or 2B	p.[H1536_V1537del];[=]	33	6	6	1
Control 1	2A	p.[S1506L];[=]	24	8	10	1.3
Control 2	2A	p.[R1597Q];[=]	39	7	9	1.3
Control 3	2B	p.[R1306W];[=]	35	<6 [a]	42	8.4
Control 4	2B	p.[V1316M];[=]	35	8	34	4.5

Mutations are reported following the guidelines of the Human Genome Variation Society (http://varnomen.hgvs.org/; accessed on April 2018). Type 2A and type 2B VWD plasma samples with VWF levels and multimeric patterns comparable to those of the proband were used as controls. VWF:Ag, von Willebrand factor antigen; VWF:GPIbM, VWF gain-of-function mutant GPIb-binding assay; VWF:RCo, VWF ristocetin cofactor activity. [a] Because the lower detection limit of the aggregometric VWF:RCo test is 6 IU/dL, a value of 5 was considered in the ratio calculation.

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CHAPTER 3

VON WILLEBRAND DISEASE TYPE 1 MUTATION p.Arg1379Cys AND THE VARIANT p.Ala1377Val SYNERGISTICALLY DETERMINE A 2M PHENOTYPE IN FOUR ITALIAN PATIENTS

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ABSTRACT

Introduction

We characterized five patients affected with von Willebrand disease (VWD) carrying the p.Arg1379Cys mutation. One was diagnosed as VWD type 1 and four as type 2M. The 2M patients also have the variant p.Ala1377Val *in cis* with p.Arg1379Cys.

Aim

To evaluate the role of p.Ala1377Val and p.Arg1379Cys von Willebrand factor (VWF) variants to explain patients' phenotype.

Methods

Conventional phenotype tests were used to evaluate patients' plasma and platelets. Direct sequence analysis of exon 28 was carried out. The allele frequency of p.Ala1377Val was evaluated using online database. pcDNA3.1-VWF-WT and mutant (A1377V, R1379C and A1377V-R1379C) expression vectors were transiently transfected in HEK293 cells. The capacity of WT and mutant recombinant (r)VWF (along with patients' plasma VWF) to bind glycoprotein Iba(GpIbα) were evaluated, using two ELISA assays. One with a wild-type (WT) recombinant (r)GpIbα at increasing ristocetin concentrations (from 0 to 1.50 mg/mL) and the other with a gain-of-function mutant rGpIbα (VWF:GPIbM).

Results

The substitution c.4130C>T (p.Ala1377Val) was reported as rare variant in online databases. At 0.25 mg/mL of ristocetin, WT, A1377V and R1379C showed 6, 7.5 and 12-fold increased binding to rGpIbα, respectively. A1377V-R1379C rVWF showed no increased binding to rGpIbα at the same ristocetin concentration and reached the highest binding, of only 3-fold increased, at 1.50 mg/mL of ristocetin. The VWF:GPIbM showed strongly reduced values for the A1377V-R1379C rVWF and the 2Mpatients' plasma.

Conclusion

Our study showed that the presence of both p.Ala1377Val and p.Arg1379Cys mutations (synergistic effect) abolishes the binding of rVWF to rGpIbα, explaining patients' 2M phenotype.

INTRODUCTION

von Willebrand disease (VWD) is a common bleeding disorder due to quantitative and/or qualitative defects of von Willebrand factor (VWF) [1]. This multimeric glycoprotein plays an important role in primary haemostasis, promoting platelet adhesion at sites of vascular injury and in secondary haemostasis serving as a carrier protein for factor VIII (FVIII) [2-3]. A revised classification distinguishes quantitative defects of VWF (types 1 and 3) from qualitative defects (types 2A, 2B, 2M, and 2N) [4]. VWD type 1 and type 3 include partial quantitative deficiency and virtually complete deficiency of VWF, respectively. VWD type 2A includes variants with decreased VWF-dependent platelet adhesion due to a loss of VWF high and intermediate molecular-weight multimers (MWM). VWD type 2B is characterized by an increased affinity for platelet glycoprotein Ibα (GpIbα). VWD type 2M includes variants with decreased VWF-dependent platelet adhesion, without a selective deficiency of high-MWM [4]. VWD type 2N is characterised by a markedly decreased binding capacity of VWF to FVIII [4-5]. Molecular analysis is a valid effort to characterise patients' phenotype in most type 2 VWF variants, caused by mutations in exon 28 (2A, 2B, and 2M) or in exons 18 to 24 (2N) [4].

In this study, we characterized five unrelated patients with VWD using biochemical and molecular analysis. Direct sequencing of exon 28 revealed the presence of p.Arg1379Cys mutation previously reported to cause VWD type 1 [6-8], in all five patients. However, only one of these patients was classified as type 1, whereas the other four were diagnosed as type 2M. The four type 2M patients had the additional substitution p.Ala1377Val *in cis* with p.Arg1379Cys previously reported as polymorphism on the VWF variants database (VWFdb; http://www.vwf.group.shef.ac.uk/vwd.html; accessed August 2016), so its role as disease-causing mutation was initially underestimated. We investigated the role of p.Ala1377Val and p.Arg1379Cys variants, in order to establish their influence in the binding of VWF to GpIbα with the goal to explain the patients' phenotype.

METHODS

Patients

All five patients were aware of the experimental nature of this study and gave informed consent, according to the declaration of Helsinki (1964). Patients bleeding history was collected using the published bleeding severity score (BSS) [9].

Blood sampling and laboratory methods

Twenty millilitres of blood was drawn in 3.13% sodium citrate (9:1 v/v) and 5 mM EDTA (9:1 v/v). FVIII coagulant activity (FVIII:C), VWF antigen (VWF:Ag) and VWF ristocetin cofactor activity (VWF:RCo) were evaluated as previously described [10]. Platelets were isolated using a density gradient and lysed using Triton X-100 [11]. Ristocetin-induced platelet agglutination (RIPA) was carried out in platelet-rich plasma (PRP) using different

ristocetin concentrations [12]. VWF collagen binding activity (VWF:CB) of patients' plasma was evaluated using collagen type I (95% type I and 5% type III; Nycomed-Horm), as previously reported [10]. Plasma VWF multimeric analysis was performed by gel electrophoresis [13] under non reducing conditions in low (1.2% HGT agarose/0.1% SDS) and intermediate resolution (1.6% LGT agarose/0.1% SDS) gels. Determination of VWF propeptide (VWFpp) was performed by ELISA assay using the anti-human VWF propeptide antibodies (Sanquin, Amsterdam, The Netherlands).

Nomenclature

The mutations are reported following the guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/recs.html; accessed August 2016).

VWF analysis

Genomic DNA was extracted from peripheral blood using standard methods. The polymerase chain reaction (PCR) was performed to amplify the *VWF* exon 28 of patients as previously reported [14] to investigate the presence of mutations in the VWF A1 domain. Oligonucleotides and PCR conditions are available on request. Short tandem repeats (STR) I and II [15] were analysed by PCR using labelled oligonucleotides as previously described [16]. Molecular analyses of patients' relatives were performed to establish the *cis/trans* state of the two mutations for patients II, IV and V and to determine the STR I and II polymorphisms linked to patients' mutated allele. As no family members of patient III were available, to evaluate the *cis/trans* state of the two variants in patient III, a PCR fragment of a portion of exon 28 (939 base pairs) was purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), cloned into the pCR®XL-TOPO® vector (TOPO® XL PCR Cloning Kit; Invitrogen, Carlsbad, CA, USA) and sequenced.

The evaluation of p.Ala1377Val allele frequency among population has been performed using Exome Variant Server (http://evs.gs.washington.edu/EVS) and 1000 Genomes database (http://www.1000genomes.org), both accessed on August 2016 [17].

In silico evaluation

The potential effect of p.Ala1377Val and p.Arg1379Cys on the destabilization of the A1 domain, was analysed using the crystal structure of the wild-type (WT) A1 domain (1AUQ.pdb; Protein Data Bank, http://www.rcsb.org/pdb/search/searchSequence.do) and the I-mutant 3.0 tool (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi) at default mode, both accessed on August 2016 [18]. PYMOL software (DeLano Scientific, San Carlos, California, USA) was used to mutagenized the 1AUQ.pdb and to analyse the impact of both variants on the secondary structure of the A1 domain in comparison with the WT.

Plasmid construction

Mutant expression vectors pcDNA3.1-VWF-A1377V, pcDNA3.1-VWF-R1379C, and pcDNA3.1-VWF-A1377V-R1379C were generated by site-directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit; Agilent Technologies, La Jolla, CA, USA), using as template the pcDNA3.1-VWF-WT vector [19] and specifically designed oligonucleotides.

Transfection experiments

Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's Modified Eagle's medium (DMEM):F12 supplemented as previously reported [19]. Cells at 80-90% of confluence were transiently transfected with 8 µg of plasmid DNA using jetPEI™ (PolyPlus-transfection, Euroclone, Pero, Italy). For WT and mutant co-transfections (hybrids) 4 µg of each vector was used. Cell media were changed before transfection and collected after 72 h. Cells were harvested and lysed as previously described [20]. WT, mutant and hybrid recombinant (r)VWF in conditioned media and cell lysates were quantified using an ELISA assay. Cell media were concentrated (10X v/v), using Aquacide II (Calbiochem, La Jolla, CA, USA). Concentrated samples were evaluated again for VWF:Ag, then evaluated for the multimeric pattern.

rGpIbα binding

The binding capacity of rVWF and patients' plasma VWF to the rGpIbα was evaluated using an ELISA assay [20] at different ristocetin concentrations (Mascia Brunelli S.p.A., Milano, Italy). rGpIbα (amino acid residues 1–305) was obtained in HEK293 cells as previously described [21]. A microtitre plate was coated with 5 µg/mL of anti-GpIbα monoclonal antibody 2D4 (Laboratory for Thrombosis Research, Interdisciplinary Research Facility Life Sciences, KU Leuven, Belgium), diluted in PBS and incubated overnight (O.N.) at 4°C. Then 100 µL of rGpIbα (1 µg/mL) was added and incubated O.N. at 4°C. Concentrated conditioned media containing rVWF, were normalized in order to allow comparisons at similar VWF:Ag levels. Similarly, the normal pooled plasma used as reference and patients' plasma were normalized for the VWF:Ag.

Fifty microlitres of each sample was seeded in duplicate for each ristocetin concentration and added with 50 µl of ristocetin (0, 0.12, 0.25, 0.50, 0.75, 1.00 and 1.50 mg/mL, final wells' concentration) in PBS. The wells were washed and bound VWF was detected with a polyclonal anti-VWF antibody conjugated with horseradish peroxidase (HRP) (DakoCytomation ApS, Glostrup, Denmark), through a colorimetric reaction. The absorbance was measured at 492 nm [20].

Gain-of-function mutant GpIbα binding (VWF:GPIbM) assay

The platelet-dependent VWF activity of rVWF and patients' plasma VWF was evaluated using a gain-of-function rGpIbα protein from amino acid residues 1–305, containing

the mutations p.Gly233Val and p.Met239Val as previously described [22]. In case of rVWF, the assay was performed on the concentrated conditioned media and the results were reported as VWF:GPIbM/VWF:Ag ratio.

Statistical analysis

The comparison between WT, mutant and hybrid rVWF levels in conditioned media and cell lysates was made using a one-way ANOVA with Bonferroni post-hoc analysis if necessary. The binding capacity of rVWF and patients' plasma VWF to the WT rGpIbα was evaluated using a two-way ANOVA with Bonferroni post-hoc analysis if necessary. P values< 0.05 were considered statistically significant.

RESULTS

Phenotypic results

Patients' laboratory data and BSS are reported in **Table 1.** Patient I showed nearly normal plasma VWF:Ag and VWF:RCo levels, whereas patient II had a nearly normal VWF:Ag value but markedly reduced VWF:RCo (<6 IU/dL). Patients III-V showed reduced VWF levels, with a VWF:RCo/VWF:Ag ratio< 0.6. The VWF:CB/VWF:Ag ratio was normal for all patients (≥ 0.6).

Multimeric analysis of patients' plasma at low resolution showed a normal pattern in patient I and a slightly loss of HMWM with smear, in patients II-V, although at different extent (**Figure 1**; upper panel). The intermediate resolution gel showed decreased or slightly decreased satellite bands of the triplet structure (i.e., diminished proteolysis) in

Table 1. Phenotype and genotype data of the five patients

Pt	ABO	BSS	FVIII:C *(IU/dL)*	VWF:Ag *(IU/dL)*	VWF:RCo *(IU/dL)*	VWF:CB *(IU/dL)*	VWF:RCo/ VWF:Ag [†]	VWF:CB/ VWF:Ag	VWFpp/ VWF:Ag
I	non-O	2	68	46	43	50	0.90	1.1	1.46
II	O	n.d.	53	38	< 6 [A]	27	0.08	0.7	2.6
III	O	3	33	17	< 6 [A]	12	0.18	0.7	3.8
IV [‡]	non-O	12	37	28	11	17	0.39	0.6	2.8
V	non-O	3	36	24	7	16	0.29	0.7	2.9
NR	-	-1 to 3	50-150	40-169 [§] 55-165 [¶]	41-160 [§] 53-168 [¶]	45-170 [§] 56-174 [¶]	> 0.6	> 0.6	0.5-1.5

Pt, patient; ABO, blood-group system; BSS, Bleeding Severity Score; FVIII:C, factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity; VWF:CB, von Willebrand factor collagen-binding activity; VWFpp, von Willebrand factor propeptide; RIPA, ristocetin-induced platelet agglutination. NR, normal range; n.a., not applicable; n.d., not determined. [†] A VWF:RCo of 3 IU/dL was considered, in those cases with

patients II-V (**Figure 1**; lower panel). RIPA was within the normal range (0.7 mg/mL; NR; 0.7-1.2 mg/mL) in patient I, slightly decreased in patient II (1.4 mg/mL) and strongly reduced (> 2.0 mg/mL) for patients III-V. The VWFpp/VWF:Ag ratio showed a clear VWF increased clearance in patients II-V. Platelet VWF levels were normal in patient I and markedly reduced in patients II-V.

VWF analysis

Molecular data of the patients are reported in **Table 1**. Direct sequencing of exon 28 led to the identification of the mutations c.4130C>T (p.Ala1377Val) and c.4135C>T (p.Arg1379Cys) in all subjects, but patient I in whom only p.Arg1379Cys was identified. Relatives' molecular analysis of patients II, IV and V showed that p.Ala1377Val and p.Arg1379Cys were *in cis*. Sanger sequencing of the cloned exon 28 PCR-fragment showed that p.Ala1377Val and p.Arg1379Cys were *in cis* also in patient III.

STR I and STR II analysis of patients II, IV, V and their relatives showed that the mutated allele (p.Ala1377Val-Arg1379Cys) was associated with the same STR I (101 bp) and STR II (170 bp). For patient III, STR I were 101 and 117 bp, whereas STR II were 162 and 166 bp.

Patient II showed the additional nucleotide substitutions c.3797C>T (p.Pro1266Leu) and c.3835G>A (p.Val1279Ile), both *in trans* with p.Ala1377Val and p.Arg1379Cys.

The minor allele frequency (MAF) of p.Ala1377Val (rs141211612) reported on the Exome Variant Server and 1000 Genomes database was <0.01 and <0.02, respectively.

RIPA *(mg/mL)*	Platelet VWF:Ag *(IU/10^9 platelets)*	Platelet VWF:RCo *(IU/10^9 platelets)*	Nucleotide Change	Amino Acid Change
0.7	0.32	0.35	c.[4135C>T];[=]	p.[Arg1379Cys];[=]
1.4	0.09	< 0.06	c.[4130C>T;4135C>T];[3797C>T;3835G>A]	p.[Ala1377Val;Arg1379Cys];[Pro1266Leu;Val1279Ile]
> 2	0.10	< 0.06	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
> 2	0.13	< 0.06	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
> 2	0.13	< 0.06	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
0.7-1.2	0.22-0.60	0.15-0.58	n.a.	n.a.

a VWF:RCo< 6 IU/dL, to perform the calculation of VWF:RCo/VWF:Ag ratio. Values are shown as a mean of three measurements in three different samples, with exception of patient IV who has been tested only once ‡ as sample was not available. Patients' platelets were isolated only once. § Range values of normal individuals with blood group O; ¶ range values of normal individuals with blood group non-O.

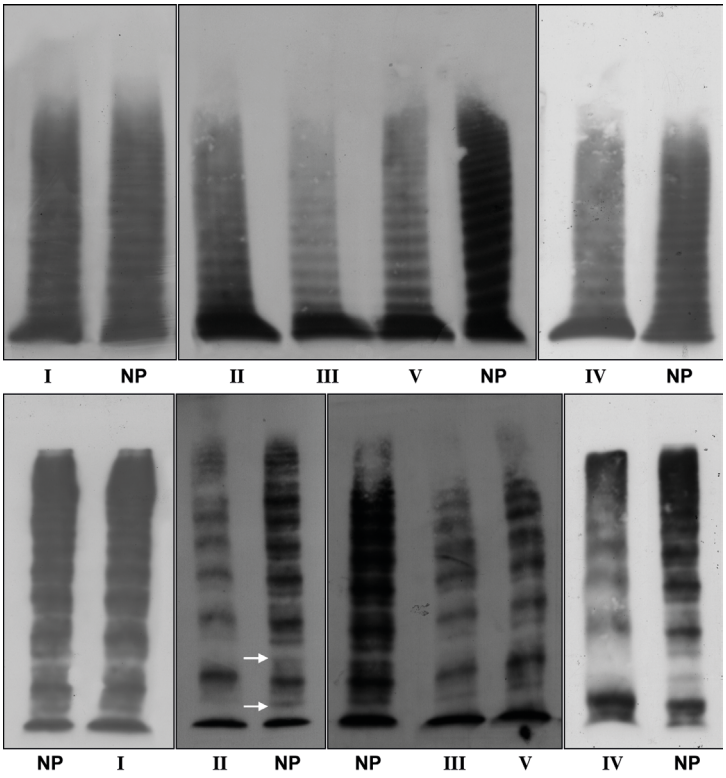

Figure 1. Multimeric analysis of patients' plasma VWF. Upper panel: Multimeric structure of plasma von Willebrand factor (VWF) visualized in a non-reducing low-resolution gel (1.2% HGT agarose/0.1% SDS) to highlight the high molecular weight multimers. All patients showed a full set of multimers, which was smeared in case of patients II-V. NP, normal plasma. Lower panel: Multimeric structure of plasma VWF visualized in non-reducing intermediate resolution gel (1.6% LGT agarose/0.1% SDS), to highlight the triplet structure. NP VWF showed the typical triplet structure and the satellite bands are indicated by the arrows. Patient I VWF showed a normal triplet structure, whereas it was decreased or slightly decreased in patients II-V. Lanes from the same gel are delimited by a black line.

Binding of patients' plasma VWF to rGpIbα

The results are reported in **Figure 2**. For each sample, the points shown in **Figure 2** correspond to the ratio between the optical density (O.D.) measured in presence of a specific ristocetin concentration and the O.D. measured with no ristocetin. The plasma VWF of patient I, showed a binding to rGpIbα similar to that of the pooled plasma at each ristocetin concentration ($P > 0.05$). The plasma VWF of patients II-V showed a reduced binding to rGpIbα than the pooled plasma (1-fold vs. 2-fold, respectively; $P > 0.05$) at 0.25 mg/mL of ristocetin. The binding of patients II-V plasma VWF to rGpIbα at 0.50 mg/mL of ristocetin was reduced, although at different extent (from 2-fold to 4-fold increased vs. 5-fold increased of normal pooled plasma).

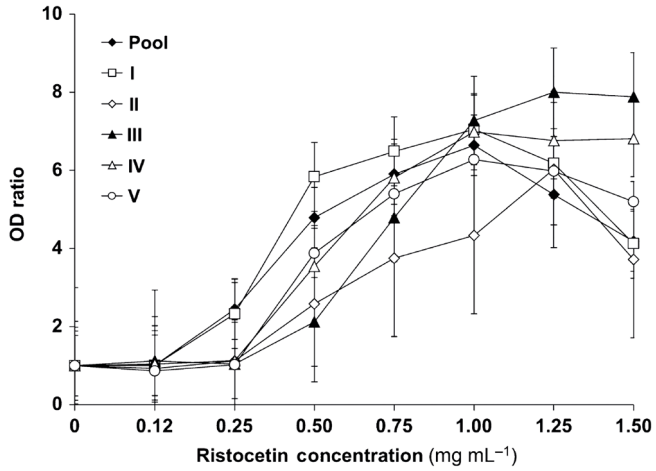

Figure 2. Binding of patients' plasma VWF to recombinant glycoprotein Ibα (rGpIbα). rGpIbα was immobilized on microtitre plates using a specific antibody. The normal pooled plasma used as reference and the patients' plasma samples were incubated in presence of increasing concentration of ristocetin (0, 0.12, 0.25, 0.50, 1.00, 1.25 and 1.50 mg/mL). For each sample, the points shown in the figure correspond to the ratio between the optical density (O.D.) measured in presence of a specific ristocetin concentration and the O.D. measured with no ristocetin. Values are plotted as the mean ± SE of two independent determinations. Pooled plasma (black diamond), patient I (square), patient II (diamond), patient III (black triangle), patient IV (triangle) and patient V (circle).

VWF:GPIbM assay performed on patients' plasma

The results are reported in **Table 2**. The VWF:GPIbM values were in line with VWF:RCo values for patients I, III, IV, and V and slightly increased for patient II (**Table 2**).

In silico evaluation

I-Mutant 3.0 predicted the decrease of A1 domain stability for both mutations with a ΔΔG of -0.91 for p.Ala1377Val and -1.36 for p.Arg1379Cys.

PYMOL showed that the substitution of the Ala1377 residue with a valine does not alter the formation of hydrogen bond with the Arg1374 residue and water. The substitution of the Arg1379 residue with a cysteine determine the loss of the hydrogen bonds with Lys1407, Lys1408 and water, whereas the hydrogen bond with Ile1410 was not disrupted.

Characterization of rVWF

rVWF in conditioned media and cell lysates were quantified as VWF:Ag, using an ELISA assay. The amount of mutant and hybrid rVWF (WT/mutants co-transfections) was expressed as a percentage of the WT rVWF referred as 100% ±SEM (**Table 3**). The amount of secreted A1377V and A1377V-R1379C rVWF was decreased to (45±10%) and (42±9%) respectively, whereas the amount of R1379C and hybrids rVWF was similar to that of WT. The amount of rVWF in cell lysates were decreased for A1377V (52±10%),

Table 2. VWF:GPIbM assay performed on patients' plasma VWF

Patient	VWF:Ag *(IU/dL)*	VWF:RCo *(IU/dL)*	VWF:GPIbM *(IU/dL)*
I	50	37	34.97
II	30	< 6	11.14
III	14	< 6	3.00
IV	19	< 6	4.25
V	26	7	6.73
NR	40-169 † 55-165 ‡	41-160 † 53-168 ‡	n.d.

Pt, patient; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity; VWF:GPIbM, VWF gain-of-function mutant GpIbα binding; NR, normal range. These assays have been performed on new patients' plasma samples. † Range values of normal individuals with blood group O; ‡ range values of normal individuals with blood group non-O; n.d., not determined.

Table 3. *In vitro* expression study of recombinant von Willebrand factor

rVWF	VWF:Ag Conditioned media (%)	VWF:Ag Cell lysates (%)
WT	100 %	100 %
A1377V	45±10 %	52±10 %
R1379C	73±18 %	68±22 %
A1377V-R1379C	42±9 %	93±11 %
A1377V/WT	75±26 %	85±13 %
R1379C/WT	80±23 %	87±29 %
A1377V-R1379C/WT	95±25 %	111±11 %

rVWF, recombinant von Willebrand factor; WT, Wild-type. VWF:Ag, von Willebrand factor antigen; The amount of mutant and hybrid rVWF (WT/mutants) were expressed as a percentage of the WT referred to 100% (± SEM).

nearly normal for R1379C (68±22%) and normal for A1377V-R1379C and hybrids rVWF. All comparison made by one-way ANOVA did not show a significant difference (P> 0.05).

All rVWF showed a full set of multimers, which was particularly smeared for A1377V-R1379C rVWF (**Figure 3**).

The rVWF binding to rGpIbα at different ristocetin concentrations was calculated as performed for patients' plasma samples and reported in **Figure 4** (upper panel). WT and A1377V rVWF showed 6 and 7.5-fold increased binding to rGpIbα (P> 0.05), respectively, at 0.25 mg/mL of ristocetin. The binding capacity of R1379C rVWF to rGpIbα was higher than that of the WT with a 12-fold vs. 6-fold increased (P> 0.05) at 0.25 mg/mL. The R1379C rVWF reached the plateau at 0.25 mg/mL, whereas WT and A1377V rVWF at 0.50 mg/mL of ristocetin. The binding capacity of A1377V-R1379C rVWF was

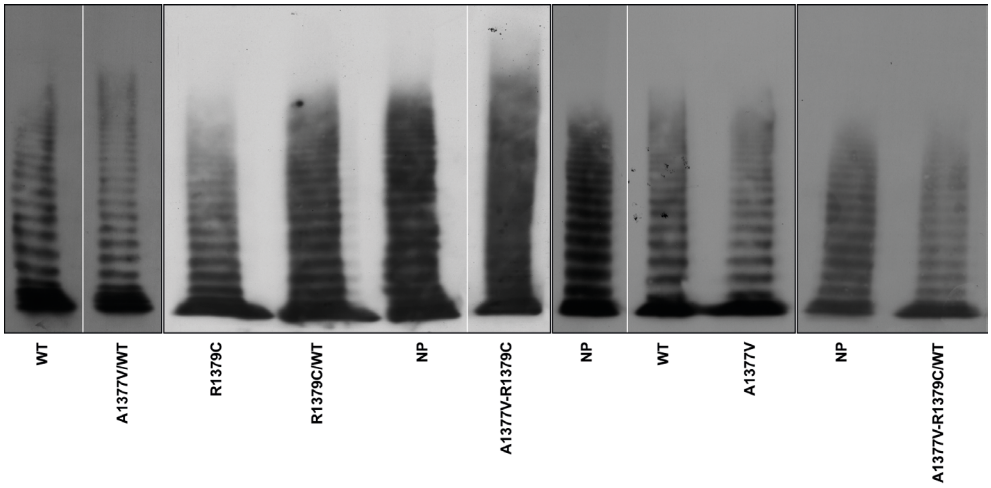

Figure 3. Multimeric structure of recombinant von Willebrand factor (rVWF) expressed in human embryonic kidney 293 cells. rVWF from conditioned media underwent electrophoresis on a 1.2% HGT agarose/0.1% SDS gel under non-reducing conditions. NP, normal plasma, WT, wild-type. Lanes from the same gel are delimited by a black line. Irrelevant lanes have been cut off (white spaces).

markedly reduced ($P < 0.05$ in the Bonferroni post-hoc analysis), showing no increasing values using ristocetin concentration varying from 0.12 to 0.75 mg/mL and reaching its highest binding capacity (only 3-fold increased) at 1.50 mg/mL. Subsequently, we analysed the A1377V-R1379C/WT rVWF binding to rGpIbα in comparison with WT and mutant A1377V-R1379C rVWF (**Figure 4**; lower panel). The hybrid rVWF showed only a slightly reduced binding capacity to rGpIbα in comparison with WT (7.6-fold vs. 10.6-fold, respectively; $P > 0.05$), at of 1.50 mg/mL of ristocetin.

The platelet-dependent VWF activity of rVWF evaluated using the VWF:GPIbM assay was reported as VWF:GPIbM/VWF:Ag ratio. The ratio was strongly reduced only for the double-mutant A1377V-R1379C rVWF (0.10) and nearly normal for the hybrid A1377V-R1379C/WT rVWF (0.86 vs. 1 of the WT). The A1377V, R1379C, A1377V/WT and R1379C/WT rVWF showed a VWF:GPIbM/VWF:Ag ratio similar to that of the WT (0.89, 0.75, 0.79, 1.00, respectively).

DISCUSSION

In this study, we described five patients carrying p.Arg1379Cys substitution already reported as VWD type 1 mutation in three different studies [6-8]. However, only one patient was diagnosed as VWD type 1. The other four patients, who also had the p.Ala1377Val variant *in cis* with p.Arg1379Cys showed a 2M phenotype.

At time of diagnosis, we investigated patient I for a type 2B mutation, due to the RIPA value (0.7 mg/mL), which was enhanced considering the VWF:RCo value (43 IU/dL). Unexpectedly, the p.Arg1379Cys mutation was identified. Only one of the previously

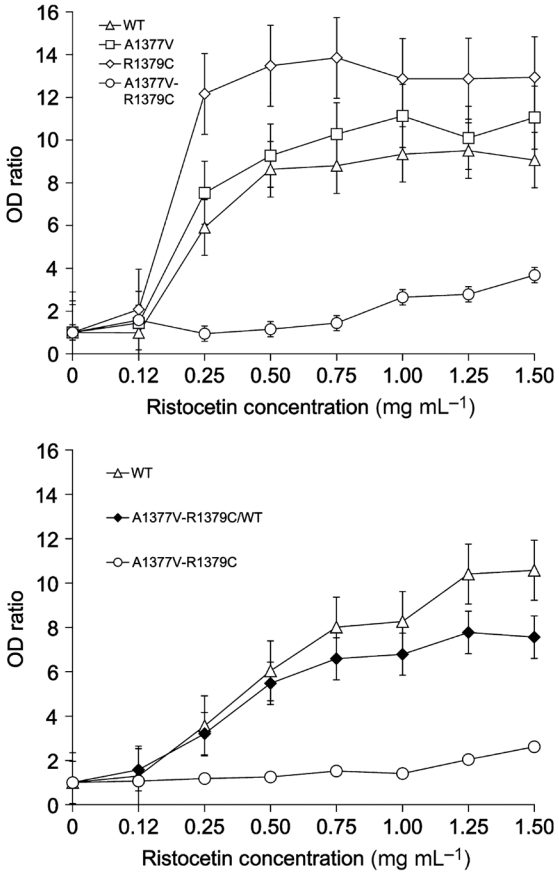

Figure 4. Binding of recombinant von Willebrand factors (rVWF) to recombinant glycoprotein Ibα (rGpIbα). rGpIbα was immobilized on microtitre plates using a specific antibody. Wild-type (WT), mutant (A1377V, R1379C, A1377V-R1379C) and hybrid A1377V-R1379C/WT rVWF were incubated in presence of increasing concentration of ristocetin (0, 0.12, 0.25, 0.50, 1.00, 1.25 and 1.50 mg/mL). For each rVWF, the points shown in the figure correspond to the ratio between the optical density (O.D.) measured in presence of a specific ristocetin concentration and the O.D. measured with no ristocetin. Values are plotted as the mean ± SE of two independent determinations. WT rVWF (triangle), A1377V rVWF (square), R1379C rVWF (diamond), A1377V-R1379C rVWF (circle) and A1377V-R1379C/WT rVWF (black diamond).

reported patients carrying p.Arg1379Cys [6] showed RIPA values similar to those of patient I. Conversely, the other two studies were not able to confirm or exclude the diagnosis of mild type 2B for patients with p.Arg1379Cys [7-8]. Therefore, we confirmed in patient I the diagnosis of VWD type 1 in agreement also with *Castaman et al.*, who reported the efficacious use of DDAVP treatment in a VWD type 1 patient carrier of p.Arg1379Cys [23].

The four 2M patients showed mild bleeding symptoms and they have been treated with desmopressin in case of minor surgeries or delivery. Patient V has been treated

once with the FVIII/VWF concentrates without bleeding complications. These patients and their affected family members (**Appendix S1**) showed similar biochemical parameters, which include a strongly reduced VWF:RCo in the presence of a nearly normal multimeric pattern. These patients showed a clearly increased VWFpp/VWF:Ag ratio, as already described for other type 2M patients [24]. Interestingly, patient II showed a markedly reduced VWF:RCo (< 6 IU/dL), but only a slightly decreased RIPA (1.4 mg/mL), probably explained by the presence of the 2B New York variant (p.Pro1266Leu) [25,26] that mitigates in RIPA assay the 2M phenotype.

Initially, the pathogenetic role of p.Ala1377Val in the 2M patients was underestimated and reported as polymorphism on the VWFdb. The search on the Exome Variant Server and 1000 Genomes database showed that p.Ala1377Val was a rare variant (MAF< 1%) generally found in individuals with African ethnicity.

The finding that patients II, IV and V showed the same STR I and II indicates a possible common ancestor, explaining the presence of both substitutions in these four unrelated patients. Then, we found that the p.Ala1377Val variant has already been reported by *Millar et al.* in two patients, who showed normal VWF:Ag levels, VWF:RCo/VWF:Ag ratios (1.0 and 1.1) and multimers [27], whereas Longsdon *et al.* showed the association of p.Ala1377Val with lower VWF levels in African-Americans individuals in a burden test [28]. These data, led us to exclude that p.Ala1377Val alone was responsible for the 2M phenotype of our patients and suggested a combined effect of p.Ala1377Val and p.Arg1379Cys mutations.

The possible impact of p.Ala1377Val and p.Arg1379Cys on the VWF A1 domain structure was performed using *in silico* tools. Both variants have been predicted to decrease the A1 domain $\Delta\Delta G<0$, whereas only p.Arg1379Cys seemed to alter the secondary structure of the A1 domain by the loss of hydrogen bonds with the nearby residues (Lys1407, Lys1408) and water. We hypothesized that the presence p.Ala1377Val and p.Arg1379Cys, may cause the destabilization of the A1 domain resulting in a reduced capacity of the double-mutant VWF to bind GpIbα.

To confirm this hypothesis, we performed the *in vitro* expression study. Transfections of mutant constructs alone, showed a reduced protein secretion for A1377V rVWF and A1377V-R1379C rVWF, whereas R1379C rVWF showed antigen values similar to the WT rVWF. The hybrids, showed antigen values similar to the WT rVWF for each variant. The results of secreted R1379C/WT rVWF were consistent with the slightly reduced VWF levels of patient I, whereas those of A1377V/WT rVWF were in line with VWF:Ag levels of the patients described by Millar *et al*. [27]. There was a discrepancy between *in vitro* results of double mutant hybrid A1377V-R1379C/WT rVWF, which mimicked patients' phenotype and the reduced patients' plasma values. This was probably due to *in vitro* over-expression of transfected vectors, the difficulty to reproduce patients heterozygous state [29] and the difference between *in vivo* and *in vitro* secretion [30]. Moreover, the increased clearance of patients II-V plasma VWF also contributes to increase this discrepancy.

We have performed a first set of experiments to clarify the role of these two mutations by evaluating the capacity of WT and mutant (A1377V, R1379C and A1377V-R1379C) rVWF to bind rGpIbα. At 0.25 mg/mL of ristocetin, the R1379C rVWF bound rGpIbα with higher affinity than the WT (12-fold vs. 6-fold increased, respectively), whereas A1377V rVWF showed a 7.5-fold increased binding. Therefore, p.Ala1377Val does not compromise the VWF capacity to bind GpIbα, in agreement with the reported patients' biochemical data [27]. As expected, A1377V-R1379C rVWF showed a strongly reduced binding to rGpIbα, demonstrating a synergistic effect of p.Ala1377Val and p.Arg1379Cys that resulted in a 2M phenotype.

We subsequently evaluated, the hybrid A1377V-R1379C/WT rVWF, that unexpectedly showed only a slightly reduced binding to rGpIbα in comparison to WT. However, we observed similar results by testing patients' plasma VWF vs. a normal pooled plasma. This led us to suppose that the static conditions of the assay might allow the patients' plasma VWF to bind rGpIbα, although patients' VWF:RCo were markedly reduced.

The platelet-dependent VWF activity of patients' plasma and rVWF was evaluated using the VWF:GPIbM assay. The VWF:GPIbM values were similar to VWF:RCo for patients I, III, IV and V, whereas it was slightly higher for patient II (probably due to the type 2B p.Pro1266Leu mutation [22]). Also in this case, the A1377V-R1379C/WT rVWF had a binding capacity similar to the WT (0.86 vs. 1 VWF:GPIbM/VWF:Ag ratio). Therefore, the results obtained by testing the A1377V-R1379C/WT are probably due to a higher proportion of the WT subunit within this hybrid rVWF. Nevertheless, the binding of the A1377V-R1379C rVWF, to both WT and gain-of-function mutant rGpIbα proteins is strongly compromised.

In conclusion, these results demonstrate that the patients' 2M phenotype is due to the presence of both mutations (synergistic effect) and not to the p.Ala1377Val alone or to an hypothetical unidentified third mutation not located in the A1 domain.

ACKNOWLEDGEMENTS

We acknowledge L. F. Ghilardini for the illustration work.

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SUPPLEMENTARY DATA

Appendix S1. Phenotype and genotype data of available patient's family members.

Family member	ABO	BSS	FVIII:C *(IU/dL)*	VWF:Ag *(IU/dL)*	VWF:RCo *(IU/dL)*	VWF:CB *(IU/dL)*	VWF:RCo/ VWF:Ag	VWF:CB/ VWF:Ag	VWFpp/ VWF:Ag
Pt II Mother	O	6	75	42	11	28	0.26	0.7	2.4
Pt IV Father	n.d.	5	42	29	11	20	0.38	0.7	3.1
Pt V Son	n.d.	10	33	14	<6 *	11	0.21	0.8	n.d
NR	-	-1 to 3	50-150	40-169 [A] 55-165 [B]	41-160 [A] 53-168 [B]	45-170 [A] 56-174[B]	> 0.6	> 0.6	0.5-1.5

ABO, blood-group system; BSS, Bleeding Severity Score; FVIII:C, factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity; VWF:CB, von Willebrand factor collagen-binding activity; VWFpp, von Willebrand factor propeptide; NR, normal range; n.a., not applicable; n.d. not determined.

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Platelet VWF:Ag *(IU/10⁹ platelets)*	Platelet VWF:RCo *(IU/10⁹ platelets)*	Nucleotide Change	Amino Acid Change
0.19	0.14	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
n.d	n.d	c.[4130C>T;4135C>T];[=]	p.[Ala1377Val;Arg1379Cys];[=]
n.d	n.d	n.d	n.d
0.22-0.60	0.15-0.58	n.a.	n.a.

* A VWF:RCo of 3 IU/dL was considered, in those cases with a VWF:RCo < 6 IU/dL, to perform the calculation of VWF:RCo/VWF:Ag ratio. Patients' plasma and platelets were tested only once. [A] Range values of normal individuals with blood group O; [B] range values of normal individuals with blood group non-O.

CHAPTER 4

THE DOMINANT p.Thr274Pro MUTATION IN THE VON WILLEBRAND FACTOR PROPEPTIDE CAUSES THE VON WILLEBRAND DISEASE TYPE 1 PHENOTYPE IN TWO UNRELATED PATIENTS

Pagliari MT, Baronciani L, Cordiglieri C, Colpani P, Cozzi G, Siboni SM, Peyvandi F.

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ABSTRACT

Background

von Willebrand factor propeptide (VWFpp) plays an important role in VWF multimerization and storage. VWFpp mutations have been previously associated with types 1, 3 and 2A/IIC von Willebrand disease (VWD).

Aims

To characterize the *novel* p.Thr274Pro variant identified in two unrelated type 1 VWD patients.

Methods

Phenotype tests were performed to evaluate patients' plasma and platelets following the current ISTH-SSC guidelines. Molecular analysis was performed using next-generation sequencing. The pcDNA3.1-VWF-WT and mutant pcDNA3.1-VWF-Thr274Pro expression vectors were transiently transfected into HEK293 cells to evaluate recombinant (r)VWF constitutive and regulated secretion. For the latter, the transfected cells were stimulated with phorbol-12-myristate-13-acetate. Immunofluorescence staining was performed to assess the localization of WT-rVWF and Thr274Pro-rVWF in endoplasmic reticulum, lysosomes, cis-/trans-Golgi and pseudo-Weibel Palade bodies.

Results

Biochemical characterization of patients' plasma samples indicated a type 1 VWD diagnosis. Both patients were heterozygous for the p.Thr274Pro variant. Hybrid Thr274Pro/WT-rVWF showed a secretion reduction of 36±4% according to patients' plasma VWF:Ag levels, whereas Thr274Pro-rVWF secretion was strongly impaired (21±2%). The amount of rVWF in cell lysates was nearly normal for both Thr274Pro (62±17%) and Thr274Pro/WT-rVWF (72±23%). The regulated secretion was impaired for Thr274Pro/WT-rVWF, whereas Thr274Pro-rVWF was not released at all. Immunofluorescence staining revealed no particular differences between WT and Thr274Pro-rVWF, although Thr274Pro-rVWF showed less pseudo-Weibel Palade bodies with a rounder shape than WT-rVWF.

Conclusions

The *novel* p.Thr274Pro mutation has a dominant effect and it is responsible of patients' type 1 VWD phenotype through a combined mechanism of reduced synthesis, impaired secretion and multimerization.

INTRODUCTION

von Willebrand disease (VWD) is a common bleeding disorder due to quantitative or qualitative defects of von Willebrand factor (VWF) and caused by mutations on *VWF*. Type 1 VWD is characterized by low VWF antigen (VWF:Ag) levels in line with VWF platelet-dependent activity and VWF collagen binding capacity (VWF:CB) in presence of a normal/nearly normal multimeric pattern [1,2].

VWF can be synthesized by endothelial cells and megakaryocytes as a pre-pro-VWF molecule of 2813 amino acids (aa) that includes a signal peptide (22-aa), a propeptide (741-aa) and the mature VWF subunit (2050-aa) [3]. Post translational modifications such as sulfation, glycosylation, carboxy-terminal dimerization, amino-terminal multimerization and the proteolytic cleavage of VWF propeptide (VWFpp) are necessary to obtain the mature VWF [4]. Then, VWF is stored within the Weibel-Palade bodies (WPBs) in endothelial cells and platelets α-granules (regulated secretion) or it can also be constitutively secreted into the bloodstream in the case of endothelial cells [5-7].

The VWFpp plays an important role in both VWF multimerization and regulated storage. Different mutations within the propeptide have been reported to be responsible for types 1, 3 and 2A/IIC VWD, although they behaved as recessive defects and *in vitro* expression studies were available only for few of them [8-15].

Here we describe the biochemical and molecular characterization of two unrelated Italian patients diagnosed with type 1 VWD. Both patients were heterozygous carriers for the *novel* missense mutation p.Thr274Pro localized in the VWFpp. In *vitro* expression studies of wild-type (WT), mutant Thr274Pro and hybrid Thr274Pro/WT recombinant (r)VWF proteins with the immunofluorescence characterization led us to demonstrate the dominant effect of this mutation and the mechanism responsible for the type 1 VWD.

MATERIALS AND METHODS

Patients

The two patients were informed of the experimental nature of this study and gave their informed consent, according to the declaration of Helsinki (1964). Patients bleeding history was collected in agreement with ISTH Bleeding Assessment Tool [16].

Blood sampling and laboratory methods

Twenty millilitres of blood was drawn in 3.13% sodium citrate (9:1 v/v) and 5 mM EDTA (9:1 v/v). Factor VIII coagulant activity (FVIII:C) was measured using the one-stage clotting assay on ACL TOP 700 analyser (Instrumentation Laboratory, Milan, Italy). VWF:Ag and VWF:CB of patient plasma were measured as previously described [17]. The platelet-dependent VWF activity was measured using a ristocetin-triggered glycoprotein-Ib binding (VWF:GPIbR) automated assay based on WT GPIb fragment and ristocetin (HemosIL von Willebrand Factor Ristocetin Cofactor Activity assay [VWF:RCo], Instrumentation

Laboratory). Measurements of VWF propeptide (VWFpp) was performed by ELISA using anti-human VWF propeptide antibodies (Sanquin, Amsterdam, The Netherlands). Platelets were isolated using a density gradient and lysed with Triton X-100 [18]. Platelet VWF content was evaluated with the VWF:Ag ELISA, whereas VWF:GPIbR was measured using HemosIL AcuStar VWF:RCo (Instrumentation Laboratory). In-house plasma VWF multimeric analysis was performed by sodium dodecyl sulphate (SDS) gel electrophoresis under non reducing conditions using gels at low-resolution (1.2% HGT agarose/0.1% SDS) and intermediate-resolution (1.6% LGT agarose/0.1% SDS) [19]. Low-resolution VWF multimeric analysis of patients' plasma and platelet lysates was also performed using the Hydragel 5 von Willebrand multimers on the semi-automated SEBIA HYDRASYS 2 SCAN (Sebia, Lisses, France). Densitometry was performed using the Phoresis 8.6.3 Software (Sebia) considering peaks 1-3 as low-molecular weight multimers (LMWM), peaks 4-7 as intermediate (I) MWM, and peaks > 7 as high (H)MWM.

VWF analysis

Genomic DNA was extracted from peripheral blood using standard methods. Patients I and II genomic DNA were sequenced using costumed next-generation sequencing (NGS) panels (Illumina [San Diego, CA] or Agilent [Santa Clara, CA]). The presence of the mutation was confirmed by Sanger sequencing of exon 7. Details about the NGS panels, oligonucleotides and PCR conditions are available on request.

In silico evaluation of VWF mutations

The potential damaging effect of the p.Thr274Pro variant was evaluated using different *in silico* tools: SIFT (http://sift.jcvi.org/), Align Grantham Variation Grantham Deviation (Align-GVGD—http://agvgd.iarc.fr/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (http://provean.jcvi.org/), PMut (http://mmb.irbbarcelona.org/PMut/) and Effect according to Russell (http://russelllab.org/aas/). The p.Thr274Pro variant was also classified using the standards of the American College of Medical Genetics guidelines [20].

Plasmid construction.

Mutant expression vector pcDNA3.1-VWF-Thr274Pro, was generated by site-directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent Technologies, La Jolla, California, USA), using as template the pcDNA3.1-VWF-WT vector [21] and specifically designed oligonucleotides.

Transfection experiments

Human embryonic kidney 293 (HEK293) cells were grown on 100-mm dishes using Dulbecco's Modified Eagle's medium (DMEM):F12 supplemented as previously reported [21]. Cells at 80-90% of confluence were transiently transfected with 8 µg of plasmid DNA using jetPEI™ (PolyPlus-transfection, Euroclone, Pero, Italy). For WT and mutant co-

transfection (hybrids) 4 µg of each vector was used. For basal secretion, cell media were replaced before transfection and collected after 72 h, whereas cells were harvested and lysed as previously described [22]. VWF:Ag in cell media and lysates were measured using an ELISA. Cell media were concentrated (10X v/v) using Aquacide II (Calbiochem, La Jolla, California, USA), evaluated again for VWF:Ag [23].

For regulated secretion, HEK293 cells were seeded in 6-wells plates and transfected with a 3 µg plasmid DNA (1.5 µg of each vector for co-transfection) using jetPEI™. Seventy-two hours after transfection the cells were washed twice with PBS and incubated for 1 hour in the release medium (OPTIMEM-I, Gibco supplemented with glutamine 1%) with or without phorbol-12-myristate-13-acetate (PMA, Sigma-Merck, final concentration 160 nM). The VWF secreted was reported as a fraction of total VWF ([VWF:Ag in the medium/ (VWF:Ag in the medium + VWF:Ag in cell lysate) x100).

Immunofluorescence

Multiplex immunostaining was performed to co-localize VWF within lysosomes, endoplasmic reticulum (ER), cis-/trans- Golgi and pseudo-WPBs (details on primary and secondary antibodies are reported in **Table S1**). HEK293 cells were seeded on poly-lysine coated 25mm diameter cover-glasses, transfected with 3 µg of WT or mutant Thr274Pro expression vectors using jetPEI™. The latter was expressed alone to emphasize the effect of p.Thr274Pro mutation, despite patients are heterozygous carriers. Cells were fixed with Formaldehyde 4%, permeabilized with Triton-X100 0.2% and blocked with a high-protein containing buffer (5% fetal calf serum, 3% bovine serum albumin [BSA], 2% goat serum, 2% donkey serum, Sigma-Merck). Primary and secondary antibody labelling were performed in 3% BSA + 1% goat serum + 1% donkey serum). Nuclear staining was performed using 4',6-diamidino-2-phenylindole (DAPI; Sigma-Merck). Cover-glasses were mounted on microscopy slides applying DABCO® mounting medium (Sigma-Merck) and imaged at high-resolution with Leica SP5 laser scanning confocal microscopy (Leica Microsystems) using a 63X/1.40 NA oil objective.

Statistical analysis

Immunofluorescence analysis was performed using an ad-hoc binary segmentation pipeline on Nis-Elements v.5.11 platform (Nikon Instruments, Florence, Italy). Statistical analysis was conducted applying GraphPad PRISM v.8 or R-studio. The comparison between WT, mutant and hybrid rVWF levels in conditioned media and cell lysates was made using a one-way ANOVA with Turkey's post-hoc analysis if necessary. $P < 0.05$ were considered statistically significant. Normality of data distribution was tested via Kolmogorov-Smirnov test prior to perform detailed analyses. Then, Mann-Whitney test was used to compare WT and mutant rVWF labeling. Pearson correlation was used to evaluate WT and Thr274Pro-rVWF co-localization within lysosomes, ER, cis/trans- Golgi and pseudo-WPBs.

RESULTS

Biochemical results

The biochemical data of the two unrelated patients showed a parallel reduction of VWF:Ag, VWF:GPIbR and VWF:CB levels in line with a type 1 VWD diagnosis (**Table 1**). The FVIII:C/VWF:Ag ratio was increased in both patients (2.2 and 3.11, respectively), whereas VWF:GPIbR/VWF:Ag and VWF:CB/VWF:Ag and VWFpp/VWF:Ag ratios were normal. Platelet VWF:Ag was at the lower limit of the normal range for patient I and reduced for patient II, whereas the platelet VWF:GPIbR was markedly reduced in both.

The in-house low-resolution multimeric analysis of patients' plasma showed a normal pattern (**Figure 1A**, left panel) with a slight decrease of high molecular weight multimers (HMWM), whereas the intermediate-resolution gel showed the presence of a normal triplet structure comparable to that of pooled normal plasma (NP; **Figure 1A**, right panel). Low-resolution multimeric analysis of patients' plasma performed using the semi-automated assay showed a slight increase of LMWM, but a decrease of IMWM and HMWM (**Figure 1B**). Platelet VWF showed a decrease of IMWM, HMWM and ultra large (UL) multimers (**Figure 1C**).

Genetic analysis and *in silico* evaluation of VWF mutation

Both patients were heterozygous carriers for a *novel* missense mutation, c.[820A>C];[=] (p.[Thr274Pro];[=]) localized within the VWFpp (D1 domain). The p.Thr274Pro was predicted as damaging by SIFT, ALIGN, GVD Polyphen 2.0, SNP&GO, Mutation Taster, Pmut, whereas PROVEAN and Effect according to Russel suggested a neutral effect. The p.Thr274Pro was classified as pathogenic following the standards of the American College of Medical Genetics guidelines.

In vitro expression studies

VWF in conditioned media and cell lysates was quantified as VWF:Ag using an ELISA. The results were expressed as a percentage of the WT-rVWF referred as 100% ± standard deviation (**Figure 2A**). The amount of secreted mutant Thr274Pro and hybrid Thr274Pro/WT-rVWF was decreased (21±2% and 36±4%, respectively). The amount of rVWF in cell lysates was slightly decreased for both mutant Thr274Pro (62±17%) and hybrid Thr274Pro/WT-rVWF proteins (72±23%). The regulated secretion from pseudo-WPBs was induced with PMA (**Figure 2B**). The WT-rVWF secretion increased from 6% to 11% ($P < 0.0001$), whereas Thr274Pro/WT-rVWF secretion slightly increased from 1% to 1.55%. Mutant Thr274Pro-rVWF was not detectable in the conditioned media even with the use of PMA, although it was detected in the related cell lysates (52±6% vs. 48±2% for unstimulated and stimulated Thr274Pro-rVWF cells, respectively; WT set as 100%). Multimeric analysis of hybrid Thr274Pro/WT-rVWF released in the conditioned media showed a full set of multimers, although slightly smeared. The mutant Thr274Pro-rVWF only showed LMWM, but no sub-bands were visible (**Figure 3**).

Table 1. Biochemical data of the two type 1 VWD patients

Pt	Age/ Sex	ISTH BAT	ABO	FVIII:C *(IU/dL)*	VWF:Ag *(IU/dL)*	VWF:GPIbR *(IU/dL)*	VWF:CB *(IU/dL)*	VWF:GPIbR/ VWF:Ag	VWF:CB/ VWF:Ag	VWFpp/ VWF:Ag	*Platelet VWF:Ag (IU/10⁹ platelets)*	*Platelet VWF:GPIbR (IU/10⁹ platelets)*‡
I	13/F	2	Non-O	69	32	27	26	0.8	0.8	1.2	0.20	0.10
II	23/F	6	O	109	35	34	29	1	0.8	0.9	0.10	0.10
N.R.	-	-	-	50-150	41-160* 55-165†	41-160* 53-168†	45-170* 56-174†	> 0.6	> 0.6	0.6-1.6	0.20-0.54	0.16-0.57

Pt, patient; Age, age at sampling time; Sex: F, female; ISTH BAT, ISTH Bleeding Assessment Tool; ABO, blood group system; FVIII:C, factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWF:GPIbR, Ristocetin-triggered GPIb binding was measured using an automated assay based on wild-type GPIb fragment and ristocetin (HemosIL von Willebrand Factor Ristocetin Cofactor Activity assay); VWF:CB, von Willebrand factor collagen-binding activity; VWFpp, von Willebrand factor propeptide; * range values of normal individuals with blood group O; † range values of normal individuals with blood group non-O. Values are shown as a mean of three measurements in three different samples. Patients' platelets were isolated once. ‡ Platelet VWF:GPIbR was measured using HemosIL AcuStar VWF Ristocetin Cofactor Activity. N.R., normal range.

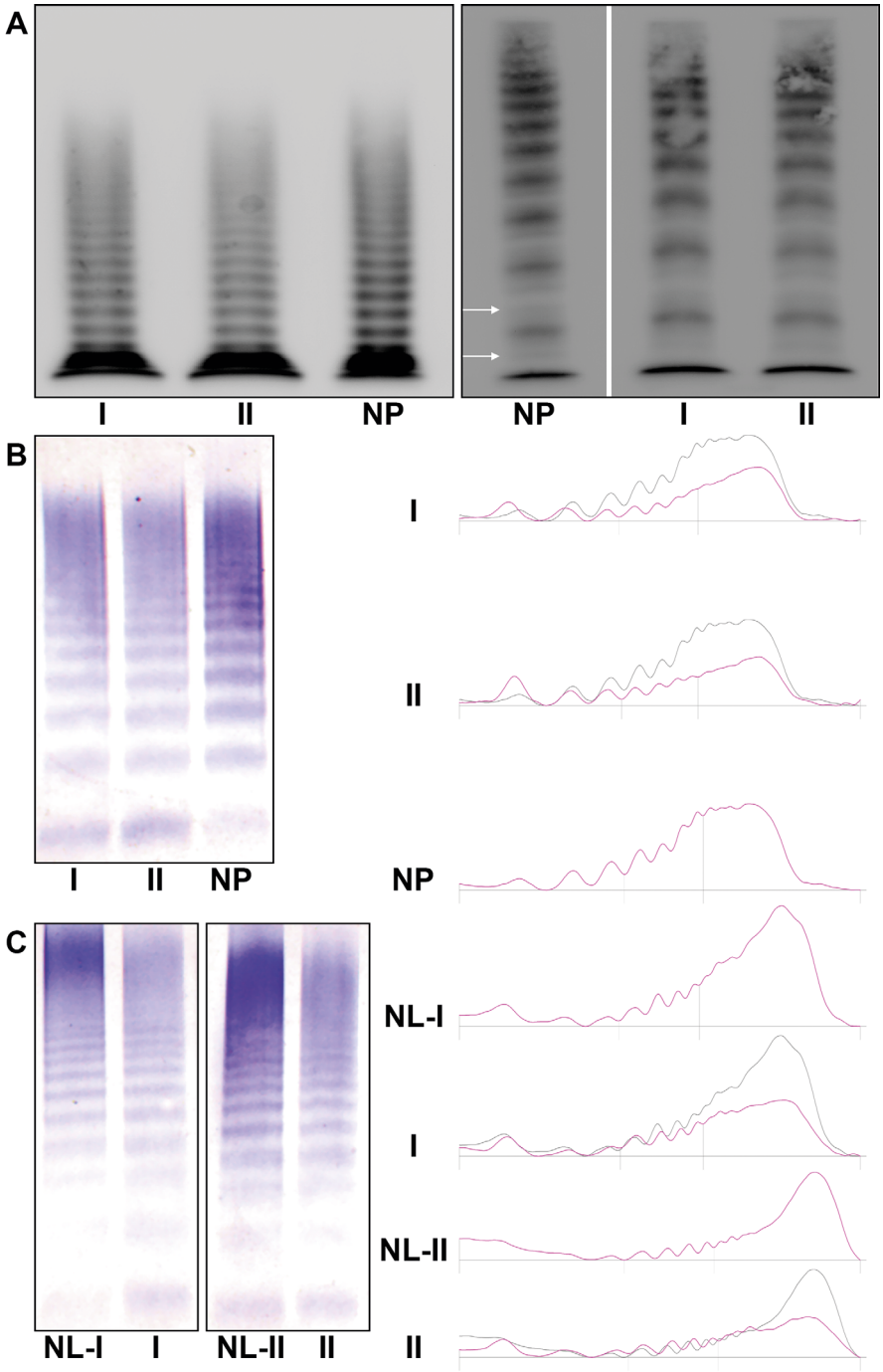
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Figure 1. Multimeric analysis of patients' plasma VWF. **(A)** Multimeric structure of plasma von Willebrand factor (VWF) visualized in a non-reducing low-resolution gel (1.2% HGT agarose/0.1% SDS) in order to highlight the high molecular weight multimers (HMWM; left). Non-reducing intermediate-resolution gel (1.6% LGT agarose/0.1% SDS) was performed to highlight the triplet structure (right). NP, normal plasma. NP-VWF showed the typical triplet structure; I, patient I; II, patient II. The satellite bands are indicated by the arrows. Lanes from the same gel are delimited by a black line. **(B)** Low-resolution multimeric analysis performed using a semi-automated assay (Sebia). Patients' multimers mainly showed a slight increase of low-MWM and a decrease of intermediate- and high- MWM confirmed by the densitogram on the right. **(C)** Multimeric pattern of platelets' VWF highlighted the quantitative loss of intermediate, high- and ultra-large multimers. Lanes from the same gel are delimited by a black line. NL, normal lysate.

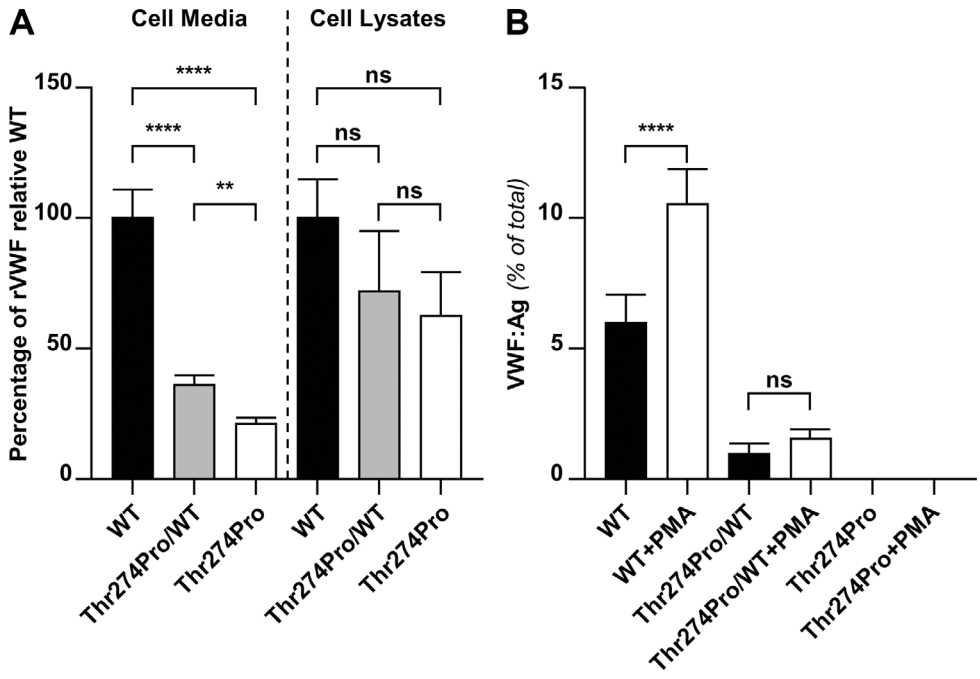

Figure 2. Basal and stimulated secretion of wild-type, hybrid and mutant recombinant von Willebrand factor. **(A)** basal secretion of wild-type (WT), hybrid (Thr274Pro/WT) and mutant (Thr274Pro) recombinant von Willebrand factor (rVWF) was measured using an ELISA. The amount of Thr274Pro/WT and Thr274Pro-rVWF were expressed as a percentage of the WT-rVWF referred as 100% ± standard deviation; **** P< 0.0001; ** P< 0.01; ns, not statistically significant. (**B)** Regulated secretion was evaluated by stimulation the transfected cells with phorbol-12-myristate- 13-acetate (PMA; final concentration 160 nM). Seventy-two hours post-transfection, HEK293 cells were washed twice and incubated with the release media with/without PMA for 1 hour at 37°C. Then, the media were collected and cells harvested. von Willebrand factor antigen (VWF:Ag) was measured using ELISA and results were reported as fraction of the total VWF (secreted plus intracellular VWF). Black bars represent the control (unstimulated release), whereas white bars represent the VWF secreted after stimulation with PMA. **** P< 0.0001; ns, not significant.

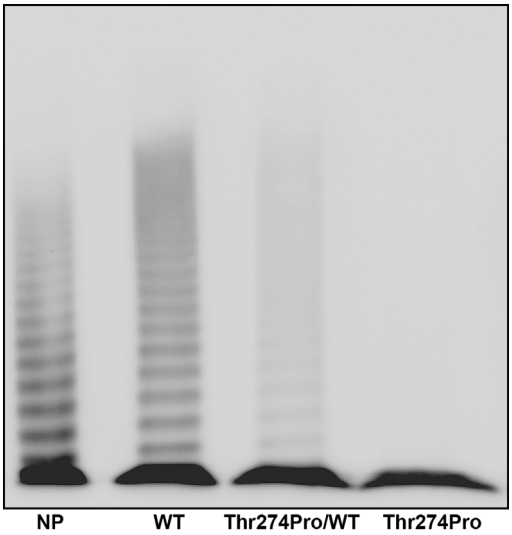

Figure 3. Multimeric analysis of rVWF. Multimeric structure of wild-type (WT), hybrid (Thr274Pro/WT) and mutant (Thr274Pro) recombinant von Willebrand factor (rVWF) were visualized in a non-reducing low-resolution gel (1.2% HGT agarose/0.1% SDS). NP, normal plasma was used as reference. Hybrid Thr274Pro/WT showed a multimeric pattern similar to that of the WT rVWF, although slightly smeared. Both WT and hybrid rVWF had ultra-large multimers. Mutant Thr274Pro-rVWF multimers consisted of a unique undefined band (no sub-bands were visible) indicating a strongly impaired multimerization process.

Immunofluorescence

Immunostaining was performed on HEK293 cells transfected with WT (reference) or mutant (Thr274Pro) expression vectors. Cells transfection efficiency, quantified on the basis of VWF positive staining (VWF^+) and measured in $n \geq 40$ field of view (FOVs) prior each analysis, was about 50%. On average, the 40% of lysosomes showed a VWF^+ without a significant difference between WT and Thr274Pro-rVWF ($P > 0.05$; **Figure 4A**). VWF^+/ER^+ was slightly lower for mutant Thr274Pro than for WT-rVWF (20% vs. 30%, $P = 0.0016$; **Figure 4A**), as confirmed by Pearson correlation (Pearson Index [PI], 0.25 and 0.37 for Thr274Pro and WT-rVWF, respectively; $P < 0.0001$). The VWF^+/cis-$Golgi^+$ was of 50% for WT-rVWF and 30% for Thr274Pro-rVWF ($P < 0.01$). Similarly, VWF^+/trans-$Golgi^+$ was slightly higher for WT than for Thr274Pro-rVWF (48% vs. 42%; $P < 0.05$; **Figure 4B**). Pearson correlation analysis was not statistically significant neither for cis-, nor for trans-Golgi ($P > 0.05$).

A total of 320 pseudo-WPBs were analysed in $n > 3500$ VWF^+ cells. The amount of rVWF co-localized within pseudo-WPBs was higher for WT than Thr274Pro-rVWF (75% vs. 65%, $P < 0.05$; **Figure 5**). The number of cells with a VWF^+/pseudo-$WPBs^+$ was slightly higher for the WT than Thr274Pro-rVWF by considering the percentage of all VWF^+ cells at first and then the mean VWF^+ cells ($FOVs \geq 35$; $P < 0.05$). The Pearson correlation did

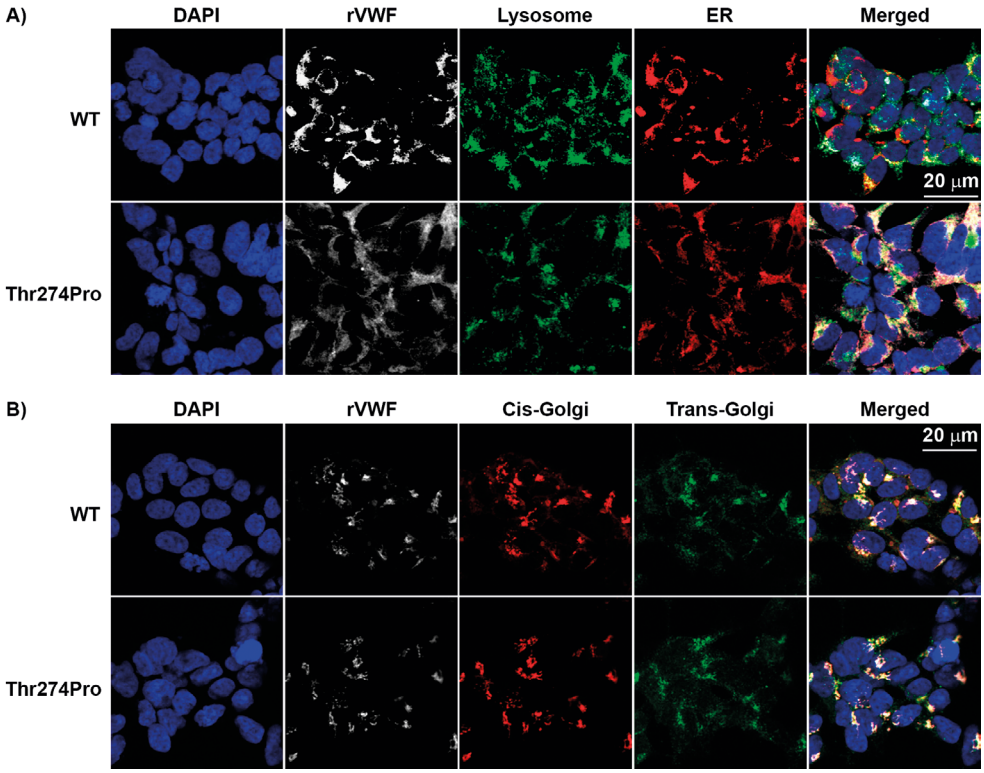

Figure 4. Intracellular localization of recombinant von Willebrand factor (VWF) in HEK293 cells using confocal microscopy. (A) HEK293 transiently transfected with wild-type (WT) or mutant Thr274Pro expression vectors were stained for VWF, lysosomes and endoplasmic reticulum (ER). Nuclei were stained using DAPI (blue). WT and Thr274Pro-rVWF (white) showed a similar localization within lysosomes (green), whereas Thr274Pro-rVWF amount was slightly lower within the ER (red). The images which compose the panel are representative examples; the results are the mean of three independent experiments, in which each condition was tested in triplicate. **(B)** HEK293 transiently transfected with wild-type (WT) or mutant Thr274Pro expression vectors were stained for VWF, cis- and trans-Golgi. Nuclei were stained using DAPI (blue). The amount of Thr274Pro-rVWF was slightly lower than that of the WT in both cis- and trans-Golgi (red and green, respectively), although these differences were not confirmed by high resolution analysis. The images which compose the panel are representative examples; the results are the mean of three independent experiments, in which each condition was tested in triplicate.

not show a statistically significant difference between WT and Thr274Pro-rVWF (PI, 0.79 vs. 0.76; P> 0.05).

Pseudo-WPBs morphology evaluation was performed by binarizing both fluorescent channel signals (VWF-AF647; WPBs-VF488). Morphological differences in the pseudo-WPBs structures, circularity, elongation and the shape factor were estimated. In brief, circularity= 1 stands for a 2D round object, elongation= 1 stands for minimum aperture of the object, again attributable to 2D round objects. Biological round objects normally

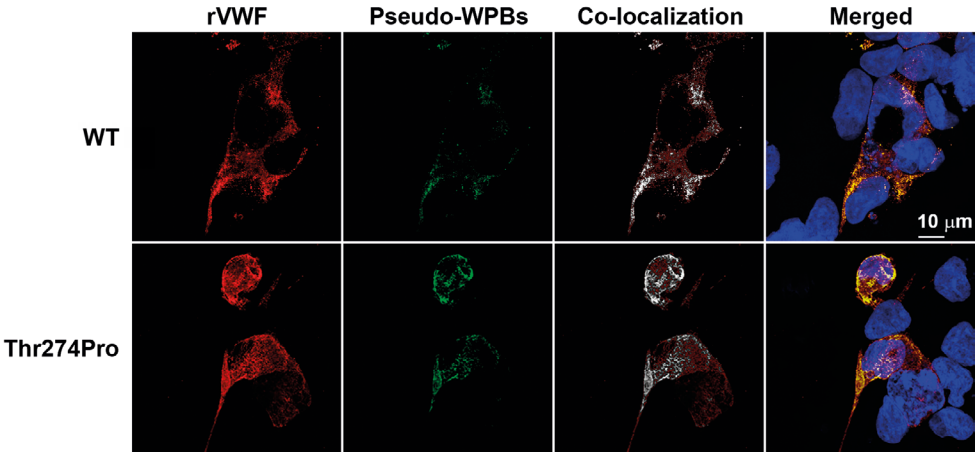

Figure 5. Pseudo Weibel Palade bodies immunofluorescence staining. HEK293 cells were transiently transfected with wild-type (WT) or mutant Thr274Pro expression vectors. Then, cells were stained for von Willebrand factor (VWF; red) and pseudo-Weibel Palade bodies (pseudo-WPBs; green) and nuclei (DAPI, blue). Thr274Pro VWF$^+$ cells presented less elongated pseudo-WPBs compartments compared to those of WT VWF$^+$ cells. The images which compose the panel are representative examples; the results are the mean of three independent experiments, in which each condition was tested in triplicate.

display 0.5-0.8 of shape factor, whereas increasing shape factors (i.e., <1) stands for multi-facetted, multi filament-bearing highly starry-like 2D structure. Both WT and Thr274Pro-rVWF showed pseudo-WPBs with similar median circularity (0.85 vs. 0.87). Thr274Pro VWF$^+$ cells showed less elongated pseudo-WPBs compartments than WT VWF$^+$ cells (medians, 1.62 vs. 1.72; P= 0.0381).

DISCUSSION

The VWFpp plays a key role in intracellular processing, multimerization and storage of VWF. To date, different mutations localized within the VWFpp have been reported as responsible of multimerization defects (type 2A/IIC), in presence or absence of reduced synthesis and/or impaired secretion (types 1 and 3) [8-15, 24]. In this study we have described two unrelated Italian patients carrying the novel missense mutation (p.Thr274Pro) localized within the VWFpp. Both patients showed reduced VWF levels compatible with the diagnosis of type 1 VWD and an increased FVIII:C/VWF:Ag ratio which suggested a reduced VWF synthesis and/or secretion as the possible pathophysiological mechanism responsible for type 1 VWD, whereas they had a normal VWFpp/VWF:Ag ratio. Previous studies demonstrated that an increased VWFpp/VWF:Ag ratio can be used to identify VWD type 1 patients and it was indicative of an increased VWF clearance [25]. However, the presence of an altered (i.e., increased) clearance cannot be excluded due to the localization of the mutation within the propeptide itself.

Patients' plasma and platelet multimers showed a modest alteration of the multimeric pattern, which was still compatible with type 1 VWD diagnosis [1, 26]. In particular, patients' plasma VWF showed a slight increase of LMWM and a decrease of IMWM and HMWM, whereas platelet VWF showed a quantitative decrease of IMWM, HMWM and UL multimers. These findings, in spite of the presence of a normal triplet structure, are indicative of an incomplete multimerization which may be explained by the incapacity of the mutant VWFpp to properly drive the multimerization process. The normal VWF:CB/VWF:Ag and VWF:GPIbR/VWF:Ag ratios suggested that the multimerization defect does not affect VWF function, although the presence of a mild functional alteration not detectable by the activity assay used cannot be completely excluded.

The potential deleterious effect of p.Thr274Pro mutation has been initially assessed using a series of in silico tools based on different algorithms. Thus, we decided to further characterize this mutation by performing an in vitro expression study using HEK293 cells. The hybrid Thr274Pro/WT-rVWF obtained by co-transfection of WT and mutant VWF expression vectors showed a reduced secretion in agreement with patients' biochemical data. The expression of the p.Thr274Pro-rVWF alone, which mimic a homozygous patient, led to a stronger reduction of secreted rVWF levels and an altered multimeric pattern. Both the hybrid Thr274Pro/WT and mutant Thr274Pro-rVWF showed a slightly reduced VWF content in the respective cell lysates, highlighting the co-presence of a reduced VWF synthesis and impaired secretion. The latter finding was confirmed by evaluating the release of rVWF after PMA stimulation. As expected, the amount of Thr274Pro/WT-rVWF secreted in the conditioned medium was lower than that of WT rVWF. Mutant Thr274Pro-rVWF secreted into the conditioned medium was undetectable, although its synthesis was confirmed by the measurable Thr274Pro-rVWF content in the respective cell lysates. This may be due to the emphasised reduction of synthesis and secretion, even though the choice to perform the experiments using a small number of cells may have played a role.

The hybrid Thr274Pro/WT-rVWF showed a multimeric pattern similar to that of the WT-rVWF. Both rVWF had UL-VWF in contrast with the quantitative decrease of IMWM and HMWM detected in patients' plasma multimers. These inconsistencies are probably due to the limits of the in vitro expression study. Indeed, the impossibility to reproduce physiological mechanisms such as VWF proteolysis and clearance may be responsible for the presence of UL in both rVWF. Differently, the Thr274Pro-rVWF only showed a single thick band that emphasized the multimerization defect exerted by this VWF mutation. This single band is probably composed by different sub-bands that cannot be further separated on a gel due to the resolution limits of the method. Nevertheless, these results led us to conclude that the p.Thr274Pro mutation is also responsible for multimerization defect, which is probably compensated by the presence of WT dimers for both hybrid Thr274Pro/WT-rVWF and patients' VWF, in line with the reduced amount of UL-multimers detected in patients' platelet lysates.

Subsequently, we performed the immunofluorescence staining on HEK293 cells transfected with WT or mutant expression vectors, in order to assess the co-localization of the respective WT or Thr274Pro-rVWF in the different cellular compartments and magnify the expected differences. Both rVWF showed a similar co-localization within the lysosomes, which led us to exclude an increased degradation of the mutant rVWF. The amount of Thr274Pro-rVWF in the ER and in cis/trans- Golgi was slightly lower than that of the WT-rVWF, although these differences were confirmed only in the ER after performing the higher resolution analysis. Mutant Thr274Pro-rVWF was able to form a lower number of pseudo-WPBs than the WT-rVWF. Moreover, the morphological analysis showed that mutant Thr274Pro-rVWF/pseudo-WPBs were less elongated that WT-rVWF/pseudo-WPBs explainable with the presence of an altered multimerization process. Taken together, these findings further supported the co-existence of a reduced synthesis, impaired secretion and altered multimerization. Indeed, the presence of the secretion defect alone would likely result in an increased accumulation of the rVWF within the ER and a disturbed pseudo-WPB formation in agreement with the data reported by other authors [27].

We already reported, along with other authors [8,9,14,24] the characterisation of propeptide mutations nearby p.Thr274Pro. None of these mutations exerted a dominant effect nor were clearly associated to the type 1 VWD [28]. The heterozygous carriers for one of these mutations have no bleeding history and their identification was inferred from to that of the respective probands. Mutation p.Arg273Trp was described in VWD patients from three different families classified as VWD type 1 or type 3. These patients were homozygous for p.Arg273Pro, showed severely reduced VWF levels and undetectable multimers or lack of high molecular weight multimers, more in line with a type 3 or a type 2A/IIC diagnosis. The probands' parents, who were heterozygous for the p.Arg273Pro, have no bleeding symptoms [8] ruling out a dominant effect of this mutation. The p.Cys275Ser was found in a type 3 patient compound-heterozygous for the stop codon p.Trp222*. The proband's mother who was heterozygous for p.Cys275Ser only showed borderline VWF levels [9], but with reduced platelet VWF levels [9]. Finally, p.Cys275Arg was inherited with the p.Pro1337Leu and produced a severe type 2B phenotype characterized by extremely low VWF:Ag and VWF:GPIbR levels (7 and 2 IU/dL, respectively). The four subjects heterozygous for the p.Cys275Arg also shown reduced plasma and platelet VWF:Ag levels. However, only one of them is compatible with a diagnosis of type 1 VWD [24]. Both p.Cys275Ser and p.Cys275Arg have been expressed in vitro [9,24]. Hybrids WT/C275S and WT/C275R-rVWFs showed a reduced secretion, similar to that described for Thr274Pro/WT-rVWF, although in presence of higher intracellular retention [9,24]. These results suggested that the reduced VWF levels measured in these patients' plasma were only due to a secretion defect. However, the use of a different cell lines (COS-7 vs. HEK293) to perform the in vitro expression studies and lack of a further characterization, such as immunofluorescence or stimulated secretion, did not allow us to perform a more detailed comparison.

In conclusion, we demonstrate that the novel p.Thr274Pro mutation exerts a dominant negative effect and it is responsible for the type 1 VWD phenotype of our patients. The *in vitro* expression studies together with the immunofluorescence staining led us to identify the co-presence of a reduced synthesis associated with a secretion defect and an impaired multimerization process as the mechanism causing patients' type 1 VWD phenotype.

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Table S1. Primary and secondary antibodies used for the immunofluorescence Staining

Target	Antibody	Manufacturer	Dilution
Lysosomes	Mouse anti-LAMP-2 [H4B4] 18822A	Santa Cruz	1:100
Endoplasmic reticulum	Mouse anti-PDI [RL90] ab2792	Abcam	1:100
cis-Golgi	Mouse anti-GOLPH4 [XY-2] sc-101054	Santa Cruz	1:100
trans-Golgi	Mouse anti-TGN46, SAB4200355	Sigma-Merck	5-10 µg/mL
pseudo Weibel-Palade bodies	Mouse anti-Rab 27a [E-8] sc-74586	Santa Cruz	1:50
von Willebrand Factor	Rabbit anti-VWF A0082	DAKO	1:1000
-	Goat anti-mouse, AlexaFluor-488, A-21141	Molecular Probes	1:1000
-	Goat anti-mouse, AlexaFluor-568, A-21134	Molecular Probes	1:1000
-	Donkey anti-rabbit AlexaFluor-647, A-31573	Molecular Probes	1:1000

PART II

TYPE 3 VON WILLEBRAND DISEASE: RESULTS FROM THE 3WINTERS-IPS

CHAPTER 5

VON WILLEBRAND FACTOR PROPEPTIDE AND PATHOPHYSIOLOGICAL MECHANISMS IN EUROPEAN AND IRANIAN PATIENTS WITH TYPE 3 VON WILLEBRAND DISEASE ENROLLED IN THE 3WINTERS-IPS STUDY

Pagliari MT, Rosendaal FR, Ahmadinejad M, Badiee Z, Baghaipour MR, Baronciani L, Benítez Hidalgo O, Bodó I, Budde U, Castaman G, Eshghi P, Goudemand J, Karimi M, Keikhaei B, Lassila R, Leebeek FWG, Lopez Fernandez MF, Mannucci PM, Marino R, Oldenburg J, Peake I, Santoro C, Schneppenheim R, Tiede A, Toogeh G, Tosetto A, Trossaert M, Yadegari H, Zetterberg EMK, Peyvandi F, Federici AB, Eikenboom J.

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ABSTRACT

Background

Type 3 von Willebrand disease (VWD) is a severe bleeding disorder caused by the virtually complete absence of von Willebrand factor (VWF). Pathophysiological mechanisms of VWD like defective synthesis, secretion and clearance of VWF have previously been evaluated using ratios of VWF propeptide (VWFpp) over VWF antigen (VWF:Ag) and factor (F)VIII coagulant activity (FVIII:C) over VWF:Ag.

Objective

To investigate whether the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios may also be applied to understand the pathophysiological mechanism underlying type 3 VWD and whether VWFpp is associated with bleeding severity.

Methods

European and Iranian type 3 patients were enrolled in the 3WINTERS-IPS study. Plasma samples and buffy coats were collected and a bleeding assessment tool was administered at enrolment. VWF:Ag, VWFpp, FVIII:C, and genetic analyses were performed centrally, to confirm patients' diagnoses. VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios were compared among different variant classes using the Mann-Whitney test. Median differences with 95% confidence intervals (CI) were estimated using the Hodges-Lehmann method. VWFpp association with bleeding symptoms was assessed using Spearman's rank correlation.

Results

Homozygosity/compound heterozygosity for missense variants showed higher VWFpp level and VWFpp/VWF:Ag ratio than homozygosity/compound heterozygosity for null variants ([VWFpp median difference, 1.4 IU/dL; 95%CI, 0.2–2.7; P= 0.016]; [VWFpp/VWF:Ag median difference, 1.4 IU/dL; 95%CI, 0–4.2; P= 0.054]). FVIII:C/VWF:Ag ratio was similarly increased in both. VWFpp level did not correlate with the bleeding symptoms (r= 0.024; P= 0.778).

Conclusions

An increased VWFpp/VWF:Ag ratio is indicative of missense variants, whereas FVIII:C/VWF:Ag ratio does not discriminate missense from null alleles. The VWFpp level was not associated with the severity of bleeding phenotype.

INTRODUCTION

Von Willebrand disease (VWD) is a common inherited bleeding disorder characterized by spontaneous mucocutaneous bleeding and caused by quantitative (types 1 and 3) or qualitative (types 2A, 2B, 2M and 2N) defects of von Willebrand factor (VWF) [1, 2]. VWF is a large multimeric glycoprotein involved in primary haemostasis, but it also acts as factor VIII (FVIII) carrier [3,4]. VWF is synthesized as pre-pro-VWF (2813 amino acids) in endothelial cells and megakaryocytes [5]. In the endoplasmic reticulum, the signal peptide of 22 amino acids is removed and pro-VWF undergoes C-terminal dimerization through cysteine disulfide bonds formation and post-translational modifications such as glycosylation [6]. Once in the Trans-Golgi apparatus, the VWF propeptide (VWFpp; 741 amino acids) is cleaved, although it remains non-covalently bound to the mature VWF [3]. In this phase, the VWFpp plays a role in the VWF multimerization which occurs through N-terminal interchain disulfide bonds [6, 7]. Mature VWF can be stored in platelet alpha granules and endothelial Weibel-Palade bodies or it can be constitutively secreted into the circulation [8]. After secretion, VWFpp dissociates from mature VWF and circulates and is cleared independently from VWF [3].

Although equimolarly secreted, VWF and VWFpp have a different half-life of 8–12 and 2 h, respectively [9]. Previous studies have demonstrated that the ratio of VWFpp over VWF antigen (VWF:Ag) along with the ratio of FVIII coagulant activity (FVIII:C) over VWF:Ag can be used to assess the properties of VWF synthesis, secretion and clearance [10, 11]. This allowed to discriminate whether reduced VWF levels in type 1 VWD patients are due to reduced synthesis, increased clearance, or a combination of both mechanisms [11]. The VWFpp/VWF:Ag ratio also discriminates between type 3 and severe type 1 VWD patients, which highlights the utility of VWFpp in the study of the pathophysiology of VWD and also in the diagnosis of VWD [12].

This study, which includes European and Iranian type 3 VWD patients enrolled in the Type *3* von *W*illebrand *Int*ernational *R*egistries *I*nhibitor *P*rospective *S*tudy (3WINTERS-IPS), aimed to assess the pathophysiological mechanisms leading to type 3 VWD using VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios in combination with the various underlying variants.

METHODS

Participants

The 3WINTERS-IPS study includes 265 patients previously diagnosed with type 3 VWD and recruited in Europe and Iran. The study was approved by the local ethical committees of all participating centers and patients gave their written informed consent. Briefly, inclusion criteria were: patients of both sexes and all ages, diagnosed by the recruiting centers as type 3 VWD; accessible information about previous bleeding episodes and treatment with concentrates containing VWF; and availability to follow-up.

For the analysis, we focus on patients who were genotyped for the underlying *VWF* variant, who were not using prophylactic concentrates containing VWF at the time of blood sampling as that precludes reliable VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios, and for whom the diagnosis of type 3 VWD was confirmed by a centrally measured VWF:Ag≤ 3 IU/dL (**Figure 1**).

Briefly, 265 patients were enrolled in the study; of these 52 patients were excluded because of the lack of DNA samples or multiple missing data. Of the 213 genotyped patients, a further 43 were excluded from analysis as they were on prophylaxis using concentrates containing VWF at the time of blood draw or because this information was missing. In the remaining group of 170 genotyped patients, 147 patients had a confirmed *VWF* genotype and this group forms the main study group, whereas in 8 patients no variant could be identified and 15 genotyped patients had a VWF:Ag level> 3 IU/dL not fulfilling the stringent diagnostic criterium for type 3 VWD.

5

Phenotypic and genotypic characterization

Plasma samples and buffy coats were collected to confirm patients' phenotype. Biochemical measurements were performed in centralized laboratories on samples taken at the time

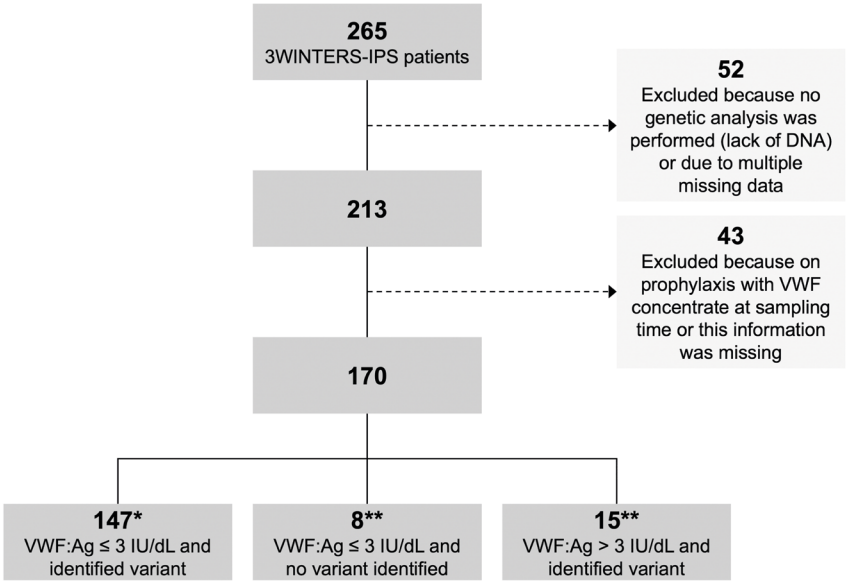

Figure 1. Flowchart of 3WINTERS-IPS patients analyzed in this study. Type 3 patients enrolled in the 3WINTERS-IPS study who met the inclusion criteria, were further divided depending on the availability of biochemical and molecular information. *Main study group included 147 fully characterized patients with VWF:Ag ≤ 3 IU/dL and a complete molecular characterization. ** The group of 8 patients with VWF:Ag≤ 3 IU/dL in whom no genetic defect could be identified and the remaining group of 15 patients with a genetic defect identified, but who did not meet inclusion criteria (VWF:Ag> 3 IU/dL) were considered separately for secondary analyses.

of recruitment. VWF:Ag was measured using a sensitive ELISA based assay. FVIII:C was measured by a one-stage clotting assay using FVIII deficient plasma (Siemens) and APTT reagent Triniclot (TCoag). VWFpp measurement was performed with an ELISA based assay using antibodies from Sanquin [9, 12]. Molecular analysis included next generation sequencing, polymerase chain reaction with Sanger sequencing and multiplex-ligation dependent probe amplification [13]. Bleeding score (BS) was calculated using a common bleeding assessment tool at time of enrolment [14].

Statistical analyses

Continuous variables were reported as median and interquartile range (IQR), whereas descriptive variables were reported as numbers with percentages.

The *VWF* gene defects identified in type 3 VWD patients were divided into missense and null defects. Missense defects included missense variants, gene conversions (not leading to null variants), small insertions and small deletions that do not alter the reading frame. Null defects included variants that introduce a stop codon, splice variants, large deletions, large insertions, small insertions, small deletions, indels, or small duplications which alter the reading frame and thereby cause a premature stop codon.

Based on these definitions, patients were grouped as homozygous/compound heterozygous for missense variants, homozygous/compound heterozygous for null variants and compound heterozygous null/missense variants. Patients with a partial molecular diagnosis because only one mutated allele was identified, were referred to as "other" and excluded from the statistical analysis due to their heterogeneity. Analyses were repeated by considering European and Iranian patients separately.

VWF:Ag, VWFpp, FVIII:C, VWFpp/VWF:Ag ratio, FVIII:C/VWF:Ag ratio, and bleeding score were analyzed in the different groups of patients classified as described above. As continuous variables were not normally distributed, comparisons (missense vs. null and missense vs. missense/null compounds) were performed using the non-parametric Mann-Whitney test. Median differences with 95% confidence intervals (CI) were estimated using the Hodges-Lehmann method. The correlation between VWFpp and bleeding score was evaluated using Spearman's rank distribution. All analyses were performed using SPSS statistics 27 (IBM Corporation).

RESULTS

The biochemical characteristics of 170 type 3 VWD patients are reported in **Table 1**. The main study group of 147 type 3 patients with a complete characterization had a median age of 27 years (IQR, 16.0-40.0) and included more women than men (59% and 41%, respectively). VWF:Ag was below the assay limit of detection (LOD, 0.8 IU/dL) in most of patients (n= 135; 92%); therefore it was arbitrarily set as 0.5 IU/dL. Median VWFpp was 1.5 IU/dL (IQR, 0.7-4.2), whereas median FVIII:C was 2.3 IU/dL (IQR, 1.8-2.9). Patients had a median bleeding score at inclusion of 14.0 (IQR, 8.0-19.0). Both VWFpp/

VWF:Ag and FVIII:C/VWF:Ag ratios were above the respective normal ranges (NR; VWFpp/VWF:Ag, NR: 0.8-2.2; FVIII:C/VWF:Ag NR: 0.6-1.9) [11].

The 8 patients in whom no *VWF* variant could be identified showed higher VWF:Ag (median, 1.8 vs. 0.5 IU/dL) and VWFpp values (median VWFpp, 2.8 vs. 1.5 IU/dL) than those of fully characterized type 3 patients, and the corresponding VWFpp/VWF:Ag ratio was lower (median, 1.6 vs. 2.8). They also had slightly lower FVIII:C (median, FVIII:C, 1.8 vs. 2.3 IU/dL), a lower FVIII:C/VWF:Ag ratio (median FVIII:C/VWF:Ag ratio 1.1 vs. 4.4) and a lower bleeding score (median 8.5 vs. 14.0) than the patients with a confirmed genotype. The 15 genotyped patients with an unconfirmed diagnosis of type 3 VWD (VWF:Ag> 3 IU/dL) were also analyzed (**Table 1**). They showed an increased median FVIII:C/VWF:Ag of 3.9, whereas the median VWFpp/VWF:Ag ratio (1.7) was within the normal range. Five patients were homozygous/compound heterozygous for null or missense variants (n= 4 and n= 1, respectively), five were compound heterozygous for null/missense variants,

Table 1. Baseline characteristics of the included patients

	Variants identified, VWF:Ag ≤ 3 IU/dL	No variants identified, VWF:Ag ≤ 3 IU/dL	Variants identified, VWF:Ag > 3 IU/dL
N	147	8	15
Age, years	27 (16-40)	18.5 (12.5-30.8)	41.0 (32-54)
Sex (female), n (%)	87 (59.0)	6 (75.0)	9 (60.0)
VWF:Ag, (IU/dL)	0.5 (0.5-0.5)	1.8 (1.2-2.4)	5.2 (4.1-6.3)
Missing, n	-	-	-
VWFpp, (IU/dL)	1.5 (0.7-4.2)	2.8 (2.3-4.4)	8.5 (3.4-15.9)
Missing, n	4	1	-
FVIII:C, (IU/dL)	2.3 (1.8-2.9)	1.8 (1.6-2.7)	19.1 (14.9-33.4)
Missing, n	1	1	
VWFpp/VWF:Ag [a]	2.8 (1.4-6.6)	1.6 (1.2-4.6)	1.7 (0.4-4.0)
Missing, n	4	1	-
FVIII:C/VWF:Ag [b]	4.4 (3.4-5.5)	1.1 (0.7-1.7)	3.9 (1.6-6.4)
Missing, n	1	1	-
Bleeding Score	14.0 (8.0-19.0)	8.5 (4.3-17.5)	11.0 (6.0-18.0)
Missing, n	6	-	-
Variant identified, n (%)	147 (100)	0 (0)	15 (100)

Note: Continuous variables were reported as median and interquartile range (IQR). Abbreviations: FVIII:C, Factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide.
[a] VWFpp/VWF:Ag, normal range 0.8–2.2, calculated as 2.5th to 97.5th percentile in 387 healthy controls [11].
[b] FVIII:C/VWF:Ag normal range 0.6–1.9, calculated as 2.5th to 97.5th percentile in 387 healthy controls [11].

Table 2. Main study group stratified by type of *VWF* variants

Patients, n (%)	VWF:Ag *(IU/dL)*	FVIII:C *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C/ VWF:Ag	VWFpp/ VWF:Ag	BS
Homozygous/compound heterozygous for missense variants, 20 (13.6)	0.5 (0.5 to 0.5)	2.6 (1.9 to 3.3)	3.6 (1.4 to 6.6)	4.4 (3.1 to 5.7)	4.9 (2.2 to 9.3)	11.5 (6.3 to 18.3)
Homozygous/compound heterozygous for null variants, 116 (78.9)	0.5 (0.5 to 0.5)	2.3 (1.8 to 2.8)	1.2 (0.6 to 3.4)	4.4 (3.4 to 5.2)	2.4 (1.2 to 5.8)	14.0 (8.0 to 19.0)
Compound heterozygous for missense-null variants 6 (4.1)	0.5 (0.5 to 1)	3.3 (2.1 to 6.1)	2.6 (1.2 to 7.8)	5.1 (4.2to 7.8)	2.8 (2.3 to 11.2)	18.5 (12.0 to 22.8)
Other, 5 (3.4)	0.5 (0.5 to 1.4)	1.9 (1.6 to 2.6)	3.1 (1.6 to 9.8)	3.8 (1.8 to 5.1)	6.2 (3.1 to 8.6)	18.5 (8.8 to 20.8)
Median Difference (95% CI), P missense vs. null	–	0.3 (–0.1 to 0.8) 0.154	1.4 (0.2 to 2.7) 0.016	0 (–0.8 to 0.8) 0.882	1.4 (0 to 4.2) 0.054	–1 (–5.0 to 3.0) 0.501
Median Difference (95% CI), P missense vs. compound missense-null	–	–0.7 (–2.3 to 0.5) 0.234	0.15 (-4.1 to 3.2) 0.838	–1.1 (3.0 to 0.8) 0.170	1.1 (–2.0 to 7.0) 0.683	–5.5 (–13.0 to 4.0) 0.234

Note: Continuous variables were reported as median and interquartile range (IQR). Comparisons between groups were performed using Mann-Whitney test. Median difference and 95% confidence intervals (CI) were estimated with Hodges-Lehmann method. Missing values: FVIII:C, n= 1; VWFpp, n= 4; FVIII:C/VWF:Ag, n= 1;VWFpp/VWF:Ag, n= 4; BS, n= 6. Abbreviations: BS, bleeding score; FVIII:C, factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide.

whereas the remaining five were referred as "other" because only one mutated allele was identified.

The main study group of 147 patients was grouped according to the molecular defects as described in the Methods section. Analyses of the VWF and FVIII parameters as well as the bleeding score are reported in **Table 2.** Type 3 patients homozygous/compound heterozygous for missense variants showed higher VWFpp levels than those homozygous/compound heterozygous for null variants (median, 3.6 vs. 1.2 IU/dL; P= 0.016, median difference of 1.4 IU/dL; 95% CI, 0.2-2.7). The group of patients compound heterozygous for a missense and a null variant showed a median VWFpp level of 2.6 IU/dL, which was intermediate between the patients homozygous/compound heterozygous for missense variants and those homozygous/compound heterozygous for null variants (**Table 2**).

VWFpp/VWF:Ag ratio was higher in those homozygous/compound heterozygous for missense variants than in those homozygous/compound heterozygous for null variants (median, 4.9 vs. 2.4, P= 0.054; median difference of 1.4 IU/dL; 95% CI, 0-4.2).

Compound heterozygosity for missense-null variants showed intermediate VWFpp/VWF:Ag ratio (2.8) between those homozygous/compound heterozygous for missense or null variants. All patients showed a similar FVIII:C/VWF:Ag ratio, regardless of the type of variant considered (**Table 2**).

Analyses were also performed by evaluating European and Iranian patients separately (**Table 3**). Patients homozygous/compound heterozygous for missense variants also showed higher VWFpp/VWF:Ag ratio than those with null variants, in both European (median, 4.7 vs. 2.4; P= 0.483; median difference of 1.0 IU/dL; 95% CI, –2.0 to 5.5) and Iranian patients (median, 5.4 vs. 2.4; P= 0.062, median difference of 1.6 IU/dL; 95% CI, –0.2 to 5.8). Compound heterozygosity for missense-null variants were mainly found among European patients (5 out of 6).

There was no association between VWFpp and the bleeding phenotype in type 3 VWD patients, assessed in the group of 147 completely characterized patients using Spearman's rank correlation (r= 0.024; P= 0.778).

DISCUSSION

Type 3 VWD is a severe recessively inherited bleeding disorder characterized by the virtual absence of VWF. It is mainly caused by homozygous or compound heterozygous *VWF* null variants [1, 2]. However, missense variants have also been identified as possible cause [15, 16]. While the pathomechanism of null variants consists of reduced VWF synthesis, the missense variants may lead to reduced VWF levels through a combination of factors like reduced synthesis, intracellular retention, impaired secretion or fast clearance from the circulation. The ratios of VWFpp/VWF:Ag and FVIII:C/VWF:Ag have previously been shown to be indicative of the pathogenic mechanism underlying the VWF deficiency for other types of VWD [10- 12].

Table 3. Main study group stratified by type of VWF variants in European and Iranian patients

European type 3 VWD patients, n (%) [a]	VWF:Ag *(IU/dL)*	FVIII:C *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C/ VWF:Ag	VWFpp/ VWF:Ag	BS
Homozygous/compound heterozygous for missense variants, 8 (13.3)	0.5 (0.5 to 1.8)	2.6 (2.2 to 4.0)	3.7 (3.0 to 7.3)	4.0 (2.8 to 4.9)	4.7 (1.7 to 12.7)	16.0 (15.0 to 24.3)
Homozygous/compound heterozygous for null variants, 43 (71.7)	0.5 (0.5 to 0.5)	2.4 (2.0 to 3.2)	1.2 (0.6 to 5.2)	4.4 (3.4 to 5.8)	2.4 (1.2 to 6.7)	18.0 (13.5 to 23.5)
Compound heterozygous for missense-null variants, 5 (8.3)	0.5 (0.5 to 1.5)	2.9 (2.0 to 7.8)	4.1 (1.1 to 8.2)	4.5 (4.0 to 7.3)	2.7 (2.2 to 13.6)	19.0 (10.5 to 24.5)
Other, 4 (6.7)	0.5 (0.5 to 1.8)	2.1 (1.8 to 2.7)	3.9 (1.2 to 11.9)	4.1 (1.5 to 5.4)	5.5 (2.4 to 9.7)	18.5 (8.8 to 20.8)
Median Difference (95% CI), P missense vs. null	-	0.3 (–0.4 to 1.1) 0.414	2.3 (–0.8 to 3.6) 0.190	–0.8 (–2 to 0.4) 0.228	1.0 (–2.0 to 5.5) 0.483	0 (–6.0 to 6.0) 0.989
Median Difference (95% CI), P missense vs. compound missense-null	-	–0.2 (–7.0 to 14.0) 0.712	0 (–0.6 to 1.4) 0.865	0.4 (–5.1 to 6.0) 0.122	–0.1 (–11.0 to 11.8) 1	–2.0 (–11.0 to 13.0) 0.607

Table 3 (continued). Main study group stratified by type of VWF variants in European and Iranian patients

Iranian type 3 VWD patients, n (%) [b]	VWF:Ag *(IU/dL)*	FVIII:C *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C/ VWF:Ag	VWFpp/ VWF:Ag	BS
Homozygous/compound heterozygous for missense variants, 12 (13.8)	0.5 (0.5 to 0.5)	2.6 (1.6 to 3.3)	2.7 (1.2 to 4.7)	5.2 (3.2 to 6.6)	5.4 (2.4 to 9.3)	7.5 (4.3 to 12.3)
Homozygous/compound heterozygous for null variants, 73 (83.9)	0.5 (0.5 to 0.5)	2.2 (1.7 to 2.6)	1.2 (0.6 to 2.7)	4.2 (3.5 to 5.2)	2.4 (1.2 to 5.4)	11.0 (4.0 to 17.0)
Compound heterozygous for missense-null variants, 1 (1.1)	0.5	3.7	2.6	7.4	5.2	15.0
Other, 1 (1.1)	0.5	1.4	3.1	2.8	6.2	-
Median Difference (95% CI), P missense vs. null	-	0.3 (–03 to 0.8) 0.271	0.8 (–0.1 to 2.9) 0.072	0.6 (–0.4 to 1.8) 0.229	1.6 (–0.2 to 5.8) 0.062	–3.5 (–7.0 to 2.0) 0.283
P missense vs. compound missense-null [c]	-	0.178	1	0.178	1	0.284

Note: Continuous variables were reported as median and interquartile range (IQR). Comparisons between groups were performed using the Mann-Whitney test. Median difference and 95% confidence intervals (CI) were estimated with the Hodges-Lehmann method. Abbreviations: BS, bleeding score; FVIII:C, factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide. [a] Missing values in European type 3 VWD patients: VWFpp, n= 2; VWFpp/VWF:Ag, n= 2, BS, n= 1. [b] Missing values in Iranian type 3 VWD patients: FVIII:C, n= 1; VWFpp, n= 2; FVIII:C/VWF:Ag, n= 1; VWFpp/VWF:Ag, n= 2; BS, n= 5. [c] Median difference cannot be calculated due to the presence of only one patient in the compound heterozygous for missense-null variants group.

In this study, we investigated whether VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios can be applied to evaluate the pathophysiological mechanism underlying type 3 VWD and to predict the type of variant. The analysis of this large group of type 3 VWD patients showed that the VWFpp level as well as the VWFpp/VWF:Ag ratio were clearly higher in patients with missense variants than those with null variants, indicating that increased clearance of secreted mature VWF plays a role in the pathogenesis of the disease in the former. Fully in line with this observation the patients compound heterozygous for a null and a missense variant had intermediate levels of VWFpp and VWFpp/VWF:Ag.

In all type 3 VWD patients, we identified strongly reduced levels of VWF measurements (VWF:Ag and VWFpp). This is expected in patients characterized by a genotype of homozygous or compound heterozygous null variants, as these variants predict a defect in VWF synthesis leading to very low or undetectable levels of both VWFpp and VWF:Ag. However, in patients characterized by a genotype of homozygous or compound heterozygous missense variants it may be expected that the mutant VWF protein is still synthesized. Therefore, the low levels of circulating VWF:Ag measured in those patients could be explained by a combination of intracellular retention, secretion defect or fast clearance of mutant protein from the circulation. In the group of type 3 patients with homozygous or compound heterozygous missense variants the VWFpp level was indeed measurable at a low level (median 3.6 IU/dL), indicating the synthesis of the protein at low level and/or intracellular retention. The VWFpp/VWF:Ag ratio in this group was clearly increased and also higher than in the group characterized by null alleles, indicating that the secreted VWF is cleared faster from the circulation. Interestingly, most of missense variants were not localized in the propeptide, therefore a direct effect on propeptide clearance and as consequence on VWFpp/VWF:Ag ratio was excluded. Moreover, these findings showed that also in type 3 VWD an increased VWFpp/VWF:Ag ratio is indicative of the presence of missense variants. Sanders *et al.* previously reported the description of a group of 15 patients with VWF:Ag levels <5 IU/dL and high levels of VWFpp (median 72 IU/dL). Of them, 14 were genotyped and 10 were heterozygous for missense variants known to be associated with (very) rapid clearance of the protein from the circulation [12]. Indeed, those heterozygous patients should actually be reclassified as severe type 1. The VWFpp/VWF:Ag ratio can thus easily discriminate between type 3 and severe type 1 VWD. In contrast to Sanders *et al.* we have now studied patients that fulfil all criteria for type 3 VWD, including being homozygous or compound heterozygous for a *VWF* defect. Even then, the increased VWFpp/VWF:Ag ratio indicates the presence of missense variants versus null variants, highlighting the utility of VWFpp in studying the pathophysiology of type 3 VWD.

Previously, we have reported that increased FVIII:C/VWF:Ag ratio can discriminate in type 1 VWD between heterozygous carriers of null variants and missense variants, where a high FVIII:C/VWF:Ag indicates a synthetic defect as the pathophysiological mechanism in the case of null variants [11, 12]. In the current cohort of type 3 VWD patients we did not find a difference in FVIII:C/VWF:Ag ratio between patients homozygous/

compound heterozygous for null or missense variants, although the ratio was higher than the normal reference range. At very low levels of VWF:Ag the capacity to bind and stabilize FVIII is severely compromised, and the circulating FVIII may partly be unbound FVIII, and thus the FVIII:C/VWF:Ag ratio in type 3 VWD may not be reliable. Based on the higher circulating levels of VWFpp and the increased VWFpp/VWF:Ag ratio in type 3 patients homozygous/compound heterozygous for missense variants, it could be possible that more mature VWF:Ag is secreted to the circulation than in patients homozygous/compound heterozygous for null variants, although VWF is cleared rapidly. Therefore, we expected a milder bleeding phenotype for patients homozygous/compound heterozygous for missense variants. This hypothesis was partially supported by the fact that these patients showed a slightly lower BS than patients homozygous/compound heterozygous for null variants (11.5 vs. 14.0; P= 0.501). However, there was no association between VWFpp level and the bleeding symptoms represented by the BS (r= 0.024; P= 0.778). Our result may be affected by the use of the bleeding score, as it is easily saturated in type 3 patients, thus preventing to discriminate small differences.

The potential correlation between VWFpp level and bleeding phenotype has also been evaluated depending on the location of the genetic defects. To this purpose patients were grouped as: (i) homozygous/compound heterozygous carriers for variants in the VWFpp; (ii) homozygous/compound heterozygous carriers for variants in mature VWF; (iii) compound heterozygous carriers for a variant in the VWFpp and a variant in mature VWF and (iv) "excluded patients" as they do not meet the requirements to be coded in the previous groups (i.e., carriers for large deletions/duplications involving both VWFpp and mature VWF; **Table S1** in supporting information). However, none of these groups showed the association between VWFpp level and the bleeding symptoms. The 3WINTERS-IPS study represents the largest population of type 3 VWD patients so far collected. However, the principal limitation of this study consists in the considerable number of excluded patients because they did not fulfil the inclusion criteria, they had multiple missing data or were on prophylaxis at sampling time. Second, we did not have information about the distance between last treatment and sampling time. Therefore, we cannot exclude that the VWF:Ag≥ 3 IU/dL measured in some patients (e.g., the 15 subjects excluded from the main analysis group despite having a complete molecular characterization) were due to residual traces of concentrate containing VWF, thus contributing to the sample size reduction. Then, the choice to focus the genetic analysis on the coding region and intron/exon boundaries may have contributed to reducing the number of completely characterized patients such as for the eight subjects with VWF:Ag≤ 3 IU/dL excluded because of the inconclusive genotyping.

Last, we had considered whether our results could be affected by the genotype heterogeneity of European and Iranian patients. Indeed, the European population showed the highest number of different variants which were distributed along the VWF, whereas the Iranian population has the highest number of homozygous carriers of variants which were mainly localized at the VWF amino-terminal end [13]. Nevertheless, the results

obtained considering the two populations separately were in line with those of the main analysis thus suggesting good generalizability of our results.

In conclusion, this study showed that an increased VWFpp/VWF:Ag ratio can be indicative of the presence of missense variants as the cause of VWD even in type 3, whereas the FVIII:C/VWF:Ag ratio failed to discriminate the presence of null alleles. Discriminating homozygosity/compound heterozygosity of missense variants could be useful clinical information as it might be indicative of a milder bleeding phenotype; however the BS as used in this study failed to show an association with VWFpp levels.

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SUPPLEMENTARY DATA

Table S1. Distribution of the 147 type 3 VWD patients based on variants' localization in the VWFpp or in mature VWF

	Homozygous/compound heterozygous for variants in VWFpp**	Compound heterozygous for a variant in VWFpp and a variant in mature VWF	Homozygous/compound heterozygous for variants in mature VWF††	"Excluded patients" who cannot be classified in the other groups‡‡
N	30	11	99	7
Age, years	26.0 (11.0-34.0)	22.0 (16.0-41.0)	26.0 (15.0-39.0)	42.0 (41.0-45.0)
Sex (female), n (%)	18 (60)	8 (73)	57 (58)	4 (57.0)
VWF:Ag, (IU/dL)	0.5 (0.5-0.5)	0.5 (0.5-0.5)	0.5 (0.5-0.5)	0.5 (0.5-0.5)
Missing, n	-	-	-	-
VWFpp, (IU/dL)	1.3 (0.6-3.4)	1.2 (0.7-8.0)	1.9 (0.8-4.9)	0.8 (0.3-1.3)
Missing, n	1	-	3	-
FVIII:C, (IU/dL)	2.3 (1.4-2.8)	2.3 (1.7-2.8)	2.3 (1.8-3.0)	2.4 (2.0-3.4)
Missing, n	-	-	1	-
VWFpp/VWF:Ag *	2.6 (1.2-5.4)	2.4 (1.0-16.0)	3.2 (1.6-7.2)	1.6 (0.6-2.6)
Missing, n	1	-	3	-
FVIII:C/VWF:Ag †	4.6 (2.8-5.7)	4.4 (3.4- 4.8)	4.3 (3.6-5.5)	4.8 (4.0-6.8)
Missing, n	-	-	1	-
Bleeding Score (BS)	14.0 (7.5-18.0)	14.0 (9.0-26.0)	14.0 (6.0-19.0)	20.0 (8.0-28.0)
Missing, n	5	-	1	-

Table S1 (continued). Distribution of the 147 type 3 VWD patients based on variants' localization in the VWFpp or in mature VWF

	Homozygous/compound heterozygous for variants in VWFpp**	Compound heterozygous for a variant in VWFpp and a variant in mature VWF	Homozygous/compound heterozygous for variants in mature VWF††	"Excluded patients" who cannot be classified in the other groups‡‡
Variant identified, n (%)	30 (100)	11 (100)	99 (100)	7 (100)
VWFpp/BS correlation‡, r (P-value)	-0.484 (0.016)	0.0336 (0.312)	0.099 (0.342)	0.036 (0.939)

Continuous variables were reported as median and interquartile range (IQR). VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide; FVIII:C, Factor VIII coagulant activity. *VWFpp/VWF:Ag, normal range 0.8-2.2, calculated as 2.5th to 97.5th percentile in 387 healthy controls; †FVIII:C/VWF:Ag normal range 0.6-1.9, calculated as 2.5th to 97.5th percentile in 387 healthy controls (Eikenboom J *et al*; Blood. 2013). ‡ The correlation between VWFpp and bleeding score was evaluated using the Spearman's rank distribution. ** Variant in VWFpp refers to all variants located in D1-D2 domains. †† Variants in mature VWF refers to all variants located in D'-CK domains. ‡‡ This group includes 7 patients who do not meet the requirements to be classified in the other groups. Of them, 6 patients are homozygous for large deletions involving both VWFpp and VWF and 1 patient is compound heterozygous for a large duplication and small deletion in exon 7.

CHAPTER 6

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

Pagliari MT, Budde U, Baronciani L, Eshghi P, Ahmadinejad M, Badiee Z, Baghaipour MR, Benítez Hidalgo O, Biguzzi E, Bodo I, Castaman G, Goudemand J, Karimi M, Keikhaei B, Lassila R, Leebeek FWG, Lopez Fernandez MF, Marino R, Oldenburg J,Peake I, Santoro C, Schneppenheim R, Tiede A, Toogeh G, Tosetto A, Trossaert M, Yadegari H, Zetterber EMK, Mannucci PM, Federici AB, Eikenboom J, Peyvandi F.

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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.

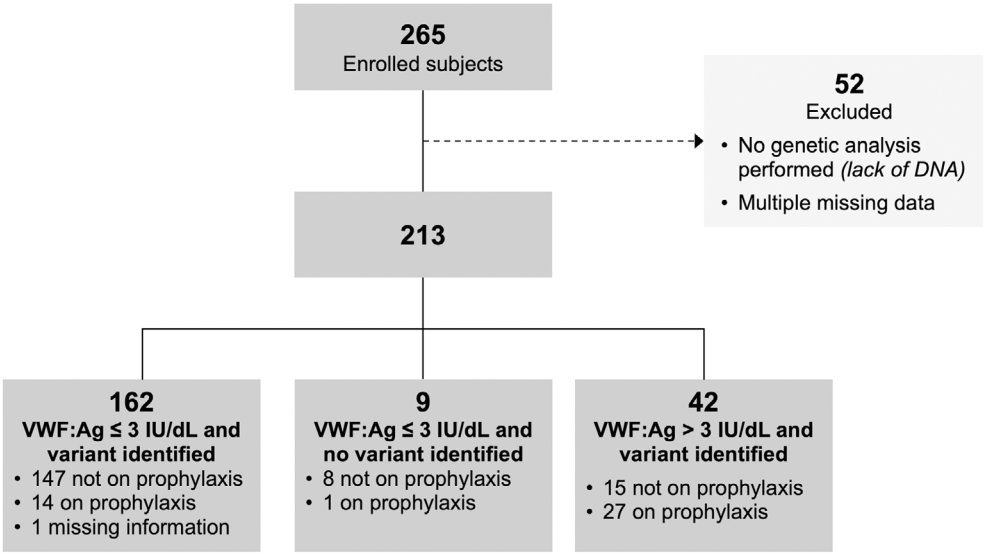

Figure 1. Flowchart of the study population. 265 subjects with a previous type 3 diagnosis were enrolled in 3WINTERS-IPS. Of them, 52 were excluded because of multiple missing data, whereas the remaining 213 subjects were tested for both non-neutralizing and neutralizing VWF antibodies using an indirect 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

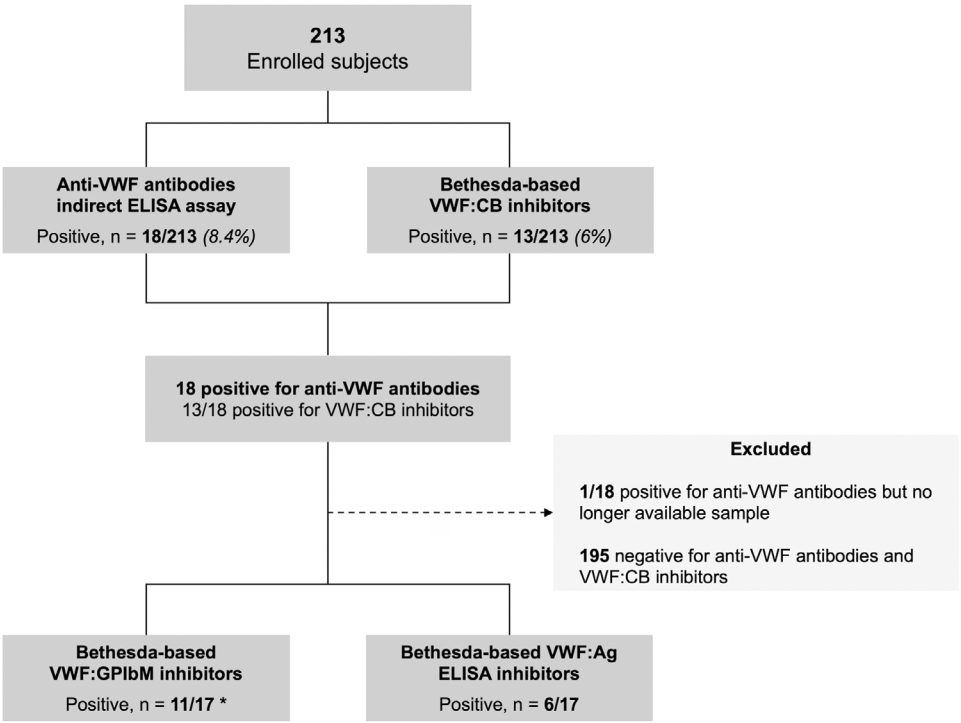

Figure 2. Sample workflow and related results. In total, 213 subjects have been screened for all antibodies against VWF (without distinguish between non-neutralizing and neutralizing antibodies) using an indirect ELISA assay. The same 213 subjects have been tested for VWF inhibitors with a Bethesda-based 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

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(**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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PART III

ROLE OF VON WILLEBRAND FACTOR IN DEEP VEIN THROMBOSIS

CHAPTER 7

ADAMTS13 ACTIVITY, HIGH VWF AND FVIII LEVELS IN THE PATHOGENESIS OF DEEP VEIN THROMBOSIS

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ABSTRACT

Background

Deep vein thrombosis (DVT) is a common multi-factorial disease with a partially understood aetiology. Although the roles of high factor (F)VIII and von Willebrand factor (VWF) levels are recognized, that of ADAMTS13 is still unclear.

Aim

To assess the association between ADAMTS13 activity levels, VWF antigen (VWF:Ag) and FVIII coagulant activity (FVIII:C) levels and DVT.

Materials and methods

365 Italian DVT patients and 292 age- and sex-matched controls were considered. Plasma ADAMTS13 activity was measured using FRETS-VWF73 assay. VWF:Ag and FVIII:C were measured using immunoassay and one-stage clotting assay (ACL TOP analyzer), respectively. Quartile analyses were performed to evaluate the individual association between ADAMTS13 activity, VWF:Ag, FVIII:C and DVT. The combined effect of high VWF levels (> 4th quartile) and low ADAMTS13 levels (< 1st quartile) was evaluated using binary variables. All models were age- and sex-adjusted. Estimated risks were reported as Odds ratio (OR) with 95% confidence intervals (CI).

Results

ADAMTS13 activity was lower in DVT patients (94% vs. 98% of controls). Patients with an ADAMTS13 activity <1st quartile (86%) showed a 1.6-fold increased risk of DVT (95%CI, 1.05-2.55). The combination of low ADAMTS13 activity and high VWF:Ag levels was associated with a 15–fold increased risk (95%CI, 7.80-33.80). VWF:Ag and FVIII:C were associated to DVT with a dose-response relationship.

Conclusions

ADAMTS13 activity < 86% was associated with a moderate risk of DVT. The co-presence of low ADAMTS13 activity and high VWF levels resulted in a strong synergistic effect on DVT risk. The association of VWF:Ag and FVIII:C with DVT was confirmed.

INTRODUCTION

Deep vein thrombosis (DVT) is a common multi-factorial thrombotic disorder caused by environmental, behavioral and genetic risk factors, high coagulant factors levels or a combination of them. Environmental factors include surgery, trauma or fracture, hospitalization, immobilization, pregnancy/puerperium, oral contraceptive use, cancer, age, sex and ethnicity [1,2]. The behavioral habits include smoke, sedentariness, obesity and long travels, whereas among the genetic risk factors there are the deficiencies of the natural anticoagulant proteins (antithrombin, protein C and protein S), Factor V Leiden, prothrombin G20210A mutation and blood group non-O [2-6]. Despite all, known risk factors can explain only a part of DVT events and a missing hereditary is still present.

ADAMTS13 is a metalloprotease which plays an important role in hemostasis due to its cleavage activity of von Willebrand factor (VWF), a large sticky multimeric glycoprotein [7,8]. Under flow conditions, VWF passes from a globular form to an elongated form, thus exposing the A2 domain. ADAMTS13 binds to VWF within A2 domain, reducing the highly thrombogenic ultra large multimers into smaller and less active molecules. This mechanism is fundamental to prevent an excessive platelets adhesion to ultra-large VWF and to dissolve VWF-platelet aggregates [9].

A severe deficiency of ADAMTS13 results in the development of thrombotic thrombocytopenic purpura (TTP) [10], a thrombotic microangiopathy characterized by VWF-mediated platelet thrombi disseminated in the microcirculation of vital organs such as heart, kidney and brain [11]. Recent finding also described the role of ADAMTS13 in the pathogenesis of other thrombotic disorders. Indeed, low and moderately low ADAMTS13 levels showed to be associated to myocardial infarction and coronary artery disease [12-14].

To date, there is few and discordant information regarding the role of ADAMTS13 in venous thromboembolism, which includes both DVT and pulmonary embolism (PE). Indeed, different authors reported the association of both high and low ADAMTS13 levels with VTE [15-17]. Furthermore, VTE was associated to low or normal ADAMTS13 activity in patients with cancer [18-20].

We hypothesized that the reduction of ADAMTS13 activity or the alteration of the equilibrium between ADAMTS13 and VWF may also play a role in DVT pathogenesis. Therefore, we decided to measure ADAMTS13 activity in a group of 365 Italian DVT patients and 292 age- and sex-matched controls with the aim to evaluate: (i) the association between ADAMTS13 activity levels and risk for DVT, and (ii) the possible synergistic effect of low ADAMTS13 activity and high VWF antigen levels as novel potential mechanism of DVT pathogenesis. In addition, we further evaluated the independent association of VWF and FVIII levels with DVT.

MATERIALS AND METHODS

Study population

We selected Italian patients with DVT among those referred to the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center in Milan (Italy) for a thrombophilia workup after a first event between 2006 and 2016. Briefly, the selection criteria included: (i) objective diagnosis of DVT of the lower limbs (i.e. by compression ultrasonography or venography); (ii) idiopathic DVT defined by the absence of cancer, surgery or immobilization; (iii) normal levels of the natural anticoagulant antithrombin, protein C and protein S; (iv) absence of FV Leiden (FVL) or prothrombin G20210A mutations. Controls, matched with cases for age (± 5 years) and sex were recruited among friends and partners who accompanied patients to the Center, agreed to be tested for thrombophilia and had wild-type FVL and prothrombin G20210A genotypes. The study was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, and all patients and controls signed their informed consent to participate into the study.

Biochemical assays

Plasma ADAMTS13 activity was measured using fluorescence resonance energy transfer on plasma samples, as previously described [21]. VWF antigen (VWF:Ag) and FVIII coagulant activity (FVIII:C) were measured using an automated immunoassay and one-stage clotting assay on ACL TOP analyzer (Instrumentation Laboratory Italy), respectively.

Statistical analysis

Continuous variables were described as median and interquartile range, whereas categorical variables were reported as counts and percentages.

A logistic regression was performed to assess the independent association of ADAMTS13 activity with risk for DVT. A first analysis was based on dichotomous exposure with an a priori cut-off set at the 5th and 1st percentiles of ADAMTS13 activity levels of the pooled control group distribution. Then, the risk of DVT was calculated across quartiles of ADAMTS13 activity, based on its distribution among controls, and using the highest quartile as reference. The measure of the relative risk was expressed as odds ratio (ORs) with the corresponding 95% confidence intervals (CI), and adjusted for age and sex.

The possible presence of a non-linear association between ADAMTS13 activity levels (kept continuous) and risk of DVT (expressed as log odds) was evaluated by using a restricted cubic spline function with three knots.

The association of VWF:Ag and FVIII:C plasma levels (divided into quartiles of their distribution among controls) with DVT, was performed using a logistic regression model with the lowest quartile of the controls' distribution set as reference. The estimated risks were expressed as OR and 95% CI. Each model was adjusted for age and sex. Binary variables with predefined cut-off points were set up to deeply evaluate the combined

effect of high VWF levels (above the 4th quartile) and low ADAMTS13 levels (below the 1st quartile) on DVT risk.

Both quartile and binary variable analyses were also performed after excluding patients in anticoagulant therapy or who had DVT event less than 3 months before blood collection, in order to avoid the possible interference of these factors on risk estimates. All the statistical analyses were performed using the statistical software R, release 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

Sample size was determined using PS Power and Sample Size Calculations (Version 3. version 3.1.6; http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize), considering a 0.05 two-tail alpha error and a power 0.8. We assumed a probability of exposure among controls of 0.2, and a correlation coefficient for exposure between matched cases and controls of 0.2. It is estimated that a minimum sample size of 212 cases and 212 controls was required to detect a true OR of 2.0.

RESULTS

The characteristics of the enrolled DVT cases (n= 365) and controls (n= 292) are reported in **Table 1**. The mean age was 48 years for cases and 47 years for controls. Both patients and controls had a higher percentage of non-O blood group than O blood group. FVIII:C levels were higher in patients than in controls (median, 148 vs. 113%), whereas the median of plasma ADAMTS13 activity was slightly lower in patients (94%; IQR 81-108) than in controls (98%; IQR, 86-112). Two out of 365 patients showed ADAMTS13 activity levels slightly below the normal range (42%; normal range 45-138%). Patients had increased VWF:Ag levels (169%; IQR, 136-209) than controls (115%; IQR, 87-148). The 47% of patients were receiving anticoagulant therapy at the time of blood sampling. The median time between DVT event and sample collection was about 9 months (IQR, 3-32).

The association between ADAMTS13 activity levels and DVT was initially calculated using as arbitrary cut-off the 1st and 5th percentile of ADAMTS13 distribution of the pooled control group. ADAMTS13 levels ≤ 5th percentile, i.e. ≤ 67.6%, were associated with an almost 2-fold increased risk for DVT (age- and sex-adjusted OR 1.63, 95% CI 0.84-3.12). Similar results were found for ADAMTS13 activity levels ≤ 1st percentile, i.e. ≤ 59% (age- and sex-adjusted OR 2.52, 95% CI 0.81-7.87). Dividing ADAMTS13 activity levels into quartiles, ADAMTS13 activity levels ≤ 86% were associated to a 1.6-fold increased risk of DVT (age- and sex-adjusted OR 1.64, 95% CI 1.05-2.55), while intermediate quartiles showed only a small association with DVT, thus excluding a clear dose-response relationship (**Table 2**).

This trend was confirmed when the relationship between ADAMTS13 levels (kept continuous) and DVT risk was evaluated with a restricted cubic spline function: a slightly non-linear component in the log-odds of DVT was visible for ADAMTS13 activity levels below 90% even after adjustment for age and sex (**Figure 1**).

Table 1. Characteristics of the study population

	Patients (n= 365)	Controls (n= 292)
Age (years), *median (IQR)*	48 (38-61)	47 (37-54)
Female sex, *n (%)*	184 (50.4%)	153 (52.4%)
ABO, *n (%)*		
O Group	50 (13.7)	95 (32.5)
Non O Group	147 (40.3)	113 (38.7)
Missing	168 (46.0)	84 (28.8)
FVIII:C (%),[a] median (IQR)	148 (124-173)	113 (93-137)
VWF:Ag (%),[b] median (IQR)	169 (136-209)	115 (87-148)
ADAMTS13 Activity (%) median, (IQR)	94 (81-108)	98 (86-112)
Anticoagulant therapy,[c] n (%)	173 (47%)	-
Progestogens, n (%)	1 (0.2)	-
Time from acute event (*months*), median (IQR)	9 (3-32)	-
Major Illness, n (%)		
liver disease	2 (0.01)	-
Kidney disease	2 (0.01)	-

ABO, blood group system; FVIII:C, FVIII coagulant activity, VWF:Ag von Willebrand factor antigen. IQR, interquartile range. [a] Available for 330 cases and 292 controls. [b] Available in 342 cases and 247 controls.
[c] Warfarin (n= 81), low molecular weight heparin (n= 54), rivaroxaban (n= 25), subcutaneous heparin (n= 6), fondaparinux (n= 5), apixaban (n = 1).

Since several previous studies have shown the association of FVIII and VWF with DVT, we performed a quartile analysis to confirm those results in our population. We found that both factors were independently associated with DVT with a dose-response effect, as showed in **Table 2.** The highest VWF:Ag levels (above the 4th quartile) conferred a 23-fold increased risk for DVT (95% CI 11.2-48.3). Similarly, FVIII:C levels above the 4th quartile were associated with a 13-fold increased risk for DVT (95% CI 7.22-25.0).

The combined effect of low ADAMTS13 activity (below the 1st quartile, i.e., ≤ 86%) and high VWF:Ag levels (above the 4th quartile, i.e., >148%) was evaluated using binary variables and results are reported in **Table 3**.

When low ADAMTS13 levels were considered alone, the estimated risk was similar to that of the main analysis (OR 1.75, 95% CI 1.04-2.96 and OR 1.64, 95% CI 1.05-2.55, respectively). High VWF:Ag levels with ADAMTS13 levels above 1st quartile were associated with an increased risk for DVT (age- and sex-adjusted OR 5.52, 95% CI 3.60-8.54). The estimated risk conferred by the combination of low ADAMTS13 and high VWF was much higher than that obtained by the sum of the two separated risks

Table 2. Risk of DVT according to various plasma levels of ADAMTS13, VWF and FVIII

	Patients	Controls	OR (95% CI)	OR[1] (95% CI)
ADAMTS13 activity	**(n= 365)**	**(n= 292)**		
≤ 5th percentile (≤67.6)	29	14	1.74 (0.89-3.31)	1.63 (0.84-3.12)
> 5th percentile (>67.6)	336	278	1 (reference)	1 (reference)
≤ 1st percentile (≤59)	13	4	2.66 (0.93-9.52)	2.52 (0.81-7.87)
> 1st percentile (>59)	352	288	1 (reference)	1 (reference)
1st quartile (≤86%)	130	76	1.73 (1.13-2.68)	1.64 (1.05-2.55)
2nd quartile (87-98%)	91	78	1.18 (0.76-1.85)	1.15 (0.74-1.80)
3rd quartile (99-111%)	72	65	1.12 (0.70-1.79)	1.10 (0.69-1.75)
4th quartile (>111%)	72	73	1 (reference)	1 (reference)
VWF:Ag	**(n= 342)**	**(n= 247)**		
1st quartile (≤87%)	10	62	1 (reference)	1 (reference)
2nd quartile (88-115%)	37	62	3.70 (1.69-8.10)	3.70 (1.69-8.10)
3rd quartile (116-148%)	69	62	6.90 (3.26-14.62)	6.92 (3.26-14.67)
4th quartile (>148%)	226	61	23.0 (11.1-47.4)	23.2 (11.2-48.3)
FVIII:C	**(n= 330)**	**(n= 292)**		
1st quartile (≤93%)	190	75	1 (reference)	1 (reference)
2nd quartile (94-113%)	79	73	2.89 (1.50-5.56)	2.95 (1.53-5.69)
3rd quartile (114-137%)	45	73	5.07 (2.71-9.49)	5.27 (2.80-9.92)
4th quartile (>137%)	16	71	12.54 (6.85-22.96)	13.43 (7.22-25.00)

VWF:Ag, von Willebrand factor antigen; FVIII:C, FVIII coagulant activity. Predefined cut-off points set at the 5th and 1st percentiles were created using the ADAMTS13 distribution of the pooled control group. ADAMTS13 activity, VWF:Ag and FVIII:C were categorized into quartiles using the control group distribution. OR, odds ratios were reported as measures of relative risk; OR[1], values adjusted for age and sex; 95% CI, 95% confidence intervals.

(combined estimated risk: OR 15.45, 95% CI 7.80-33.80 vs. expected OR 1+ [1.75 - 1] + [5.52 - 1] = 6.27), indicating a synergistic effect of the two risk factors.

Finally, we performed a sensitivity analysis restricting our study population to the 167 patients whose blood samples were collected at least three months after the DVT event or not during anticoagulant therapy (**Table 4**). The association between ADAMTS13 levels <86% and DVT was similar to that of the main analysis (age- and sex-adjusted OR 1.69, 95% CI 0.98-2.91). VWF:Ag and FVIII:C still showing a clear increasing trend of DVT risk, which reached the maximum association for the highest quartiles (**Table 4**).

Most importantly, the sensitivity analysis performed to evaluate the combined effect of low ADAMTS13 activity levels and high VWF levels did confirm the main analysis results with a 13-fold increased risk for DVT (95%CI 5.80-29.22, **Table 5**).

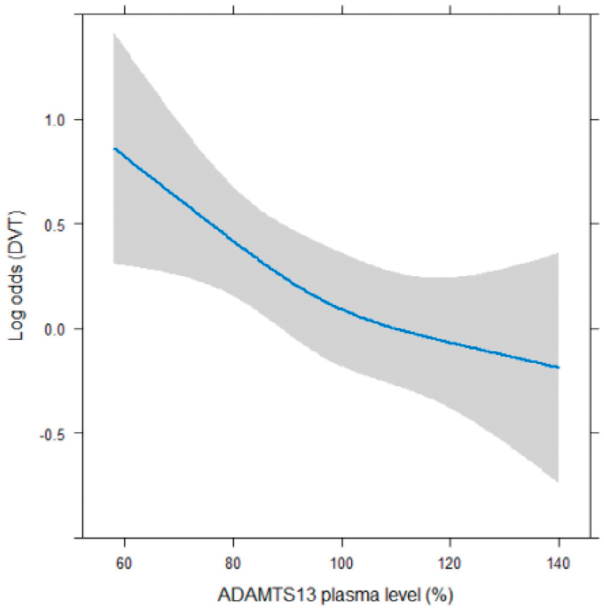

Figure 1. Restricted cubic spline curve. Restricted cubic spline curve showing the model-predicted log odds of DVT against ADAMTS13 activity levels, adjusted for age and sex (solid line). The grey area represents 95% confidence intervals.

Table 3. Risk of DVT in relation to the combination of low ADAMTS13 and high von Willebrand factor plasma levels

Low ADAMTS13 (<1st quartile)	High VWF (> 4th quartile)	Patients, n	Controls, n	OR[1] (95% CI)
-	-	75	142	1 (reference)
+	-	39	43	1.75 (1.04-2.96)
-	+	149	52	5.52 (3.60-8.54)
+	+	79	10	15.45 (7.80-33.80)

OR, odds ratios were reported as measures of relative risk; OR[1], values adjusted for age and sex; 95% CI, 95% confidence intervals. Binary variables were created to evaluate: low ADAMTS13 plasma levels, below the 1st quartile and low (+/-); high VWF plasma levels, above the 4th quartile (+/-), or both (+/+). ADAMTS13 activity VWF:Ag levels were available in 342 cases and 247 controls.

DISCUSSION

In this study, we evaluated the role of low ADAMTS13 activity levels as possible new risk factor of DVT. ADAMTS13 cleaving activity of ultra large VWF multimers is fundamental to maintain a proper hemostasis. Furthermore, the role of this metalloprotease has been previously described in the pathogenesis of other thrombotic disorders such as myocardial

Table 4. Sensitivity analyses for DVT risk according to various plasma levels of ADAMTS13, VWF and FVIII

	Patients	Controls	OR (95% CI)	OR[1] (95% CI)
ADAMTS13 activity	(n= 167)	(n= 292)		
1st quartile (≤86%)	59	76	1.67 (0.98-2.83)	1.69 (0.98-2.91)
2nd quartile (87-98%)	40	78	1.10 (0.63-1.92)	1.01 (0.63-1.93)
3rd quartile (99-111%)	34	65	1.12 (0.63-2.01)	1.11 (0.62-2.00)
4th quartile (>111%)	34	73	1 (reference)	1 (reference)
VWF:Ag	(n= 152)	(n= 247)		
1st quartile (≤87%)	5	62	1 (reference)	1 (reference)
2nd quartile (88-115%)	21	62	4.2 (1.49-11.85)	4.18 (1.48-11.80)
3rd quartile (116-148%)	35	62	7.00 (2.57-19.05)	7.01 (2.57-19.10)
4th quartile (>148%)	91	61	18.50 (7.03-48.66)	18.88 (7.12-50.02)
FVIII:C	(n= 167)	(n= 292)		
1st quartile (≤93%)	86	75	1 (reference)	1 (reference)
2nd quartile (94-113%)	40	73	3.08 (1.41-6.76)	3.05 (1.39-6.71)
3rd quartile (114-137%)	30	73	4.11 (1.91-8.26)	4.16 (1.92-9.02)
4th quartile (>137%)	10	71	9.10 (4.37-48.87)	9.15 (4.67-19.87)

Samples were collected at least 3 months after DVT event and in the absence of any anticoagulant treatment. VWF:Ag, von Willebrand factor antigen; FVIII:C, FVIII coagulant activity. ADAMTS13 activity, VWF:Ag and FVIII:C were categorized into quartiles using the control group distribution. OR, odds ratios were reported as measures of relative risk; OR[1], values adjusted for age and sex; 95% CI, 95% confidence intervals.

Table 5. Sensitivity analysis of DVT risk in relation to the combination of low ADAMTS13 and high von Willebrand factor plasma levels

Low ADAMTS13 (<1st quartile)	High VWF (> 4th quartile)	Patients, n	Controls, n	OR[1] (95% CI)
-	-	40	142	1 (reference)
+	-	19	43	1.66 (0.86-3.18)
-	+	59	52	4.14 (2.45-6.99)
+	+	34	10	13.01 (5.80-29.22)

Odds ratios were reported as measures of relative risk; OR[1], values adjusted for age and sex; 95% CI, 95% confidence intervals. Binary variables were created to evaluate: low ADAMTS13 plasma levels, below the 1st quartile and low (+/-); high VWF plasma levels, above the 4th quartile (+/-), or both (+/+). ADAMTS13 activity VWF:Ag levels were available in 152 cases and 247 controls. Samples were collected at least 3 months after DVT event and in the absence of any oral anticoagulant treatment.

infarction and ischemic stokes [12-14]. Based on this background, we decided to evaluate the role of ADAMTS13 activity levels in 365 DVT patients referred to our Center and 292 controls with the following aims: (i) to evaluate the association between ADAMTS13 activity and risk for DVT (ii) to evaluate the possible synergistic effect of low ADAMTS13 and high VWF levels as potential novel mechanism in the pathogenesis of the disease; and (iii) to confirm the association between DVT risk and high levels of FVIII and VWF, as previously reported by other authors [22-24].

We found that DVT patients had a moderately lower median ADAMTS13 activity levels than controls. First evidence of ADAMTS13 association with DVT was showed for levels below the 5th and 1st percentile (OR 1.63, 95% CI 0.84-3.12 and OR 2.52, 95% CI 0.81-7.87, respectively). When ADAMTS13 activity were categorized into quartiles, we showed that even a modest reduction of ADAMTS13 activity (below 86%) was associated with an increased risk of DVT (OR 1.64, 95% CI 1.05-2.55). Interesting results were obtained by the evaluation of the combined effect of low ADAMTS13 activity and high VWF antigen levels, which resulted in a 15-fold increased risk for DVT. As the combined estimated risk was clearly higher than the sum of the separated risks (15-fold vs. 6-fold, respectively), a synergistic effect of these two factors was plausible. Therefore, these data showed that a modest reduction of ADAMTS13 activity, especially when combined with high VWF levels (ADAMTS13-VWF equilibrium alteration) plays a role in the onset of DVT events.

Interestingly, highest VWF levels showed a higher association to DVT than highest FVIII levels (23-fold vs. 13-fold, referred to the main analyses). This finding led us to speculate that VWF association with DVT is not related to its protective role of FVIII [22], but it acts as independent risk factor [23].

To date, the role of ADAMTS13 levels was evaluated in few studies referred to VTE patients. Our results were in accordance with those reported by Karakaya *et al,* who described low ADAMTS13 antigen and high VWF levels, although referred to a small study population of 30 VTE patients and 30 controls [15]. Low ADAMTS13 antigen levels, below the 5th and 10th percentile, have also been associated with VTE event in Spanish women, but not in men, who had a first VTE event before 70 years of age [16]. Our results, were in contrast with those of Mazzetto *et al*, who described a high ADAMTS13 and VWF levels in VTE patients, in presence of high inflammatory markers long time after the VTE event. These authors speculated that high ADAMTS13 levels could be due to a kind of compensatory mechanism against increased VWF levels following thrombotic events [17]. However, the lack of information about inflammatory markers in our study population did not allow a proper comparison with that study.

The mechanism involving ADAMTS13 in DVT pathogenesis is not fully understood. Because of the nature of the study design, we cannot establish whether the slightly reduced ADAMTS13 activity levels and the increased VWF are the cause or the consequence of DVT event. We can speculate that the reduction of ADAMTS13 activity levels are due to increased VWF antigen levels (i.e. excessive ADAMTS13 consumption), thus resulting in an excess of ultra-large VWF, which is more prone to bind platelets. Furthermore,

the genetic component should also play a part in the alteration of VWF-ADAMTS13 balance. Indeed, our group previously described that carriers of one rare single nucleotide of *ADAMTS13* had lower activity levels than non-carriers [25].

This study could suffer of some limitations that require to be mentioned. First, blood sampling was not performed at the same time point (distance from the acute event) in all patients. This aspect may have introduced some variability on VWF, FVIII and ADAMTS13 levels. On the other hand, the sampling procedure was performed at a median of 9 months after the DVT event, leading us to exclude that the proteins levels were still a consequence of the DVT event, at least for VWF and FVIII levels. Indeed, different authors have previously reported that both FVIII [24, 26] and VWF levels [24] remain stable for years after the DVT event, whereas this information is still to be clarified for ADAMTS13. Second, the continuation of anticoagulant therapy at the time of blood collection for half of patients could have influenced the results, although an effect on FVIII, VWF:Ag and ADAMTS13 levels is unlikely [24]. However, to rule out these hypotheses, analyses were repeated after excluding patients whose samples were collected less than three months from DVT event and/or during anticoagulant therapy. Neither the quartile analyses of ADAMTS13, VWF and FVIII nor the combined effect of ADAMTS13 and VWF seemed to be affected, thus confirming our results. Third, the lack of consecutive samples did not allow us to directly evaluate the potential variation over time of ADAMTS13, VWF and FVIII levels in our population or to assess whether ADAMTS13 and VWF levels may be helpful to predict a recurrence of DVT, as already described for FVIII levels [27]. Lastly, because the levels of inflammatory markers were not determined, we cannot exclude that inflammatory events other than acute DVT may have contributed to the increased VWF/FVIII levels and decreased ADAMTS13 levels. However, our study aimed to establish the association of slightly decreased ADAMTS13 levels (alone or combined with VWF levels) with DVT and to confirm that with high VWF and FVIII levels with DVT, rather than explaining the mechanisms which cause them.

CONCLUSIONS

We evaluated the effect of ADAMTS13 activity levels as adjunctive risk factor for DVT. We showed that a modest reduction of ADAMTS13 activity is enough to have a 1.6-fold increased risk of DVT. Furthermore, we found that the combination of low ADAMTS13 levels and high VWF levels resulted in a clearly increased risk. This led us to hypothesize that a synergistic effect between these two factors plays an important role in the DVT pathogenesis, although the causative mechanism which involves them still needs to be clarified.

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CHAPTER 8

ROLE OF ADAMTS13, VWF AND F8 GENES IN DEEP VEIN THROMBOSIS

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ABSTRACT

Background

We previously described the association between rare *ADAMTS13* single nucleotide variants (SNVs) and deep vein thrombosis (DVT). Moreover, DVT patients with at least one rare *ADAMTS13* SNV had a lower ADAMTS13 activity than non-carriers.

Aims

To confirm *ADAMTS13* variants association with DVT and reduced plasma ADAMTS13 activity levels in a larger population. To investigate the role of *VWF* and *F8* variants.

Methods

ADAMTS13, VWF and *F8* were sequenced using next-generation sequencing in 594 Italian DVT patients and 571 controls. Genetic association testing was performed using logistic regression and gene-based tests. The association between rare *ADAMTS13* variants and the respective plasmatic activity, available for 365 cases and 292 controls, was determined using linear regression. All analyses were age-, sex- adjusted.

Results

We identified 48 low-frequency/common and 272 rare variants. Nine low-frequency/common variants had a P<0.05, but a false discovery rate between 0.06 and 0.24. Of them, 7 were found in *ADAMTS13* (rs28641026, rs28503257, rs685523, rs3124768, rs3118667, rs739469, rs3124767; all protective) and 2 in *VWF* (rs1800382 [risk], rs7962217 [protective]). Rare *ADAMTS13* variants were significantly associated with DVT using the burden, variable threshold (VT) and UNIQ (P<0.05), but not with C-ALPHA, SKAT and SKAT-O tests. Rare *VWF* and *F8* variants were not associated with DVT. Carriers of rare *ADAMTS13* variants had lower ADAMTS13 activity than non-carriers (ß -6.2, 95%CI -11,-1.5). This association was stronger for DVT patients than controls (ß -7.5, 95%CI -13.5,-1.5 vs ß -2.9, 95%CI -10.4, 4.5).

Conclusions

ADAMTS13 and *VWF* low-frequency/common variants mainly showed a protective effect, although their association with DVT was not confirmed. DVT patients carrying a rare *ADAMTS13* variants had slightly reduced ADAMTS13 activity levels, but a higher DVT risk. Rare *VWF* and *FVIII* variants were not associated with DVT suggesting that other mechanisms are responsible for the high VWF and FVIII levels measured in DVT patients.

INTRODUCTION

Deep vein thrombosis (DVT) is a common life-threatening thrombotic disorder caused by the shift of the hemostatic equilibrium toward the blood clot formation. DVT along with pulmonary thromboembolism constitute venous thromboembolism (VTE), which is characterized by an annual incidence of 2-3 per 1000 individuals [1]. Part of DVT events is explained by a strong genetic component that includes the deficiencies of natural anticoagulants proteins (antithrombin, protein C and protein S), factor V Leiden (FVL), prothrombin G20210A mutation, fibrinogen gamma chain C10034T mutation [2]. The dissemination and improvement of new sequencing approaches including genome-wide association studies (GWAS) along with meta-analyses of GWAS data, led to the identification of new loci which mainly refer to genes involved in the hemostatic pathways [3,4]. However, these genetic risk factors contribute to explain only 50-60% of DVT genetic hereditability [5-7]. Our group previously reported the results obtained using DNA next-generation sequencing (NGS) to evaluate the role of the disintegrin and metalloprotease with thrombospondin type 1 motif, number 13 (ADAMTS13) and von Willebrand factor (VWF) genes which encode two proteins involved in the maintenance of the equilibrium between hemostasis and thrombosis. In particular, we showed that DVT patients had an excess of rare *ADAMTS13* single nucleotide variants (SNVs) compared with controls. Moreover, DVT patients carrying at least one rare SNV showed a lower ADAMTS13 activity than non-carrier patients [8]. The functional effect of these variants has been evaluated by performing *in vitro* expression studies, which confirmed a reduction of ADAMTS13 activity for 3 out of 9 variants (p.V154I, p.D187H and p.R421C) [9].

Based on this background, we aimed to confirm our previous results which suggested an association between rare *ADAMTS13* variants and DVT (odds ratio [OR] 4.8; 95% confidence interval [CI] 1.6–15.0) [8]. To this purpose, a total of 594 Italian DVT patients and 571 controls were sequenced using NGS. Then, the association between carrier-ship of a rare *ADAMTS13* variant and ADAMTS13 activity levels was evaluated. The study also focused on *VWF* and *F8* due to the strong role of the respective encoded proteins in the maintenance of hemostasis and thrombosis equilibrium and because increased VWF and FVIII levels have been already described as associated with an increased DVT risk [10].

MATERIALS AND METHODS

Study population

We selected Italian DVT patients from those referred to the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center in Milan (Italy) between 2006 and 2016. Briefly, the selection criteria included: (i) objective diagnosis of DVT of the lower limbs (i.e., by compression ultrasonography or venography); (ii) idiopathic DVT defined by the absence of cancer, surgery or immobilization; (iii) normal levels of the natural anticoagulant proteins antithrombin, protein C and protein S; (iv) absence of FVL or prothrombin G20210A mutations. Controls, matched with cases for age (+/- 5 years) and sex were recruited among

friends and partners who accompanied patients to the Center, agreed to be tested for thrombophilia and had wild-type FVL and prothrombin G20210A genotypes [9].

ADAMTS13 activity, previously measured using the FRET-VWF73 assay [11], was available in 365 patients and 292 controls [12]. The study was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico. All study subjects were aware of the content of this study and gave a written informed consent.

Next generation sequencing, quality control and data analyses

NGS experiments were performed in two stages. For 298 DVT cases and 298 controls, the coding region, intron-exon boundaries, and 3' and 5' untranslated regions (UTRs) of *ADAMTS13, VWF* and *F8* were sequenced as part of a larger panel, as previously reported [13]. Briefly, a multiplexed NGS (Human Genome Sequencing Center, Baylor College of Medicine, Houston, USA) was performed using unique barcode-sequencing tags, to create library pools of 8–20 samples, which were captured and sequenced in parallel using the Illumina HiSeq 2000 sequencing platform (Illumina, San Diego, USA). Reads were mapped to reference genome GRCh37/hg19 by use of the Burrows–Wheeler aligner (BWA)-MEM, resulting in BAM files per sample [13].

A second NGS panel (Illumina, San Diego, USA) limited to the coding region, intron-exon boundaries, and 3' and 5' untranslated regions (UTRs) of *ADAMTS13, F8* and *VWF* was subsequently designed to increase our study population by sequencing additional 296 cases and 273 controls. The sequences were aligned to the same reference genome (GRCh37/hg19) using the BWA-MEM algorithm. Picard and GATK tools were applied to sort index and recalibrate the BAM files of the individual subjects. Then, BAM files obtained from both sequencing panels underwent variant calling, performed using Haplotype Caller (GATK). The single gVCF files were assembled in a unique file and the variants were filtered according to the guidelines reported by the Broad Institute (https://software.broadinstitute.org/gatk/best-practices/). Variants' annotation was performed using wANNOVAR (http://wannovar.wglab.org/). A further quality control was performed using KGGSeq (http://grass.cgs.hku.hk/limx/kggseq/), excluding variants with an average reading depth< 10, Phred score< Q30 and Hardy-Weinberg equilibrium P< 1.0 E-04. Variants were divided on the basis of their minor allele frequency (MAF) in low-frequency/common (MAF≥ 1%) and rare (MAF< 1%).

Statistical analysis

Continuous variables were described as median and interquartile range (IQR), whereas categorical variables were reported as counts and percentages. ADAMTS13 activity, von Willebrand factor antigen (VWF:Ag) and factor VIII coagulant activity (FVIII:C) levels in DVT patients and controls were compared using the non-parametric Mann-Whitney test. P-values< 0.05 were considered statistically significant. Low-frequency and common variants were individually analyzed using a logistic regression model age- and sex- adjusted

and considering a model of additive inheritance with PLINK (http://zzz.bwh.harvard.edu/plink/contact.shtml). Results were reported as odds OR with the corresponding 95% CI. The False discovery rate (FDR) was used to correct for multiple testing and variants with an FDR< 0.25 were further considered. Rare variants were analyzed using seven different cumulative association tests based on different analytical approaches: (i) unidirectional tests which include burden [14], variable threshold (VT [15]), UNIQ [16] and SUMSTAT tests [17]; (ii) bi-directional variance component tests which allow different variants effects (neutral/deleterious) such as C-ALPHA [18] and the Sequence Kernel Association Test (SKAT [19]); and (iii) a combination of both unidirectional and bi-directional variance-component tests, represented by SKAT-O [20]. All gene-based association tests were performed with the PLINK/SEQ suite (https://atgu.mgh.harvard.edu/plinkseq/) and adjusted for age and sex [14].The association between rare *ADAMTS13* variants and ADAMTS13 activity levels was evaluated using linear regression models adjusted for age and sex, by considering: (i) all variants identified in ADAMTS13; (ii) the variants potentially affecting ADAMTS13 activity such as missense mutations, frameshift mutations, deletions and insertions; (iii) the variants potentially affecting ADAMTS13 activity also predicted as damaging by CADD algorithm (score> 20) [21].The analyses were performed in all individuals with an available measurement of ADAMTS13 activity, then stratifying by case-control status. Results were reported as beta coefficients with 95% CI. Statistical analyses were performed using the statistical software R, release 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Baseline characteristics of the study population, which include 594 cases and 571 controls, are reported in **Table 1**. About 60% of DVT patients were women with a mean age of 47 years, similar to controls. DVT patients showed higher median VWF:Ag (169% vs. 115%; P< 0.0001) and FVIII:C levels (148% vs. 113%, P< 0.0001) plasma levels than controls (**Table 1**). ADAMTS13 activity, available in 365 DVT patients and 292 controls, was slightly reduced in DVT patients than controls (94% vs. 98%, respectively; P= 0.02) [12].

NGS identified a total of 320 variants distributed in the in the *ADAMTS13*, *VWF* and *F8*. These SNVs were mainly localized in the coding regions (77.5%) and introns (16%), whereas only 6.5% was found in the UTRs of sequenced genes. Forty-eight low-frequency/ common variants, all missense, were distributed as follows: 18 in *ADAMTS13*, 27 in the *VWF* and 3 in the *F8* (**S1 Table**). Of 272 rare variants (MAF< 1%), 210 were localized in exons and included 126 missense, 80 synonymous mutations, 1 in-frame insertion, 2 in-frame deletions and 1 frameshift mutation, whereas the remaining 62 variants were found in introns and UTRs. Seventy out of 272 variants were referred as *novel* because not present in dbSNP or other databases. The complete list of rare variants is reported in the **S2 Table**.

A total of 89 rare variants were found in *ADAMTS13* and spanned across the gene. Of them, 48 were found in DVT patients, 28 in controls and 13 were found in both. Of the remaining 183 rare variants, 149 were in *VWF* and 34 in *F8*. Rare *VWF* and *F8* variants were homogeneously distributed among cases and controls (93 vs. 105 for *VWF*; 17 vs. 20 for *F8*). A total of 3 tri-allelic variants were identified (n= 1 in *ADAMTS13* and n= 2 in *VWF*).

Low-frequency/common variants

The QQ plot of the observed P-values vs. the expected distribution showed a signal inflation due to high linkage disequilibrium of variants (**Figure 1**). Of 48 low-frequency/ common SNVs, 9 showed a P< 0.05, but an FDR between 0.06 and 0.24 after correction for multiple testing (**Table 2**). Seven out of these 9 SNVs were located in ADAMTS13 and two in the VWF. None of the 3 SNVs identified in F8 were found to be associated with DVT. Low-frequency/ common ADAMTS13 variants showed a protective effect on DVT onset, with size effect estimates ranging from an OR 0.53 to 0.82. ADAMTS13 SNVs rs3124768, rs3118667 and rs739469 showed the same OR of 0.78, explained by the high linkage disequilibrium among them (r^2 between 0.74 and 1 for each combination). Similar results were found for ADAMTS13 SNVs rs28641026 and rs28503257, which were associated to DVT with an OR of 0.53 (r^2= 1). VWF SNV rs1800382 had a strong association with DVT (OR 3.26, 95% CI 1.18-8.98; P= 0.02), whereas the rs7962217 had a protective effect on disease onset (**Table 2**).

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Rare variants

The association between rare variants and DVT was assessed using different gene-based tests and results are summarized in **Table 3**. Because these tests consider the variant position, the three tri-allelic variants are not included in the count herein reported. *ADAMTS13* SNVs (n= 88) were found to be associated with DVT using the burden test (P= 0.02), VT (P= 0.03), and UNIQ (P= 0.04), but not with C-ALPHA, SUMSTAT, SKAT tests and SKAT-O (P> 0.05).

Both burden and VT tests remained significant after performing a restriction analysis on potentially damaging variants (n= 45; P= 0.02 and P= 0.04, respectively). The same results were obtained by restricting the analysis to the 15 potentially damaging *ADAMTS13* variants also predicted to be damaging by CADD> 20 (burden, P= 0.003; VT, P= 0.005). SKAT-O showed a significant association after restriction analyses to potentially damaging variants (P= 0.05) and potentially damaging variants with CADD> 20 (P= 0.008). None of these tests showed an association between rare *VWF* variants and DVT, neither considering all identified variants nor by performing a restriction analysis on potentially damaging variants. SKAT and SKAT-O showed a significant association after restriction analysis to *VWF* potentially damaging variants with a CADD> 20. Rare *F8* variants were not associated with DVT, even after restriction analyses (**Table 3**).

Table 1. Baseline characteristics of the study population

	DVT Patients	Controls	P*
N	594	571	-
Age (years), mean *(SD)*	47±15	45±14	-
Female sex, n *(%)*	328 (57)	313 (55)	
ADAMTS13 activity (%), median *(IQR)* [a]	94 (81-108)	98 (86-112)	P= 0.02
VWF antigen, median *(IQR)* [b]	169 (136-209)	115 (87-148)	P< 0.0001
FVIII coagulant activity, median *(IQR)* [c]	148 (124-173)	113 (93-137)	P< 0.0001
Time from acute event (months), median *(IQR)*	9 (3-32)	-	-
Patients treated at time of sampling, n *(%)* [d]	174 (48)	-	-

[a] Available in 365 cases and 292 Controls. [b] Available in 342 cases and 247 controls.
[c] Available in 330 cases and 292 Controls. [d] Warfarin (n= 81), low molecular weight heparin (n= 54), Rivaroxaban (n= 25), unfractionated heparin (n= 6), Fondaparinux (n= 5), Apixaban (n= 1).
* Mann-Whitney test was performed. P-values< 0.05 were considered statistically significant.

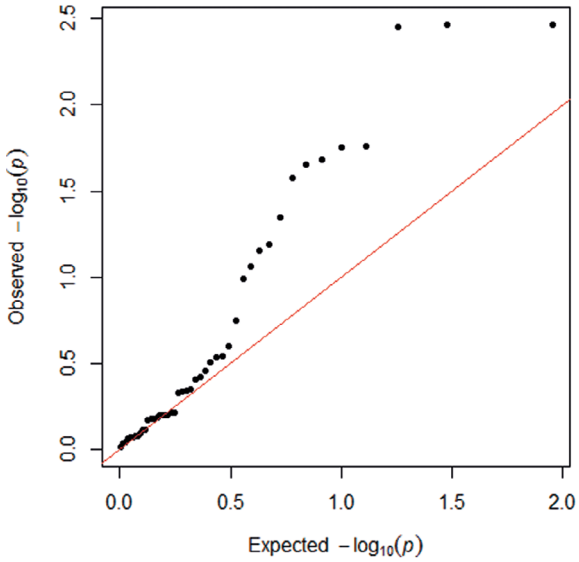

Figure 1. Quantile-Quantile plot. P value distributions referred to the 48 low-frequency and common variants (MAF≥ 1%) identified in Italian patients with DVT and matched healthy controls sequenced by next generation sequencing.

Association between rare ADAMTS13 SNVs and ADAMTS13 activity levels

This part of the study was performed by selecting sequenced cases and controls (n= 365 and n= 292, respectively) who had available ADAMTS13 activity levels, measured by the FRET-VWF73 assay. The association between the presence of at least one rare

Table 2. Low-frequency and common variants associated with DVT

Gene	rsID	Chr	Position	Class	Ref. Allele/ Risk Allele	Amino Acid Position	MAF Risk allele (Cases/ Controls)	OR (95%CI) [a]	P	FDR
ADAMTS13	rs28641026	9	136314952	exonic	C/T	p.V970V	0.02/0.04	0.53 (0.31-0.89)	0.02	0.15
ADAMTS13	rs28503257	9	136319589	exonic	G/A	p.A1033T	0.02/0.04	0.53 (0.31-0.89)	0.02	0.15
ADAMTS13	rs685523	9	136310908	exonic	C/T	p.A900V	0.07/0.10	0.74 (0.55-0.99)	0.05	0.24
ADAMTS13	rs3124768	9	136304497	exonic	G/A	p.T572T	0.43/0.50	0.78 (0.67-0.92)	0.003	0.06
ADAMTS13	rs3118667	9	136291063	exonic	T/C	p.A140A	0.36/0.43	0.78 (0.66-0.92)	0.003	0.06
ADAMTS13	rs739469	9	136298729	intronic	C/G	.	0.43/0.49	0.78 (0.67-0.92)	0.004	0.06
ADAMTS13	rs3124767	9	136308542	exonic	T/C	p.G760G	0.45/0.50	0.82 (0.69-0.97)	0.02	0.15
VWF	rs1800382	12	6128388	exonic	C/T	p.R1399H	0.01/0.001	3.26 (1.18-8.98)	0.02	0.15
VWF	rs7962217	12	6061559	exonic	C/T	p.G2705R	0.05/0.07	0.67 (0.47-0.95)	0.03	0.16

Chr, chromosome; Ref. allele, Reference allele; MAF, Minor allele frequency; OR – odds ratio; 95% CI – confidence interval; FDR – false discovery rate; [a] age/sex adjusted.

Table 3. The association between *ADAMTS13, VWF* and *FVIII* rare variants and DVT assessed using different gene-based tests

	Rare variants				Potential Damaging [a] mutations				CADD > 20			
Gene	N	Test	P	I	N	Test	P	I	N	Test	P	I
ADAMTS13	88 [b]	BURDEN	0.02	0.001	45 [b]	BURDEN	0.02	0.001	15	BURDEN	0.003	0.0003
		C-ALPHA	0.14	0.003		C-ALPHA	0.11	0.004		C-ALPHA	0.09	0.0003
		SUMSTAT	0.18	0.003		SUMSTAT	0.30	0.004		SUMSTAT	0.18	0.0003
		VT	0.03	0.003		VT	0.04	0.004		VT	0.005	0.0003
		SKAT	0.19	-		SKAT	0.15	-		SKAT	0.09	-
		UNIQ	0.04	0.003		UNIQ	0.08	-		UNIQ	0.09	0.006
		SKAT-O	0.06	-		SKAT-O	0.05	-		SKAT-O	0.008	-
VWF	147 [b]	BURDEN	0.21	0.019	72 [b]	BURDEN	0.31	0.032	35 [b]	BURDEN	1	0.200
		C-ALPHA	0.26	0.024		C-ALPHA	0.10	0.008		C-ALPHA	0.06	0.005
		SUMSTAT	0.5	0.024		SUMSTAT	0.30	0.008		SUMSTAT	0.08	0.005
		VT	1	0.024		VT	0.64	0.008		VT	1	0.005
		SKAT	0.13	-		SKAT	0.06	.		SKAT	0.02	-
		UNIQ	1	0.200		UNIQ	1	0.200		UNIQ	1	0.200
		SKAT-O	0.23	-		SKAT-O	0.14	-		SKAT-O	0.04	-

Table 3 (continued). The association between *ADAMTS13, VWF* and *FVIII* rare variants and DVT assessed using different gene-based tests

	Rare variants				Potential Damaging [a] mutations				CADD > 20			
Gene	N	Test	P	I	N	Test	P	I	N	Test	P	I
F8	34	BURDEN	0.71	0.167	11	BURDEN	1	0.200	3	BURDEN	0.83	0.200
		C-ALPHA	0.86	0.167		C-ALPHA	0.06	0.010		C-ALPHA	0.46	0.130
		SUMSTAT	0.71	0.167		SUMSTAT	0.08	0.005		SUMSTAT	0.54	0.043
		VT	1	0.167		VT	1	0.005		VT	0.38	0.174
		SKAT	0.64	-		SKAT	0.36	-		SKAT	0.35	-
		UNIQ	0.7	0.167		UNIQ	1	0.200		UNIQ	0.83	0.200
		SKAT-O	0.81	-		SKAT-O	0.31	-		SKAT-O	0.47	-

P, P value based on permutation. I, proportion of null replicates for which the best test statistic was tied. All tests were adjusted for age and sex.[a] Potentially damaging mutations include missense, frameshift mutations, deletions and insertions; N, number of variants analysed. VT= variable threshold. [b] Analyses have been done by considering variant position. Therefore, the tri-allelic variant identified in ADAMTS13 and VWF genes (n= 1 and n= 2, respectively) were not included in the count. The complete list of annotated variants is reported in the supplemental materials (**S2 Table**).

ADAMTS13 variant and the protein activity levels was assessed using linear regression adjusted for age and sex.

The first analysis was performed by considering together patients and controls carriers for rare *ADAMTS13* variants (**Table 4**).

Carriers of at least one rare variant (n= 71) showed reduced ADAMTS13 activity levels compared with non-carriers (n= 586; β -6.2, 95% CI -11, -1.5; P= 0.01). The association became stronger considering those variants potentially affecting ADAMTS13 activity (missense mutations, frameshift mutations, deletions and insertions [n= 33; β -11.0, 95% CI -18.0, -4.3; P= 0.001]) or variants potentially affecting ADAMTS13 activity with a CADD> 20 (n= 13; β -28, 95% CI -39.0, -18.0; P< 0.001). Secondly, we considered patients and controls, separately (**Table 4**). The association for patients was similar to that of the main analysis for each category of variants evaluated, with the highest association for those variants with CADD> 20 (n= 13; β -25.9, 95% CI -36, -16; P< 0.001). Conversely, no association was found in controls (n= 28; β -2.9, 95% CI -10.4, 4.5; P= 0.4).

DISCUSSION

In the last years, the role of the *ADAMTS13* gene as a genetic risk factor for both arterial and venous thrombosis has been explored. Different authors reported the existing link between *ADAMTS13* variants and stroke or other cardiovascular diseases [22, 23], whereas our group was the first to describe an excess of rare *ADAMTS13* variants in a small group of 94 DVT patients and 98 controls. Furthermore, we showed that patients carrying at least one rare ADAMTS13 variant had lower ADAMTS13 activity levels than non-carriers [8].In the present study, we aimed to confirm our previous results in a larger population of 594 Italian DVT patients and 571 controls, focusing on *ADAMTS13* along with *VWF* and *F8* genes. We found a total of 320 variants which included 272 rare and 48 low-frequency/common variants mainly located in *ADAMTS13* and *VWF*, whereas those in *F8* were only a few. Similar to our previous study, DVT patients showed an excess of rare ADAMTS13 variants than controls (61 vs. 41). These variants spanned across the gene, without highlight clusters [8].

A total of 15 rare SNVs were predicted as potentially damaging with CADD > 20 (**S2** and **S4 Tables**). Of them, 2 have already been identified in our previous studies and their effect on ADAMTS13 activity was confirmed by *in vitro* expression studies [9]. In addition, 1 DVT patient carrying the p.P1218C mutation showed an ADAMTS13 activity slightly below the normal range (42% vs. 45%). The association between rare variants and DVT was tested using different cumulative association methods [14–20]. All rare ADAMTS13 variants were associated with DVT using the burden and VT tests and the association was maintained even after restriction analyses to potentially damaging variants or potentially damaging variants with CADD > 20. The restriction analyses results were also confirmed by SKAT-O, a powerful method that combines the assumptions of unidirectional and bi-directional variance component methods. Differently, UNIQ showed a significant

Table 4. Association between rare *ADAMTS13* variants and ADAMTS13 activity levels in the whole population and after stratifying on case-control status

	Carriers		Non Carriers		Linear Regression [a]	
Variant Class	All, n	ADAMTS13 Activity (%)	All, n	ADAMTS13 Activity (%)	ß (95% CI)	P
All rare	71	91 (80-106)	586	96 (84-109)	-6.2 (-11.0, -1.5)	0.01
Potential damaging [b]	33	89 (71-100)	624	96 (84-109)	-11.0 (-18.0, -4.3)	<0.001
CADD> 20	13	63 (58-81)	644	96 (84-109)	-28.0 (-39.0, -18.0)	<0.001
Variant Class	Cases, n	ADAMTS13 Activity (%)	Cases, n	ADAMTS13 Activity (%)	ß (95% CI)	P
All rare	43	90 (72-98)	322	95 (81-108)	-7.5 (-13.0, -1.5)	0.014
Potential damaging [b]	24	82 (60-94)	341	94 (82-108)	-14.5 (-22.0, -6.8)	<0.001
CADD> 20	13	63 (58-81)	352	94 (82-108)	-25.9 (-36.0, -16.0)	<0.001
Variant Class	Controls, n	ADAMTS13 Activity (%)	Controls, n	ADAMTS13 Activity (%)	ß (95% CI)	P
All rare	28	98 (85-112)	264	98 (86-111)	-2.9 (-10.4, 4.5)	0.4
Potential damaging [b]	9	100 (86-114)	283	98 (86-111)	-2.4 (-10.0, 15.0)	0.7
CADD> 20	0	-	292	98 (86-111)	-	-

Analyses have been performed by considering at first all carriers of at least one rare ADAMTS13 variant and then by dividing carriers in DVT patients (cases) and controls. ADAMTS13 activity (expressed as percentage) was reported as median and interquartile range. Linear regression results were reported as beta coefficients (ß) and 95% confidence intervals (CI). [a] The model was adjusted for age and sex. [b] Potentially damaging mutations include missense mutations, frameshift mutations, deletions and insertions.

association only when used to test all rare ADAMTS13 variants, whereas SUMSTAT never showed any association. These inconsistencies can be related to the limits of the tests used. Indeed, both UNIQ and SUMSTAT have lower mean power than burden and VT tests [24]. The finding of an association with burden and VT (unidirectional methods), but not with C-ALPHA and SKAT (bi-directional variance- component methods) are probably due to an excess of deleterious variants. Indeed, burden and VT tests are most powerful when the majority of rare variants have the same "unidirectional" effect (i.e., deleterious), whereas SKAT and C-ALPHA are mostly powered when both neutral and deleterious variants are present [18, 19, 24].

DVT patients carrying at least one rare *ADAMTS13* variant showed a reduction of ADAMTS13 activity levels, which was more pronounced considering variants predicted to be damaging (ß -25.9%, 95% CI -36.0, -16.0), in agreement with our previous study [8]. Conversely, controls carrying at least one rare *ADAMTS13* variant showed a slight and non-statistically significant reduction of ADAMTS13 activity levels compared with non-carriers. Unfortunately, none of the 5 controls carrying a rare potentially damaging variant with CADD> 20 had available plasma samples. As final step, to exclude that the association was biased by additional factors such as ADAMTS13 consumption during DVT event or drug administration, we repeated the analyses considering the 167 DVT cases whose plasma sample were collected≥ 3 months from the acute event and/or not during anticoagulant therapy confirming our results (**S3 Table**).

The use of cumulative tests to assess the association between rare *VWF* variants and DVT did not show any association by considering all rare *VWF* variants or by restricting the analysis to potentially damaging variants. Only SKAT and SKAT-O showed a significant association after restriction to potentially damaging variants with CADD> 20. These results were unexpected as both methods should be optimal to test a mix of neutral and deleterious variants (i.e., before restriction analyses). However, the negative results obtained with the other methods, independently from the group of variants tested, led us to exclude an association between rare *VWF* variants and DVT. These findings, together with the absence of rare *F8* variants associated with DVT, led us to speculate that the increased VWF and FVIII levels measured in DVT patients could be due to other mechanisms such as the presence of mutations in regulatory regions or other genes involved in the VWF clearance [25–27].

Differently from our previous study, we identified 9 low-frequency/common variants potentially associated with DVT (P< 0.05) as the adjustment for multiple testing resulted in a high FDR (between 0.06 and 0.24). Of them, 7 were previously reported as *ADAMTS13* polymorphisms and showed a protective effect. This was partially explained considering that two groups of SNVs were in high linkage disequilibrium and showed the same association signal ([rs28641026, rs28503257; OR 0.53, 95% CI 0.31–0.89] and [rs3124768, rs3118667, rs739469; OR 0.78, 95% CI 0.67–0.92]). The effect of these SNVs was previously evaluated using *in vitro* expression studies (with the exception of

the intronic variant rs739469) and none of them showed a severe reduction of ADAMTS13 activity levels [28, 29].

The remaining 2 SNVs localized in *VWF*, showed opposite effects and inconsistencies with previous reported studies. The rs1800382 showed the strongest association with DVT (OR 3.26, 95% CI 1.18–8.98), whereas it was previously described in VWD patients as responsible for a collagen type IV binding defect, hence resulting in a prolonged bleeding time instead of a thrombotic effect [30]. However, we cannot exclude that rs1800382 is in linkage disequilibrium with another *VWF* defect located in a region not covered by our study design, such as deep intronic or regulatory regions. The rs7962217 showed a protective effect. Nevertheless, its role is still uncertain because reported as associated with increased Factor VIII coagulant activity levels, but not with VWF antigen levels in a larger cohort of non-European Americans and African-Americans subjects [31]. Of the low-frequency/common variants identified, only three were located in F8 and none of them was associated with DVT. This was in agreement with previous findings, demonstrating that increased FVIII levels were not due to the presence of common polymorphisms in F8 [32].

This study could suffer from some limitations such as the relatively small sample size. However, the choice of striking criteria finalized to exclude the DVT patients who already had known genetic (e.g., FV Leiden and prothrombin G20210A mutations) or environmental risk factors for DVT, contributed to magnify the power. A second limitation could be related to the sequencing approach that was focused on the coding regions avoiding the identification of variants potentially located in the regulatory or intronic regions. Third, ADAMTS13 activity measurements were available for a part of the enrolled subjects. Finally, we cannot assume the generalizability of our findings in other different populations, as commonly reported in genetic studies.

In conclusion, rare *ADAMTS13* variants were associated with an increased risk for DVT. We confirmed that patients carrying a rare *ADAMTS13* variant had lower ADAMTS13 activity levels than non-carriers. These findings support the hypothesis that rare *ADAMTS13* variants may increase the DVT risk thru a mechanism that includes the reduction of ADAMTS13 activity. Conversely, 8 out of 9 top SNVs with low/common frequency showed a protective effect, although their association with DVT needs to be confirmed. Rare *VWF* and *F8* variants were not associated with DVT risk, suggesting that the increased plasma VWF and FVIII levels are caused by other mechanisms.

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SUPPLEMENTARY DATA

The supporting information listed below are available at the following web address: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0258675#sec016

S1 Table. Common variants identified in Italian DVT patients and controls.

S2 Table. Rare variants identified in Italian DVT patients and controls.

S3 Table. Association between rare ADAMTS13 variants and ADAMTS13 activity levels in DVT patients whom blood sampling was performed not during treatment and at least three months after the DVT event.

S4 Table. DVT patients and controls carrying one out of 15 potentially damaging ADAMTS13 SNVs with CADD> 20.

CHAPTER 9

GENERAL DISCUSSION AND PERSPECTIVES

This thesis discusses the role of mutated *VWF* as the cause of VWD, a common bleeding disorder caused by mutations within *VWF* and it also explores VWF's role in the pathogenesis of deep vein thrombosis (DVT) with a particular interest on the ADAMTS13-VWF equilibrium.

PART I: GENETICS OF VON WILLEBRAND DISEASE

VWF is located on chromosome 12 and spans about 178 Kilobases. It is composed of 52 exons encoding for a 2813 amino acids (aa) protein that includes a signal peptide (22 aa), a propeptide (741 aa) and the VWF (2050 aa). Each VWF monomer (2050 aa) is made up of conserved domains arranged in a specific order (D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C6-CK) [1]. The mature VWF molecules are obtained through a complex mechanism that includes monomer dimerization, dimer multimerization and several post-translational modifications (O- and N- glycosylation, sialylation, sulfatation and propeptide cleavage [2, 3]). Then, the VWF can be stored in the Weibel Palade bodies (WPBs) within endothelial cells, in platelets' α-granules or it can be released in the circulation [2, 3].

VWF mutations are responsible for VWD, an inherited bleeding disorder. The classification of VWD distinguishes between VWF quantitative defects that can be partial or complete (type 1 and type 3 VWD, respectively) and qualitative defects that may affect different VWF function (type 2 VWD) [1].

Type 1 *VWF* variants are mostly missense, although other genetic defects have been identified such as small insertions and deletions, splice variants and nonsense mutations. These variants act following three main mechanisms: reduced VWF synthesis, increased intracellular VWF retention and increased VWF clearance or a combination thereof [1]. Type 3 VWD is mainly due to null variants leading to a complete lack of VWF, whereas missense variants are less common.

Differently from type 1 and type 3 variants, that may be found across the whole gene without a specific localization, the qualitative type 2 variants show a target distribution in specific VWF domains. Type 2A can be considered the more complex subtype as mutations can be localized in different functional domains, although they all result in an altered multimeric pattern [4]. These variants are mostly found within the A2 domain, but they can be also found within D1-D3 and CK domains [5, 6]. Type 2B variants are responsible for an increased affinity for the GPIbα receptor and are localized in the A1 domain [7]. Type 2M variants cause a decreased VWF affinity for GPIbα and are localized in the A1 domain, however in some cases the variants cause a collagen binding defect and are located in the A3 domain [8, 9].

Contrary to the other type 2 variants, those causing type 2N are inherited recessively. These variants impair the capacity of VWF to bind FVIII and can be identified in a small portion of *VWF* corresponding to the D' and part of D3 domains [5].

Performing *VWF* genetic analysis can be a valid support to corroborate the VWD diagnosis. The diffusion of new genotype techniques such as next-generation sequencing (NGS) represents a huge advantage, especially for type 1 and 3 defects. On the other

hand, these techniques also led to the identification of a large number of *novel* variants with an unconfirmed causal role [10].

In silico tools can be helpful to predict the possible pathogenic role of these variants. To date, several tools based on different algorithms have been freely released and they can be applied to either single nucleotide variants (SNVs) or they permit testing if a variant may impact the mRNA causing alternative splicing. Since these tools suffer from specific weaknesses that may affect the prediction results, it is recommendable to apply different tools and combine the obtained results [10]. However, in spite of their utility in filtering potentially damaging variants, they are not affirmative for pathogenicity and other approaches are required to proof variants' effect.

From heterologous systems to endothelial colony forming cells (ECFCs)

The use of heterologous systems represents the most common approach to evaluate the pathogenic effect of a *novel* variant. Briefly, the expression vector containing the wild-type (WT) or mutant VWF (obtained by site direct mutagenesis) cDNA are transiently transfected in non-endothelial cell lines. Then, the respective recombinant (r) proteins released in cell media or present in cell lysates are collected and characterized [11]. So far, different cell lines characterized by different properties, have been used to study VWF. Among all, Human Embryonic Kidney (HEK) 293 cells are those preferred due to their capacity to form pseudo-WPBs, allowing for evaluation of VWF structure, its storage within cells, and its secretion [12, 13].

The extensive application of this method raised some limitations that result in discrepancies between patients' phenotypes and *in vitro* results such as: (i) the overexpression of recombinant (r)VWF due to vectors' strong promoter; (ii) the impossibility of completely reproduce the synthesis and secretion pathways and (iii) the difficulty to reproduce patients' heterozygous state by co-transfecting the same amount of WT and mutant expression vectors. Despite all, heterologous systems still contribute to increase the knowledge about the mechanisms responsible for VWF synthesis, storage and secretion as reported in Chapters 3 and 4 of this thesis.

A valid alternative approach is the use of ECFCs, previously referred as blood outgrowth endothelial cells (BOECs) [14]. These cells can be isolated from the peripheral blood mononuclear cells by performing a density gradient [15, 16]; they show the classical cobblestone morphology typical of endothelial cells, express endothelial markers (CD31, CD34, CD51/61, CD144, CD146, CD309) and form WPBs [17]. ECFCs can be isolated from VWD patients allowing to study naturally mutant VWF [18] overcoming the limitation of the heterologous systems mentioned above, and they can be used to evaluate other VWF functions such as its role as an angiogenesis regulator [19].

Even in this case, some limitations have been identified, starting from the variable isolation rate that has been recently resized from 80% [16] to 50% [20]. Moreover, the studies so far published showed variability of ECFCs parameters including ECFCs

morphology, VWF expression and angiogenic properties, [19-21] highlighting the necessity to reach a consensus either on both ECFCs specific characteristics as well as the methods used to characterize these cells [19, 20].

Nevertheless, ECFCs represents a promising research tool in the field of personalized treatments such as the selective inhibition of the mutant allele using small-interfering (si) RNA [22] or to perform VWF CRISPR editing [23].

PART II: TYPE 3 VON WILLEBRAND DISEASE: RESULTS FROM THE 3WINTERS-IPS

Type 3 VWD is a recessively inherited bleeding disorder due to the complete absence of VWF [1, 24, 25]. Despite the rarity of this disorder, with a reported prevalence between 0.1 and 5.3 per million [26], the severe clinical manifestations along with the requirements of replacement therapy and the related complications such as inhibitor development and anaphylactic reactions make it extremely relevant from the clinical point of view.

The 3WINTERS-IPS is the largest cohort of type 3 patients so far collected that includes 265 patients with a previous diagnosis of type 3 VWD who were enrolled in Europe and Iran. At enrolment, patients underwent blood sampling and a bleeding questionnaire was administered. Blood samples were used to confirm phenotype and genotype diagnosis, and by the bleeding questionnaire their bleeding history was evaluated. The evaluation of patients' bleeding phenotype was compared to that of type 1 VWD patients enrolled in the frame of another study, the Molecular and Clinical Markers for the Diagnosis and Management of type 1 von Willebrand Disease (MCMDM-1VWD) [27].

As expected, type 3 VWD patients showed a more severe bleeding manifestation than type 1 VWD patients, with a bleeding score that increased with age. In addition, some bleeding manifestations such as central nervous system bleeding seem to be typical of type 3 patients, whereas cavity bleeding, hemarthroses, and deep hematomas were found to be from 7 to 10-fold most frequently reported than in type 1 VWD patients.

The authors also evaluated if some bleeding symptoms may cluster together resulting in non-random bleeding patterns. The results showed two patterns, one including hemarthroses, gastrointestinal bleeding and epistaxis, whereas oral cavity bleeding was reported together with post-surgical and post-extraction bleeding [27]

The 87% of type 3 patients enrolled in the 3WINTERS-IPS have been genotyped using NGS in combination with Sanger sequencing and multiplex ligation-dependent amplification techniques. The overall analyses showed that European and Iranian type 3 patients have different genotypes and a different variant distribution throughout the gene. Indeed, most of the variants found in Iranian patients were located at the NH2 terminal of the pro-VWF, whereas the variants found in European patients span across the pro-VWF [28, 29]. These data were in line with previous studies on Indian, French and the one recently published on American populations indicating that geographical localization and consanguinity played a role [29-31]. In most of the cases, null alleles

or putative null alleles have been found (79%), whereas the remaining 21% consist of missense variants mainly found among European patients. Of them, about one-half of these variants cause the loss of cysteines suggesting a deleterious effect. A small portion of patients had incomplete or inconclusive genotyping, probably due to technical limitations that do not allow for the identification of variants localized in deep intronic regions or gene conversion [28].

The 3WINTERS-IPS cohort of patients has been described in two chapters of this thesis. At first, we focused on the evaluation of VWFpp over VWF:Ag and FVIII:C over VWF:Ag ratios to explain the pathophysiological mechanism underlying type 3 VWD (Chapter 5). This approach was based on previous studies performed on type 1 VWD patients reporting that the VWFpp/VWF:Ag ratio can be used to attribute the reduction of VWF levels to a reduced secretion and/or increased clearance from circulation. Similarly, the FVIII:C/VWF:Ag was indicative of a reduced VWF synthesis [32].

We found out that the VWFpp/VWF:Ag ratio was significantly higher in type 3 patients carrying missense variants than in those carriers for nulls alleles, indicating that the remaining circulating VWF in patients with missense variants has an increased clearance and suggesting that the reduced VWF level was at least partly due to an increased protein clearance. Therefore, VWFpp/VWF:Ag ratio may also be used to discriminate patients carrying missense variants from those with null defects. Differently, FVIII:C/VWF:Ag ratio was similar among all patients, independently of the type of genetic defect identified, thus in type 3 VWD this FVIII:C/VWF:Ag ratio cannot be used to identify a defective VWF synthesis [33].

Type 3 VWD treatment requires the use of plasma derived concentrates containing VWF and different amounts of FVIII or recombinant VWF. The use of these products may be responsible for serious side effects such as the development of VWF inhibitors, which make the treatment ineffective, and the occurrence of anaphylactic reactions in a minor percentage of patients [34,35]. Up to now, the available data were limited and mainly related to small groups or single type 3 patients. Moreover, the identification of VWF inhibitors is complicated by the limited accessibility of the laboratory tests used to detect them and because of the lack of a standardized method [36]. Based on this background, we assessed the prevalence of alloantibodies in the cohort of 3WINTERS-IPS as reported in Chapter 6. The type 3 patients from 3WINTERS-IPS cohort were tested for the presence of VWF alloantibodies. Of them, 8.4% were found to be positive for alloantibodies using an indirect ELISA able to detect all alloantibodies against VWF. The detection of alloantibodies with inhibitory effect was performed with a modified version of the Bethesda assay using VWF:CB ELISA assay and resulted in a prevalence of 6% inhibitors. Subsets of samples have been evaluated with two other Bethesda-based methods. Samples positive for non-neutralizing anti-VWF antibodies were tested with a Bethesda-based method using the semi-automated gain-of-function glycoprotein-Ib binding (VWF:GPIbM) assay. Patients positive for VWF:GPIbM inhibitors also had VWF:CB inhibitors, but one only had

VWF:GPIbM inhibitors. These results point out that the estimation of inhibitor prevalence is related to the type of assay performed (i.e., by the different epitopes recognized by alloantibodies). The Bethesda assay using VWF:Ag ELISA was limited to samples positive for VWF:CB inhibitors, and was able to detect VWF:Ag inhibitors in half of the patients highlighting its poor sensitivity.

The inhibitor data were also interpreted based on the genotype characterization confirming that they are mainly found in patients carrying null alleles [37-39]. Nevertheless, not all carriers of the same variants developed VWF inhibitors thus confirming a variable penetrance of inhibitor development [40].

Altogether, the data obtained from the 3WINTERS-IPS allowed us to highlight new information about the pathogenesis of type 3 VWD and corroborate the previously reported data obtained on smaller populations. The information that will stem from the ongoing perspective phase of this project, will lead to a better knowledge of type 3 VWD starting from the diagnostic process to the management of patients' treatment.

PART III: ROLE OF VON WILLEBRAND FACTOR IN DEEP VEIN THROMBOSIS

Deep vein thrombosis is a common life-threatening disorder that can be caused by environmental, behavioral and genetic risk factors [41]. However, part of its etiology remains unclear.

In this part of the thesis, we evaluated the equilibrium existing between the metalloprotease ADAMTS13 and VWF as a possible mechanism involved in DVT onset. This hypothesis is plausible considering that in physiological conditions, ADAMTS13 cleaves VWF multimers at the cleavage site in the A2 domain reducing its size and as consequence its procoagulant activity [42]. The alteration of ADAMTS13 levels is the cause of thrombotic thrombocytopenic purpura (TTP), a thrombotic microangiopathy characterized by VWF-mediated platelet microthrombi [43]. Moreover, the synergistic effect of low ADAMTS13 activity and high VWF levels were reported to be associated with myocardial infarction and coronary artery disease in a meta-analysis [44].

Initially, high VWF levels were found to be associated with arterial thrombosis [44-46], whereas their role in venous thrombosis started to be considered later. Moreover, most of these studies refer to venous thromboembolism which includes both DVT and pulmonary embolisms.

In 1995, Koster et al., were the first to describe that increased VWF levels were associated with an increased risk of VTE [47]. Over the years, these results have been replicated in different case-control studies. Recently, Rietvield et al. [48], showed that the association of VWF and FVIII with VTE was stronger than that found considering thrombin, FVII, FIX, FX and FXI, in a large case-control study. The same authors also reported that the increased VWF and FVIII levels are stable over time, contributing to sustains a direct causal effect [48]. Indeed, most of the studies evaluating VWF and FVIII

association with DVT, as well as ours, are case-control studies in which sample collection occurred after the thrombotic event. Therefore, the causality of the association cannot be proven.

Further confirmations also derive from prospective studies showing that the increased levels of VWF and FVIII are also associated with an increased dose-dependent risk of recurrent VTE [49]. This association was stronger for those patients with unprovoked VTE [50].

Even in our study, we found an association between increased VWF and FVIII levels with DVT (Chapter 7). We also showed that a moderate decrease in ADAMTS13 activity was sufficient to increase the risk for DVT in comparison to controls. However, the most interesting result was obtained by analysing the combined effect of low ADAMTS13 activity levels and high VWF levels that lead to a dramatic 15-fold increase in DVT risk. This latter finding suggested that ADAMTS13 and VWF act synergistically and even a small alteration of this equilibrium is enough to contribute to DVT risk [51].

To date, the role of ADAMTS13 levels has been evaluated in a few studies, involving smaller populations of VTE patients [52-54]. Of them, two were in line with our results, although they measure ADAMTS13 antigen levels instead of activity [53-54]. Differently, another study refers to high ADAMTS13 and VWF levels in presence of high inflammatory markers for a long time after VTE events [52]. This information was not available in our study population and therefore a proper comparison cannot be made.

The genetic component of DVT

DVT and more in general VTE have a strong genetic component that accounts for 50-60% of the hereditability, although part of it remains unexplained [55,56]. Most well-known genetic risk factors are factor V Leiden (FVL), prothrombin G20210A mutation, fibrinogen gamma chain C10034T mutation and the deficiencies of natural anticoagulants proteins (antithrombin, protein C and protein S) [57].

Large genome-wide association studies (GWAS) and meta-analyses contributed to identify *novel* common variants in hemostasis-related genes, even if they were performed in populations of VTE patients rather than DVT subjects alone [58,59]. Among them, different common variants on *VWF* and other genes involved in the modification of its plasma levels have been reported. Sabater-Lleal et al., performed a meta-analysis of GWAS results from 46354 individuals of European, African, East Asian, and Hispanic ancestry and identified 13 *novel* loci associated with FVIII and VWF levels. The same authors further evaluated the association between VWF level and VTE performing two-sample mendelian randomization in the population of 7507 VTE cases and 52632 controls enrolled in the frame of the INVENT consortium, confirming a causal role of VWF levels [60].

GWAS are not able to identify rare variants and therefore a different approach is required. Desch et al. [61], performed a whole-exome study of 393 individuals with unprovoked VTE and 6114 controls. Results showed an excess of rare variants in *PROS1*, *STAB2*, *PROC*, and *SERPINC1* in VTE patients than in controls. Then, the authors focused

on *STAB-2* because of its role in the clearance of VWF and *in vitro* characterized 7 missense variants, proving that they are responsible for a reduced expression of STAB-2 protein on the surface of endothelial cells. Patients carrying one of these variants had higher VWF levels than non-carriers. Taken together, these data support that the impaired VWF clearance may contribute to increased VWF levels [61].

In our study, reported in Chapter 8, we used the NGS to sequence the coding region and intron/exon boundaries of *ADAMTS13, VWF* and *F8* in a population of DVT cases and controls. The evaluation of common/low frequency variants showed inconsistent results. Only 9 common/low frequency variants were found to be associated with *DVT*, 7 in *ADAMTS13* and 2 in *VWF*. Nevertheless, the adjustment for multiple testing resulted in high false discovery rate. Instead, we confirmed the association between rare *ADAMTS13* variants and DVT [62], as previously reported by Lotta et al., [63]. Moreover, patients carrying at least one rare *ADAMTS13* variant showed lower ADAMTS13 activity than non-carriers. Conversely, there was no association between rare *VWF* and *F8* variants and DVT [62]. These results together with those already discussed above may further suggest that the defective VWF clearance is an adjunctive mechanism underlying the increased VWF levels.

Targeting VWF as a therapeutical approach in thrombosis

VWF represents an attractive target in the treatment and prevention of both arterial and venous thrombosis. This may be achieved through the regulation of VWF levels including its synthesis or modulating VWF interaction with specific targets such as GPIb, collagen and ADAMTS13.

Conventional drugs routinely used for other scopes may be taken into account as they unselectively target VWF. Heparin, usually administrated as an anticoagulant, also exerts an anti-platelet effect blocking the interaction between the A1 domain and GPIb [64].

A meta-analysis showed that statins, lipid-lowering drugs, may reduce VWF levels, although their use as anti-thrombotic agents is still debated [65]. N-acetylcysteine used to reduce mucins multimer size for the treatment of chronic obstructive pulmonary disease can also reduce UL-VWF multimers by reduction of the intrachain disulfide bonds. In addition, experiments performed in ADAMTS13 deficient mice demonstrated that N-acetylcysteine can rapidly reduce thrombus size, suggesting a potential use for TTP treatment [66].

Among the proposed specific target, there is the regulation of WPBs size. Indeed, smaller WPBs correspond to shorter VWF strings characterized by a reduced capacity to recruit and bind platelets on the endothelial cell surface. Recently, Ferraro et al., evaluated the capacity of 37 compounds to reduce WPBs size performing *in vitro* studies on HUVEC cells. These authors showed that both the pharmacological and physiological (e.g., hyperglycemia) status should be monitored and considered in a way to control VWF pro-coagulant activity [67].

Another class of potential drugs consists of specific VWF antagonists, as they may have the potential to reduce therapy side effects. Different monoclonal antibodies targeting VWF-GPIb have been tested in animal models. Results showed a good antithrombotic action, with limited side effects in terms of bleeding and thrombocytopenic [64].

Aptamers are single-stranded DNA/RNA oligonucleotides characterized by a specific and stable three-dimensional structure that allows for the recognition of a target with high specificity. Aptamers targeting VWF-GPIb interaction or VWF–collagen binding have been proposed to treat other thrombotic disorders [68,70]. Among them, the aptamer BT200 which inhibits VWF platelet dependent function has been shown to prevent arterial occlusion in non-human primates [71].

Nanobodies are small functional fragments of single-chain antibodies with a potential application in several fields. Of them, Caplacizumab, approved in 2018 for the treatment of TTP, specifically binds the VWF-A1 domain and thereby inhibits VWF-GPIb interaction [72]. However, there are no data about application of nanobodies in VTE.

The administration of rADAMTS13 may also be considered. Positive results have been reported in preclinical studies in TTP patients [73], whereas mice models of ischemic stroke highlighted a protective effect from inflammation and ischemic damages [74]. However, at the moment there is no information regarding their application to treatment of venous or arterial thrombosis.

CONCLUSIONS AND REMARKS

The increasing use of next-generation sequencing approaches simplified the genotyping of *VWF* in the frame of the VWD diagnostic process and led to the identification of several *novel* VWF variants with an unconfirmed causal role. In this thesis, we showed that *in silico* tools and conventional heterologous systems still contribute to evaluating the effect of variants, thus explaining patients' phenotype. More in general, we showed that the combination of phenotype and genotype tests continues to be necessary to properly classify VWD patients.

In the second part of this thesis, we focused on the characterization of type 3 VWD using conventional phenotyping tests to evaluate the mechanisms at the basis of the most severe form and we evaluated the prevalence of potential side effects due to replacement therapy. These data along with those generated in the frame of the 3WINTERS-IPS study add information on the characterization and management of type 3 VWD.

In the last part of this thesis, VWF has been evaluated as a risk factor for DVT. We found that a modest variation of the equilibrium between ADAMTS13 activity and VWF antigen levels increases the DVT risk. While the modest reduction of ADAMTS13 activity may be explained by the excess of rare *ADAMTS13* variants found in DVT patients, rare *VWF* and *F8* variants do not seem to play a role. The results reported by other authors suggested that the increased VWF levels may be partially due to variants localized in genes

involved in VWF clearance. Nevertheless, the comprehension of underlying mechanisms responsible for DVT and more in general in venous thrombosis requires further effort to be elucidated.

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CHAPTER 10

ENGLISH SUMMARY
SAMENVATTING
SOMMARIO

ENGLISH SUMMARY

von Willebrand factor (VWF) is a multimeric glycoprotein mainly known to be involved in primary hemostasis recruiting platelets at the site of damaged vessels and acting as factor VIII (FVIII) carrier.

Quantitative or qualitative alteration of VWF protein is responsible for von Willebrand disease (VWD). Conversely, increased VWF levels have been found to be associated to different thrombotic disorders such as arterial thrombosis, ischemic stroke and venous thromboembolisms. The dual role of VWF in both bleeding and thrombosis, with a focus on VWD and deep vein thrombosis (DVT), are discussed in this thesis.

In the first part, we described the approach used to perform VWD diagnosis, including the use of *in silico* tools and heterologous cell systems to confirm the disease-causing role of VWF variants.

In **Chapter 2**, we described the approach used to perform a differential diagnosis between type 2A and 2B VWD in a pediatric patient carrying a *de novo* and novel variant. The biochemical characterization allowed us to exclude type 2M due to a loss of high molecular weight multimers and reduced VWF:CB. The finding of a *novel* deletion, despite its localization in the A2 domain, did not allow for discrimination between type 2A and 2B VWD. The conventional approach would require to perform the ristocetin-induced platelet aggregation (RIPA) to discriminate type 2B VWD from the other type 2. This assay needs a relatively large amount of fresh blood, which is especially undesirable in the case of a pediatric patient. Therefore, we opted for an alternative approach using a platelet-dependent VWF activity (VWF:GPIbM) ELISA. This assay is able to discriminate type 2B VWD patients from the other type 2 VWD patients and it can be done using a small amount of frozen plasma sample. This led us to confirm that our patient was affected by type 2A VWD.

In **Chapters 3** and **4**, we combined the use of predictive *in silico* tools and heterologous cell systems to prove the pathogenic role of the VWF variants identified in our VWD patients. In both chapters, *in vitro* expression studies have been performed by transient transfection of wild type and mutant expression vector into HEK293 cells. In **Chapter 3**, we evaluated the effect of the previously reported type 1 variant p.Arg1379Cys, identified in five unrelated patients. Of them, one was diagnosed as affected with type 1 VWD, whereas the other four had a type 2M diagnosis. These latter patients also carried a polymorphism p.Ala1377Val, which role has been initially underestimated. The *in silico* evaluation showed that both variants destabilize the A1 domain, leading us to hypothesize a synergistic effect resulting in a decreased capacity of VWF to bind GPIb. Then, we tested the capacity of the wild-type, mutant, and hybrid recombinant (r) VWF to bind recombinant glycoprotein Ibα (rGpIbα) in presence of an increasing concentration of ristocetin. In this way, we were able to confirm that the synergistic effect exerted by these two variants was responsible for patients' type 2M phenotype.

Chapter 4 described the characterization of two unrelated Italian patients diagnosed as affected by type 1 VWD. Both were found to be heterozygous carriers for the same *novel* variant p.Thr274Pro localized in the VWF propeptide (VWFpp). This variant showed a dominant negative effect in contrast with other previously reported variants found nearby it, which have been described to be responsible for type 3 or type 2A/IIC VWD.

Therefore, we decided to perform an *in vitro* study including immunofluorescence with the aim to elucidate the disease-causing mechanism. Our results showed that the p.Thr274Pro was responsible for patients' phenotypes through a combined mechanism including defective synthesis, secretion and an impaired multimerization process.

The second part of this thesis focused on the characterization of type 3 VWD, the rarest and most severe manifestation of this bleeding disorder. In **Chapter 5** we evaluated if the VWFpp over VWF antigen (VWF:Ag) ratio and FVIII coagulant activity (FVIII:C) over VWF:Ag ratio can be used to determinate the pathophysiological mechanism of this disease. We showed that the VWFpp/VWF:Ag ratio was able to identify homozygous/compound heterozygous carriers for a missense variant as already described for type 1 VWD patients. Moreover, it also indicated that the extremely reduced VWF levels measured in these patients were at least partly due to a faster VWF clearance from the circulation. On the contrary, FVIII/VWF:Ag ratio failed to discriminate carriers for null defects from those carrying missense variants.

Treatment for type 3 VWD patients includes the administration of concentrates containing VWF or rVWF. The development of VWF alloantibodies and/or anaphylactic reactions are rare but important side effects of replacement therapy administrated to type 3 VWD patients.

In **Chapter 6**, the prevalence of alloantibodies against VWF in the 3WINTERS-IPS cohort has been assessed. Because of the lack of a gold-standard, we chose to carry out an indirect ELISA assay able to detect all anti-VWF antibodies and a Bethesda-based method using VWF:CB. Anti-VWF alloantibodies were found in 8.4% of the study population. We confirmed that the development of neutralizing antibodies represents a rare event with a prevalence of 6% and it is mainly found in type 3 patients homozygous for null defects. Two other Bethesda-based methods using either VWF:GPIbM or VWF:Ag ELISA were performed in subgroups of patients. Both methods were able to identify VWF inhibitors in a lower number of patients. However, the Bethesda-based method using VWF:GPIbM identified a further patient who tested negative for VWF:CB inhibitors. Taken together, these data confirmed that prevalence estimation is strongly affected by the epitope recognized by the alloantibodies and therefore by type of assay used.

Last part of the thesis aimed to evaluate whether the reduction of ADAMTS13 activity or the alteration of ADAMTS13-VWF equilibrium may play a role in DVT pathogenesis.

In **Chapter 7**, we performed a case-control study to evaluate the association between ADAMTS13, VWF, and FVIII plasma levels and DVT. We showed that a slight decrease in ADAMTS13 activity levels was associated with a moderately increased risk for DVT,

whereas we confirmed the strong association between increased VWF:Ag and FVIII:C levels and DVT.

More interestingly, we showed that the combination of slightly reduced plasma ADAMTS13 activity levels and increased VWF levels was responsible for a markedly increased DVT risk. These latter findings were further confirmed performing a sensitivity analysis after the exclusion of patients whose samples were collected less than three months from DVT event and/or during anticoagulant therapy.

Subsequently, we decided to investigate if variants localized in *ADAMTS13*, *VWF*, and *F8* genes may contribute to explain the altered levels of the respective, encoded proteins, as described in **Chapter 8**. For this purpose, a larger population of DVT cases and healthy volunteers has been sequenced using NGS. We confirmed that rare *ADAMTS13* variants alone are associated with DVT risk. Moreover, DVT patients carrying a rare *ADAMTS13* variant had a lower ADAMTS13 activity than non-carriers. In contrast, neither rare *VWF* nor *F8* variants were associated with DVT, thus indicating that other mechanisms are responsible for increased VWF and FVIII plasma levels.

Finally, **Chapter 9** includes a general discussion and future perspectives about the topics described in this thesis.

SAMENVATTING

Von Willebrand factor (VWF) is een multimeer glycoproteïne dat vooral betrokken is in de primaire hemostase bij het aantrekken van bloedplaatjes naar de plaats van vaatbeschadiging en tevens functioneert VWF als dragereiwit voor stollingsfactor VIII (FVIII). Kwantitatieve of functionele veranderingen van VWF zijn verantwoordelijk voor de ziekte van Von Willebrand (VWD). Omgekeerd is gebleken, dat verhoogde concentraties van VWF geassocieerd zijn met verschillende trombotische aandoeningen, zoals arteriële trombose, herseninfarct en veneuze trombo-embolie. De dubbele rol van VWF bij zowel het ontstaan van bloedingen als trombose, met een focus op VWD en diep veneuze trombose (DVT), wordt in dit proefschrift besproken.

In het eerste deel van de thesis hebben we de diagnostische benadering van VWD beschreven, inclusief het gebruik van *in silico* tools en heterologe celsystemen om de oorzakelijkheid van *VWF* varianten bij het ontstaan van VWD vast te stellen.

In **Hoofdstuk 2** hebben we de diagnostische benadering beschreven hoe er onderscheid gemaakt kon worden tussen type 2A en 2B VWD bij een kind dat drager bleek van een *de novo* en niet eerder gerapporteerde *VWF* variant. Op basis van biochemische karakteristieken, namelijk het ontbreken van hoog moleculair gewicht VWF multimeren en een verlaagde VWF collageen binding (VWF:CB), kon VWD type 2M worden uitgesloten. Het identificeren van een nieuwe, nog onbekende deletie in het A2 domein, maakte het niet mogelijk om op basis van de mutatie onderscheid te maken tussen type 2A en 2B VWD. De conventionele benadering om type 2B van de andere types 2 VWD te onderscheiden vereist het verrichten van de zogenaamde ristocetine geïnduceerde plaatjes aggregatie (RIPA) test. Deze assay vereist echter een relatief groot volume vers afgenomen bloed, hetgeen met name bij kinderen minder wenselijk is. Daarom hebben we een alternatieve benadering gekozen, waarbij gebruik gemaakt is van een plaatjes-afhankelijke VWF activiteitsbepaling (VWF:GPIbM ELISA). Deze bepaling kan, met slechts een kleine hoeveelheid ingevroren plasma, onderscheid maken tussen type 2B VWD en de overige types 2. Met deze benadering konden we bevestigen dat er bij onze patiënt sprake was van type 2A VWD.

In **Hoofdstukken 3** en **4** hebben we voorspellende *in silico* modellen en heterologe celsystemen toegepast om de pathogene rol te bewijzen van VWF varianten die waren gevonden bij VWD patiënten. In beide hoofdstukken zijn *in vitro* expressie studies verricht met behulp van transiente transfectie in HEK293 cellen van wild type en mutante expressievectoren. In **Hoofdstuk 3** hebben we het effect bestudeerd van de eerder beschreven type 1 variant p.Arg1379Cys, die we gevonden hadden in vijf niet verwante patiënten. Van deze vijf patiënten was er één gediagnosticeerd als type 1 VWD, terwijl de andere vier getypeerd waren als type 2M VWD. Deze laatste vier waren ook drager van een polymorfisme, p.Ala1377Val, waarvan de bijdrage aanvankelijk was onderschat. De *in silico* analyse toonde, dat beide varianten het A1 domein destabiliseren, hetgeen ons deed hypothetiseren dat beide varianten mogelijk een synergistisch effect hebben

leidend tot een verlaagde VWF bindingscapaciteit voor GPIb. Daarna hebben we de capaciteit van wild type, mutant en hybride recombinant (r)VWF voor binding aan recombinant glycoproteïne Ibα (rGpIbα) getest in de aanwezigheid van oplopende concentraties ristocetine. Via deze aanpak waren we in staat om te bevestigen, dat het synergistische effect van de twee varianten verantwoordelijk is voor het type 2M fenotype in de patiënten.

In **Hoofdstuk 4** is de karakterisering beschreven van twee niet-verwante Italiaanse patiënten die gediagnostiseerd waren als type 1 VWD. Beiden bleken heterozygoot drager van dezelfde nieuwe VWF variant p.Thr274Pro gelokaliseerd in het VWF propeptide (VWFpp). Deze variant toonde een dominant-negatief effect in tegenstelling tot eerder beschreven varianten in hetzelfde gebied die verantwoordelijk zijn voor type 3 of type 2A/IIC VWD. Derhalve hebben we *in vitro* expressie studies verricht, inclusief immunofluorescentie, met als doel het ziektemechanisme op te helderen. Onze resultaten toonden aan, dat p.Thr274Pro verantwoordelijk is voor het fenotype van de patiënt via een gecombineerd mechanisme van verstoorde synthese, secretie en een verminderd multimerisatie proces.

Het tweede deel van dit proefschrift richtte zich op de karakterisering van type 3 VWD, de zeldzaamste en ernstigste vorm van deze bloedingsziekte. In **Hoofdstuk 5** hebben we onderzocht of de ratio van VWFpp over VWF antigeen (VWF:Ag) en de ratio van FVIII stollingsactiviteit (FVIII:C) over VWF:Ag gebruikt kunnen worden om het pathofysiologische mechanisme van de ziekte vast te stellen. We hebben laten zien, dat de VWFpp/VWF:Ag ratio homozygote/compound heterozygote dragers van een missense variant kan identificeren, zoals ook was beschreven voor type 1 VWD. Bovendien wees de VWFpp/VWF:Ag ratio er ook op, dat de sterk verlaagde VWF concentratie bij deze patiënten op zijn minst gedeeltelijk verklaard wordt uit snellere klaring van VWF uit de circulatie. Daarentegen bleek de FVIII:C/VWF:Ag ratio niet geschikt om dragers van null defecten te onderscheiden van dragers van missense varianten.

Behandeling van type 3 VWD patiënten bestaat uit de toediening van concentraten die plasma VWF of rVWF bevatten. De ontwikkeling van VWF alloantilichamen en/of anafylactische reacties zijn zeldzame maar ernstige bijwerkingen van de substitutietherapie toegepast bij type 3 VWD patiënten.

In **Hoofdstuk 6** is de prevalentie van alloantilichamen tegen VWF in het 3WINTERS-IPS cohort vastgesteld. Vanwege het ontbreken van een goud-standaard is er gekozen voor een indirecte ELISA assay die alle anti-VWF antilichamen kan detecteren en voor een Bethesda-gebaseerde methode die gebruik maakt van de VWF:CB. Anti-VWF alloantilichamen werden vastgesteld in 8,4% van de studie populatie. We hebben bevestigd, dat het ontwikkelen van neutraliserende antilichamen een zeldzame gebeurtenis is met een prevalentie van 6% en dat ze voornamelijk voorkomen bij type 3 patiënten die homozygoot zijn voor null allelen. Twee andere Bethesda-gebaseerde methodes die gebruik maken van ofwel VWF:GPIbM of VWF:Ag ELISA zijn uitgevoerd bij

een subgroep patiënten. Beide methodes waren in staat om VWF remmers te identificeren in een kleiner aantal patiënten. Echter, de VWF:GPIbM gebaseerde Bethesda-methode identificeerde een extra patiënt die negatief getest was voor VWF:CB remmers. Tezamen bevestigden deze gegevens dat de geschatte prevalentie van alloantilichamen tegen VWF sterk beïnvloed wordt door het epitoop waartegen de antilichamen gericht zijn en dat de prevalentie dus afhankelijk is van het type assay dat gebruikt wordt.

Het laatste deel van het proefschrift richtte zich op de vraag of verlaging van de ADAMTS13 activiteit dan wel verandering in het ADAMTS13-VWF evenwicht een mogelijke rol speelt in de pathogenese van DVT.

In **Hoofdstuk 7** hebben we een case-controle onderzoek verricht waarin de associatie tussen plasma ADAMTS13, VWF en FVIII concentraties en DVT is geëvalueerd. We toonden aan dat een geringe daling van ADAMTS13 activiteit geassocieerd is met een matig verhoogd risico op DVT, terwijl we een sterke associatie tussen verhoogde VWF:Ag en FVIII:C spiegels en DVT bevestigden. Interessante bevinding was dat een licht verlaagde plasma ADAMTS13 activiteit in combinatie met een verhoogde VWF spiegel geassocieerd bleek met een sterk verhoogd DVT risico. Deze bevindingen werden verder bevestigd na een sensitiviteitsanalyse waarbij patiënten werden geëxcludeerd waarvan de bloedmonsters binnen drie maanden na de DVT waren verzameld of tijdens antistollingsbehandeling.

Zoals beschreven in **Hoofdstuk 8**, hebben we vervolgens onderzocht of varianten gelokaliseerd in de *ADAMTS13*, *VWF*, en *F8* genen bijdragen aan de veranderde plasma concentraties van deze eiwitten. Met dit doel is een grotere populatie van DVT patiënten en gezonde controles gesequenced met behulp van NGS. We vonden, dat zeldzame varianten van *ADAMTS13* alleen geassocieerd zijn met risico op DVT. Bovendien hadden DVT patiënten die drager zijn van een zeldzame *ADAMTS13* variant een lagere ADAMTS13 activiteit dan patiënten zonder die variant. In tegenstelling waren noch *VWF*, noch *F8* varianten geassocieerd met DVT, hetgeen suggereert dat andere mechanismen verantwoordelijk zijn voor de verhoogde VWF en FVIII plasmaspiegels.

Tot slot bevat **Hoofdstuk 9** een algemene discussie en toekomstperspectieven betreffende de onderwerpen beschreven in dit proefschrift.

SOMMARIO

Il fattore von Willebrand (VWF) è una glicoproteina multimerica nota per essere coinvolta nell'emostasi primaria, in quanto media l'adesione delle piastrine al sito di danno vascolare, ma anche per il suo ruolo di carrier del FVIII. L'alterazione quantitativa o qualitativa del VWF causa la malattia di von Willebrand (VWD), un disordine emorragico comune. Al contrario, un aumento dei livelli di VWF è risultato essere associato a diversi disturbi trombotici, come la trombosi arteriosa, l'ictus ischemico e trombosi venosa.

In questa tesi è stato discusso il duplice ruolo del VWF sia nelle nel versante emorragico che in quello trombotico, con particolare attenzione alla VWD e alla trombosi venosa profonda (TVP).

Nella prima parte, abbiamo descritto l'approccio utilizzato per eseguire la diagnosi di VWD, compreso l'uso di strumenti *in silico* e di sistemi d'espressione eterologhi per confermare il ruolo causale delle varianti del VWF nell'insorgenza della VWD.

Nel **capitolo 2**, abbiamo descritto l'approccio utilizzato per effettuare una diagnosi differenziale tra VWD di tipo 2A e 2B in un paziente pediatrico portatore di una variante *de novo*. La caratterizzazione biochimica ci ha permesso di escludere la VWD tipo 2M data la perdita dei multimeri ad alto peso molecolare e la ridotta capacità di legare il collagene. La scoperta di una delezione non riportata in letteratura, nonostante la sua localizzazione nel dominio A2, non ha permesso di discriminare tra VWD di tipo 2A e 2B. L'approccio convenzionale prevede l'esecuzione dell'aggregazione piastrinica indotta da ristocetina (RIPA) per discriminare la VWD di tipo 2B dagli altri difetti di tipo 2. Questo test necessita di una quantità relativamente elevata di sangue fresco, soprattutto nel caso di pazienti pediatrici. Pertanto, abbiamo optato per un approccio alternativo utilizzando un ELISA per valutare la capacità del VWF plasmatico di legare la proteina GPIbα ricombinante caratterizzata dalla presenza di due mutazioni gain-of-fuction (VWF:GPIbM). Questo test, che può essere eseguito utilizzando un volume ridotto di plasma congelato, ci ha portato a confermare che il nostro paziente era affetto da VWD di tipo 2A.

Nei **capitoli 3** e **4**, abbiamo combinato l'uso di strumenti predittivi *in silico* e di sistemi eterologhi per dimostrare il ruolo patogenico delle varianti del VWF identificate nei nostri pazienti VWD. In entrambi i capitoli sono stati eseguiti studi di espressione *in vitro* mediante trasfezione transiente dei vettori di espressione wild-type (WT) e mutati in cellule HEK293. Nel **capitolo 3**, abbiamo valutato l'effetto della variante p.Arg1379Cys, precedentemente associata alla VWD di tipo 1, identificata in cinque pazienti non imparentati. Di questi, uno era stato diagnosticato come affetto da VWD di tipo 1, mentre gli altri quattro avevano una diagnosi di tipo 2M. Quest'ultimi erano anche portatori eterozigoti del polimorfismo p.Ala1377Val, il cui ruolo è stato inizialmente sottovalutato. La valutazione *in silico* ha mostrato che entrambe le varianti destabilizzano il dominio A1, portandoci a ipotizzare un effetto sinergico che si traduce in una ridotta capacità del VWF mutato di legare la GPIbα. Abbiamo quindi testato la capacità delle proteine VWF ricombinanti WT, mutati e dell'ibrido di legare la rGpIbα

ricombinante in presenza di concentrazioni crescenti di ristocetina. In questo modo, abbiamo potuto confermare che l'effetto sinergico esercitato da queste due varianti è responsabile del fenotipo di tipo 2M dei pazienti.

Il **capitolo 4** descrive la caratterizzazione di due pazienti italiani non imparentati, diagnosticati come affetti da VWD di tipo 1. Entrambi sono risultati portatori eterozigoti della stessa variante p.Thr274Pro localizzata nel propeptide del VWF (VWFpp). Questa variante ha mostrato un effetto dominante negativo in contrasto con quello delle altre varianti trovate nelle sue vicinanze, che erano responsabili della VWD di tipo 3 (recessiva) o della VWD di tipo 2A/IIC.

Pertanto, abbiamo deciso di eseguire uno studio *in vitro* che includeva immunofluorescenza allo scopo di chiarire il meccanismo che causa la malattia. I nostri risultati hanno dimostrato che la p.Thr274Pro è responsabile del fenotipo dei pazienti attraverso un meccanismo combinato che comprende i difetti di sintesi e di secrezione e un processo di multimerizzazione alterato del VWF.

La seconda parte di questa tesi si è concentrata sulla caratterizzazione della VWD di tipo 3, la forma più rara e grave di questa malattia. Nel **capitolo 5** abbiamo valutato se i rapporti tra VWFpp e antigene VWF (VWF:Ag) e tra attività coagulante del FVIII (FVIII:C) e VWF:Ag potessero essere utilizzati per determinare il meccanismo fisiopatologico di questa malattia. Abbiamo dimostrato che il rapporto VWFpp/VWF:Ag è in grado di identificare i portatori omozigoti/eterozigoti composti di varianti missenso, come già descritto per i pazienti con VWD di tipo 1. Il rapporto VWFpp/VWF:Ag suggerisce quindi che i livelli di VWF estremamente ridotti misurati in questi pazienti sono dovuti a una sua rapida eliminazione dalla circolazione. Nei pazienti tipo 1, un rapporto FVIII:C/VWF:Ag aumentato è solitamente riscontrato nei pazienti portatori di un allele nullo ed è quindi indicativo di una ridotta sintesi. Tuttavia, tutti i pazienti tipo 3 avevano un FVIII:C/VWF:Ag incrementato, ma simile indipendentemente dal tipo di difetto genetico.

La terapia dei pazienti con VWD di tipo 3 prevede la somministrazione di concentrati contenenti VWF o VWF ricombinante (rVWF). Lo sviluppo di alloanticorpi contro il VWF e/o di reazioni anafilattiche sono effetti collaterali della terapia sostitutiva rari, ma importanti. Nel **capitolo 6** è stata valutata la prevalenza degli alloanticorpi anti-VWF nella corte del 3WINTERS-IPS. A causa della mancanza di un metodo di riferimento (gold-stantard), abbiamo scelto di eseguire un test ELISA indiretto in grado di rilevare tutti gli anticorpi anti-VWF. Per identificare gli anticorpi neutralizzanti (inibitori) abbiamo scelto un metodo basato su Bethesda utilizzando il test che valuta la capacità residua del VWF di legare il collagene, il VWF:CB. Gli alloanticorpi anti-VWF sono stati riscontrati nell'8,4% della popolazione. Abbiamo confermato che lo sviluppo di anticorpi neutralizzanti rappresenta un evento raro (prevalenza del 6%) e si riscontra principalmente nei pazienti di tipo 3 portatori omozigoti di difetti nulli. I pazienti positivi per gli anticorpi anti-VWF sono stati ulteriormente testati con altri due metodi basati su Bethesda, che utilizzano rispettivamente il VWF:GPIbM e il VWF:Ag ELISA. Entrambi i metodi sono stati in grado

di identificare gli inibitori del VWF in un numero inferiore di pazienti rispetto al metodo basato su Bethesda che utilizza il VWF:CB. Tuttavia, il metodo basato su Bethesda che utilizza VWF:GPIbM ha identificato un ulteriore paziente che era risultato negativo agli inibitori del VWF:CB. Nel complesso, questi dati hanno confermato che la stima della prevalenza è fortemente influenzata dall'epitopo riconosciuto dagli alloanticorpi e quindi dal tipo di test utilizzato.

L'ultima parte della tesi mirava a valutare se la riduzione dell'attività dell'ADAMTS13 e l'alterazione dell'equilibrio ADAMTS13-VWF giocano un ruolo nella patogenesi della TVP. Nel **capitolo 7**, abbiamo condotto uno studio caso-controllo per valutare l'associazione tra i livelli plasmatici dell'ADAMTS13, VWF, FVIII e la TVP. Abbiamo dimostrato che una lieve diminuzione dei livelli di attività dell'ADAMTS13 è associata a un moderato aumento del rischio di TVP, mentre abbiamo confermato la forte associazione tra l'aumento dei livelli di VWF:Ag e FVIII:C e l'aumentato rischio di TVP.

Inoltre abbiamo provato che una leggera riduzione dell'attività dell'ADAMTS13 in combinazione con l'incremento dei livelli plasmatici del VWF porta ad un notevole incremento del rischio di TVP.

Questi risultati sono stati ulteriormente confermati dopo l'esclusione dei pazienti i cui campioni erano stati raccolti a meno di tre mesi dall'evento di TVP e/o durante la terapia anticoagulante.

Successivamente, abbiamo deciso di indagare se le varianti localizzate nei geni ADAMTS13, VWF e F8 potessero contribuire a spiegare i livelli alterati delle rispettive proteine codificate, come descritto nel **capitolo 8**. A tale scopo, una popolazione più ampia di pazienti TVP e di volontari sani è stata sequenziata mediante NGS. Abbiamo confermato che le varianti rare dell'ADAMTS13 sono associate ad un incremento del rischio di TVP. Inoltre, i pazienti con TVP portatori di una variante rara dell'*ADAMTS13* presentavano un'attività dell'ADAMTS13 inferiore rispetto ai non portatori. Diversamente, né le varianti rare di *VWF* né quelle di *F8* sono state associate alla TVP, indicando così che altri meccanismi sono responsabili dell'aumento dei livelli plasmatici di VWF e FVIII.

Infine, il **capitolo 9** comprende una discussione generale e prospettive future sugli argomenti descritti in questa tesi.

APPENDIX A

ACKNOWLEDGMENTS

CURRICULUM VITAE

PUBLICATION LIST

PHD PORTFOLIO

ACKNOWLEDGMENTS

Even this adventure has come to the end. Flora, Jeroen, and Frits thank you for giving me the support and instruments to make this dissertation possible. Flora, my thank goes beyond the work done together for this thesis. Jeroen and Frits, it has been a great opportunity to spend a year in the Netherlands, although the pandemic has changed our plans.

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Finally, I thank my family and friends. You have a lot of patience with me.

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CURRICULUM VITAE

Maria Teresa Pagliari was born on 29 April 1986 in Crema, Italy. After achieving a technical diploma in Chemistry, she moved to Pavia for her bachelor's degree in Biotechnologies at the Università degli Studi di Pavia. She completed her internship in virology at the Department of Morphological, Eidological, and Clinical Sciences, Section of Microbiology, Università degli Studi di Pavia, focusing on the genotyping of papillomavirus types in HIV-positive women. After graduating in 2008, she started her master in Medical Biotechnologies at the Università degli Studi di Pavia. Her internship focused on cell models and genotyping applied to the characterization of endocrine disorders in the Laboratory for Endocrine Disruptors, directed by Professor Luca Chiovato. She received her Master's degree in September 2010.

Since 2011 she is working as a researcher at A. Bianchi Bonomi Haemophilia and Thrombosis Center, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano under the supervision of Prof. Flora Peyvandi. Her research focused on the biochemical, molecular, and in vitro characterization of von Willebrand disease and thrombotic disorders, such as deep vein thrombosis.

Maria Teresa started her PhD program in May 2019 as part of a joint PhD between the Leiden University Medical Center and the Università degli Studi di Milano under the supervision of Professor Frits Rosendaal, Professor Jeroen Eikenboom, and Professor Flora Peyvandi. After her PhD defense, Maria Teresa will continue her research activity as a Postdoc in the group directed by Prof. Peyvandi in Milan.

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PHD PORTFOLIO

PhD period: May 2019 - February 2023

Promotores: Prof. Dr H.C.J. Eikenboom, Prof. Dr. F. R. Rosendaal

Mandatory courses	Year	Hours
PhD introductory meeting	2020	5
Basic Methods and reasoning in Biostatistics	2020	42
BROK Course (exempted)	-	-
Disciplinary courses		
International Course on Clinical Epidemiology (Schiermonnikoog)	2019	42
Prediction modelling and intervention research	2020	84
Causal Inference Course	2020	84
Population Health: Study Design (Coursera)	2020	29
Population Health: Responsible Data Analysis (Coursera)	2020	21
Regression Analysis	2020	22
Meta-Analysis	2021	28
Analysis of Repeated Measurements 2021	2021	42
2D, 3D and 4D iPSC-derived cellular models to study neuromuscular diseases (University of Milan)	2021	10
Hemostasis and inflammation: from molecular aspects to organ damage (University of Milan)	2022	10
Other courses and transferable skills*		
Scopus for the calculation of H-Index	2021	2
Managing your reference using Endnote	2021	3
Research Integrity II, 35° cycle	2021	2
Fundaments of academic Writing, 36° cycle	2021	4
Dal brevetto all'impresa	2021	3
Language coaching: Presentation skills, 36° cycle	2021	4
Communication on new media, 34° cycle	2021	4
Language coaching: Interpersonal Skills- Interview skills, 35° cycle	2021	4
Lezione propedeutica di base su Ip e brevetti, 36°cycle	2021	4
Lezione avanzata sull'utilizzo dell'IP per fare innovazione, 34° cycle	2021	4
Academic Writing: Research Paper, 35° cycle	2021	4
Valorizzare Creando impresa: fare spin-off, 34° cycle	2021	8
Grantsmanship I, 35° cycle	2021	4
Grantsmanship II 35° cycle	2021	4
Open science e valutazione della ricerca, 36° cycle	2021	4

PhD Portfolio (continued)

	Year	Hours
Self-Branding, 35° cycle	2021	3
4EU+ Training Session "What is Open Science?"	2021	1.5
4EU+ Cycle Of Scientific Publication: An Overview	2021	1.5
4EU+ What are my funders requirements on Open Science? A focus on Plan S	2021	1.5
Behind the Scene of a peer-reviewed Journal	2021	3
4EU+ Strategies for publishing in Open Access Journals.	2021	1.5
4EU+ Training: Data management plans, one tool with many applications	2022	1.5
Laboratorio per la preparazione di un piano di disseminazione	2022	2
4EU+ Training: Research Data Management-Introduction to FAIR and Open Data	2022	1.5
4EU+ Open Research Software	2022	1.5
Data Management	2022	3
4EU+ Citizen Science: producing data with people for innovating research	2022	1.5
CV e tecniche di selezione: come compilare un buon CV e approcciarsi alle tecniche di selezione aziendale	2022	3
Other activities		
Attendance Rembrandt Symposium 2019	2019	9
Attendance Rembrandt Symposium 2020	2020	9
Managing your references using Mendeley	2021	3
Seminars on reproducible results: Rational and benefits	2021	2
Seminars on reproducible results: Theoretical session	2021	2
Conoscere E Difendersi Dal Nuovo Coronavirus Sars-Cov-2	2021	4
Il Decreto Legislativo n. 81/08 –Formazione Specifica Profilo di rischio A Sanitari	2021	14
Congress presentation and posters	**Year**	
ISTH 2019 Oral Presentation and poster	2019	
ISTH 2020 Poster Presentation (virtual)	2020	
Prizes, Awards and Grants	**Year**	
Young Investigator Award, 58th Scientific and Standardization Committee of the ISTH; Liverpool, UK	2012	
Ricerca Finalizzata Giovani Ricercatori (GR-2011-02351977), Italian Ministry of Health. Principal Investigator	2016-2019	
Early Career Travel Grant. ISTH 2019; Melbourne, Australia.	2019	
Prize Guelfo Marcucci (Kedrion Biopharma and Carlo Erba Foundation)	2020	

* Seminaries, lectures, workshops attended as request by the University of Milan PhD regulations.