Chapter 5

Biogenesis and exocytosis of Weibel-Palade bodies is affected by naturally occurring variants within the von Willebrand factor A1-A3 domains

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Summary

Background: Release of von Willebrand factor (VWF) from Weibel-Palade bodies (WPB) in response to desmopressin (DDAVP), a specific agonist of WPB exocytosis, is clinically applied to raise VWF plasma levels in patients with von Willebrand disease (VWD). A subset of patients with VWD type 1 shows a reduced response to DDAVP, suggesting the absence of recruitable WPB. The VWF propeptide (D1-D2 domains) and the D'D3 domains are necessary for the tubular assembly of VWF, which drives WPB formation. Some variants in the VWF A1-A3 domains have been linked to reduced DDAVP responsiveness, suggesting that determinants for WPB formation may reside outside the D1-D2-D'-D3 domains. We hypothesized that a reduced tendency to assemble into VWF tubules underlies the defective DDAVP response in VWD type 1 patients with mutations in the A domains.
Methods: Human Embryonic Kidney (HEK) 293 cells were transiently transfected with plasmids coding for full length VWF to study its storage and secretion. Six mutations in the VWF A1-A3 domains were studied.
Results: We found that all mutants, except p.Arg1583Trp, showed impaired formation of elongated pseudo-WPB. Upon co-transfection with WT-VWF, this was partly restored. The p.Leu1307Pro and p.Val1822Gly showed reduced constitutive secretion, even in the heterozygous state. Both mutations, together with the p.Ser1285Pro, also showed impaired regulated secretion.
Conclusion: Our data shows that mutations within the A domains of VWF can cause defects in WPB formation. These defects may be the underlying cause of the poor response to DDAVP infusion seen in VWD type 1 patients.

Introduction

Von Willebrand factor (VWF) is a multimeric glycoprotein. Upon vascular damage VWF serves as an adhesion molecule for platelets thereby initiating platelet plug formation. VWF is also the carrier protein of coagulation factor VIII (FVIII). VWF is synthesized in endothelial cells and megakaryocytes and is either secreted constitutively into plasma or stored in cell-specific organelles; the Weibel-Palade bodies (WPB) in endothelial cells or α-granules in megakaryocytes. The highest molecular weight VWF multimers are stored and are released from endothelial cells in response to secretion stimuli like thrombin, stress, and vasopressin or its synthetic analogue desmopressin (DDAVP) [1].

A decreased concentration or abnormal function of VWF is responsible for von Willebrand Disease (VWD), the most common inherited bleeding disorder with a prevalence ranging from 3-4 per 100.000 to 1.3% of the population [1,2]. Type 1 VWD (partial quantitative deficiency) is the most common type, approximately 50-75% of all cases. Mutations identified in type 1 VWD are predominantly missense mutations and only 15% of mutations lead to null alleles (ISTH-SSC VWF mutation database up to 2010 www.vwf.group.shef.ac.uk/). These mutations may lead to decreased VWF plasma levels via decreased synthesis, impaired secretion, increased clearance or a combination of these conditions.

A subset of type 1 VWD patients shows a reduced response to DDAVP, a specific agonist for release of WPB, which is clinically applied to raise VWF plasma levels [3]. The lack of response to DDAVP suggests the absence of recruitable WPB. Generally, it is assumed that VWF propeptide (D1-D2 domains) and D'D3 domains are involved in the tubular assembly of VWF, which drives WPB formation [4,5]. Interestingly, a reduced response to DDAVP was mainly found among patients with mutations in the A1-A3 domain of VWF [3]. This suggests that determinants for WPB formation can reside outside the D1-D2-D'-D3 domains. We hypothesize that a reduced tendency to assemble into VWF tubules may underlie the defective DDAVP response in VWD type 1 patients with mutations in the A1 and A3 domains.

Materials and methods

Patients and Mutations

The mutations (Table 1) we have studied were originally identified in type 1 VWD patients [6-9]. Except for p.Arg1583Trp and p.Tyr1584Cys, the mutations were associated with minor multimer abnormalities which were much more subtle than in regular type 2A VWD [10].

Plasmid Constructs

Recombinant pCI-neo expression plasmid containing full-length wild-type human VWF (WT-VWF) cDNA has been described [11]. Mutations were introduced into pCI-neo WT-VWF plasmid with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). DNA was purified using the PureYield™ Plasmid Maxiprep System (Promega) and preparations were verified by sequencing.

Cell culture and transfections

HEK293 cells from ATCC (Rockville, USA) were cultured and transfected as described [11]. Briefly, using FuGENE HD transfection reagent (Roche Diagnostics, Mannheim, Germany) plasmid constructs were transiently transfected into HEK293 cells at a final concentration of 0.66 µg/mL in the medium (0.33 µg/mL WT-VWF and mutant VWF plasmids, respectively, for co-transfections). All data were collected 72 h post-transfection.

Immunostaining

Cells were stained with immunofluorescent antibodies and analyzed by Leica SL confocal laser scanning microscopy with a 63X/1.40 NA oil objective [11]. Polyclonal FITC-conjugated sheep anti-human VWF antibody (Abcam, Cambridge, UK) and polyclonal rabbit anti-human Protein Disulfide Isomerase (PDI) antibody A66 (obtained from Prof. I. Braakman, Department of Chemistry, Utrecht University, Utrecht, The Netherlands) were used as primary antibodies against VWF and the endoplasmic reticulum (ER) marker PDI, respectively. Alexa 594-conjugated secondary antibody was purchased from Invitrogen.

Structural analysis of VWF

VWF multimer analysis was performed as previously described [12]. Processing of VWF was analysed by reducing SDS-PAGE and Western blot analysis for the conditioned media and cell lysates. Samples were reduced using 20 mM dithiothreitol, separated by Novex 6% Tris-Glycine gel electrophoresis (Invitrogen), immunostained with polyclonal rabbit anti- human VWF conjugated to horseradish peroxidase (DAKO, Glostrup, Denmark) and visualized with Supersignal WestFemto (Thermo Scientific, Rockford, IL, USA).

Table 1. Summary of defects caused by mutations in the A domain of VWF compared to WT-VWF

Domain	*Variant*	*DDAVP response patients**	*Multimers**	*ER retention*	*Pseudo WBP formation†*		*Secretion of VWF*		*Secreted multimers*
					No.	Elongated shape	Basal	Stimulated	
1	p.Ser1285Pro	Unknown	Abnormal	↑	Normal	↓↓	Normal	↓	Normal
A1	p.Leu1307Pro	Unknown	Abnormal	↑↑	↓	↓↓	↓↓↓	↓↓	Lack of HMW multimers
A1	p.Arg1374His	Poor	Abnormal	-	Normal	↓↓	↓↓	↓	Normal
A2	p.Arg1583Trp	Good	Normal	-	Normal	Normal	Normal	Normal	Normal
A2	p.Tyr1584Cys	Good	Normal	-	Normal	↓↓	Normal	Normal	Normal
A3	p.Val1822Gly	Poor	Abnormal	↑	↓	↓	↓↓	↓↓	Normal

*According to the MCMDM-1VWD study [3,10].

†Based on immunofluorescent images.

Basal and regulated secretion of VWF

Basal secretion of VWF was determined in conditioned media and cell lysates collected 72-hours post transfection [11,12]. To analyze the regulated secretion of VWF, 72-hours post transfection cells were incubated for 60 minutes with release medium containing 160 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St louis, MO, USA) or vehicle (DMSO). VWF:Ag in the media and cell lysates was determined by ELISA [13]. Regulated secretion is expressed as secreted VWF as a fraction of total VWF (medium plus lysate). Statistical analyses were performed by the Student's *t*-test using GraphPad Prism (version 4.02).

Transmission electron microscopy

Transfected cells grown on 35-mm petri dishes were fixed and prepared for transmission electron microscopy (TEM) as described [11]. Briefly, osmium tetroxide and tannic acid treated cells were serially dehydrated with ethanol and embedded in Epon. Samples were cut into thin sections of 70-100 nm, were stained with uranyl acetate and lead citrate, and then analyzed with a Tecnai 12 at 120 kV equipped with a 4kx4k CCD camera (Model Eagle, Fei Company, The Netherlands).

Results

Pseudo Weibel-Palade body formation in VWF transfected cells

We have analyzed the formation and intracellular distribution of pseudo-WPB in HEK293 cells by transiently transfecting cells with VWF variants. Cells transfected with WT-VWF or VWF p.Arg1583Trp formed numerous elongated pseudo-WPB (Figure 1). p.Ser1285Pro, p.Leu1307Pro, p.Arg1374His, p.Tyr1584Cys and p.Val1822Gly were able to form these organelles, but in most cases the pseudo-WBP were shorter and more round compared to those formed by WT-VWF (Table 1 and Figure 1, left panels). Upon co-transfection of mutant and normal VWF (Figure 1, right panels) the defected elongation of pseudo-WPB by those mutations was only minimally corrected. Retention of VWF in the ER, identified by the co-localization of VWF and PDI (yellow colour), was present in about 50% of the cells expressing p.Leu1307Pro and 25-35% of the cells expressing p.Ser1285Pro and p.Val1822Gly as compared to 10% in WT-VWF (Figure 1).

Pseudo-WPB formation was further studied using TEM. In WT-VWF transfected cells, elongated and electron dense pseudo-WPB were observed with the typical striations seen in endothelial cells [14] (Figure 2A). p.Arg1583Trp showed authentic

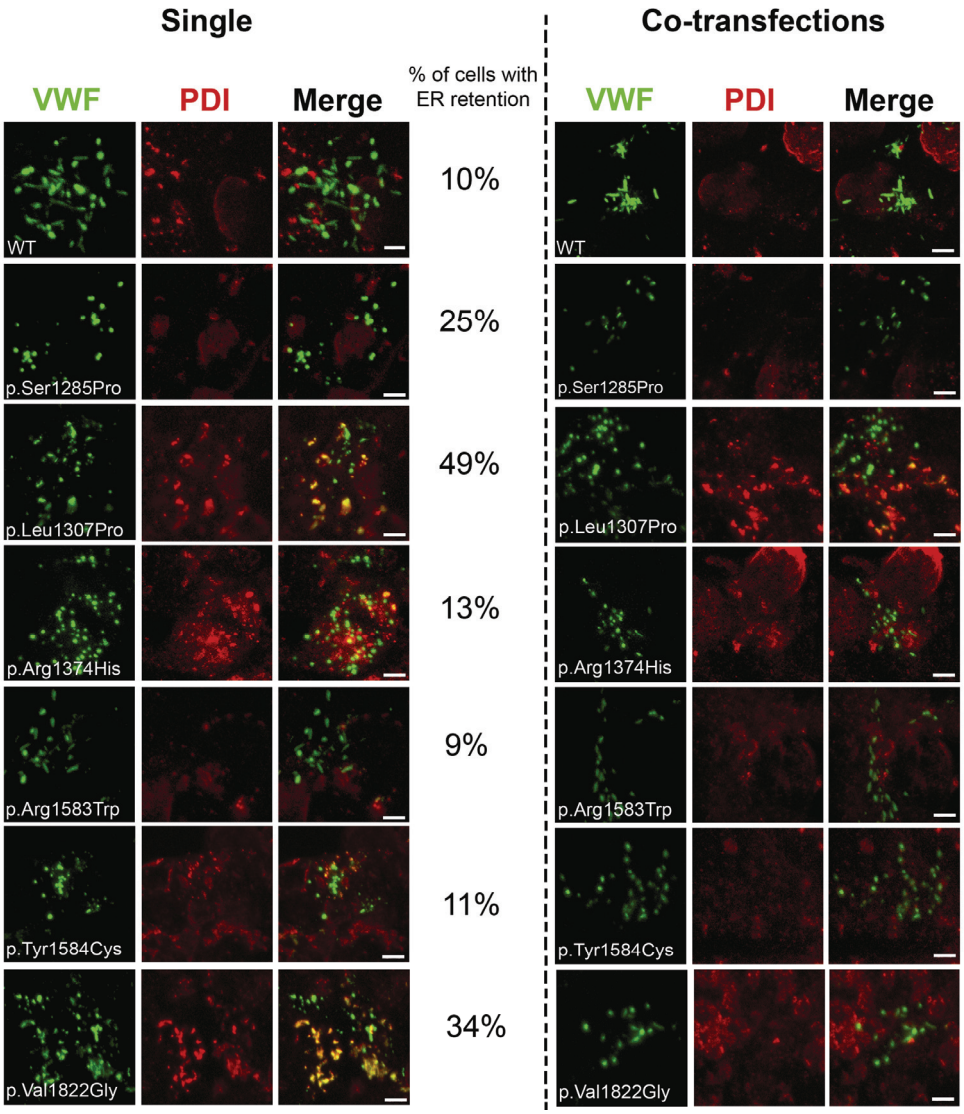

Figure 1. Intracellular localization of VWF in transfected HEK293 cells. HEK293 cells were transiently transfected with WT-VWF or mutant VWF (single transfections, left panel) or with WT- and mutant VWF (co-transfections, right panel). Cells were fixed for immunofluorescence imaging 72 hours post transfection and stained for VWF (green channel) and PDI (an ER marker, red channel). In the merged images, pseudo-WPB show up in green (VWF staining only), and VWF in the ER shows up as yellow (double staining of VWF and PDI). Scale bars = 5 μm. The percentage of cells (single transfections) that showed ER retention of VWF was determined in two independent experiments in 200-260 cells expressing each of VWF variants.

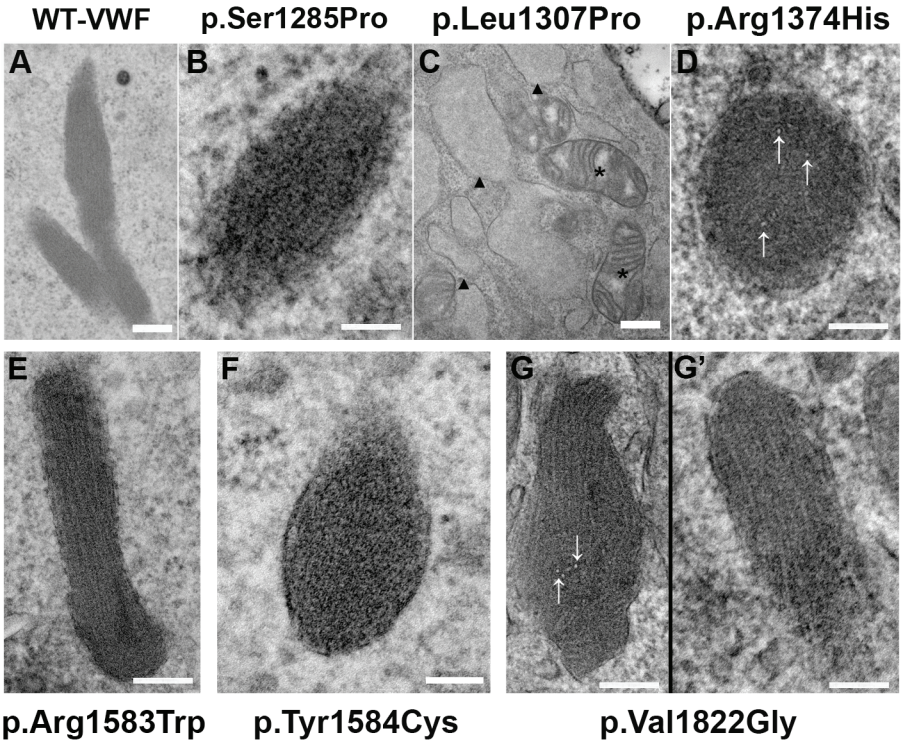

Figure 2. Ultrastructure of transfected HEK293 cells visualized by TEM. HEK293 cells were transiently transfected as indicated. Pseudo-WPB displaying VWF tubules were formed by WT-VWF (A) and the mutants (B, D-G'). Arrows indicate clearly visible cross sections of tubules (D, G). For the p.Leu1307Pro we were not able to visualize with certainty organelles with internal tubules, however, dilated ER was particularly observed (C). The arrowheads and stars indicate ER and mitochondria, respectively (C). Scale bars in panel A, C = 200 nm; scale bars in panel B, D-G' = 100 nm.

cigar-shaped pseudo-WPB with typical VWF striations (Figure 2E). Shorter and more round pseudo-WPB were found in cells expressing the other VWF variants (Figure 2B,D,F,G), except for p.Leu1307Pro where we were not able to visualize organelles with internal tubules (Figure 2C). The round structures are, however, recognized as pseudo-WPB as they contain disorganized tubular structures indicating storage of VWF tubules. Grossly dilated ER was frequently observed in cells expressing the p.Leu1307Pro variant (Figure 2C), in accordance with the observed ~50% ER retention (Figure 1).

Retention of VWF was further investigated by evaluating the amount of processed VWF with Western blotting under reducing conditions. VWF secreted into the

medium consisted mainly of fully processed mature VWF subunits (Figure 3A). Cell lysates showed both mature VWF subunits and unprocessed proVWF. The p.Ser1285Pro, p.Leu1307Pro and p.Val1822Gly variants showed relatively more uncleaved proVWF compared to WT-VWF as indicated by the ratio between mature VWF and proVWF (Figure 3B, upper panel). Upon co-transfection with WT-VWF more processed mature VWF was observed in these variants (Figure 3B, lower panel). The relative increase of proVWF in the VWF variants indicates retention in the ER and lack of cleavage in the Golgi apparatus, where furin cleaves the propeptide [15-17].

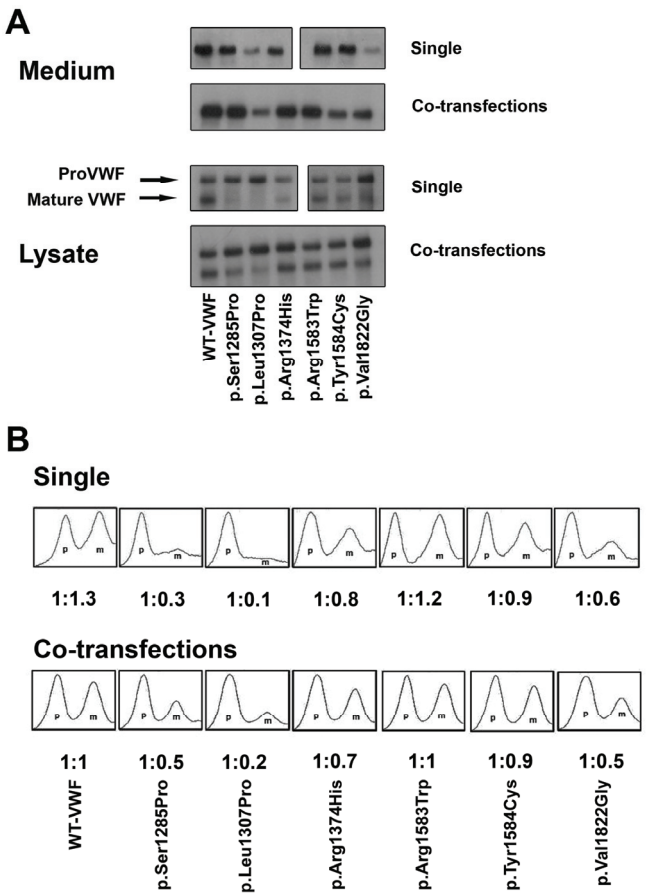

Figure 3. Subunit composition of VWF under reducing conditions. VWF secreted into the medium or VWF retained in lysates was reduced with DTT and analyzed with SDS-PAGE and Western blot (A). In panel B, the ratio of proVWF (p) and mature VWF subunit (m) in the cell lysate was analyzed with Image J (NIH software, version 1.44P).

Table 2. Expression of VWF variants in transfected HEK293 cells

Variant	Transfection	VWF:Ag* Medium		VWF:Ag* Lysate		Total (medium + lysate)	Ratio†
		mU	%	mU	%	mU	
WT-VWF		71±2.3	100	32±2.0	100	102±3.7	2.2±0.1
p.Ser1285Pro	Single transfection	73±3.1	103±4.5	39±2.7	123±1.2	112±5.8	1.9±0.1
	Co-transfection	71±2.4	100±1.2	35±2.2	112±2.1	106±3.9	2.0±0.1
p.Leu1307Pro	Single transfection	28±1.2	40±2.3	42±3.3	132±2.9	70±4.4‡	0.7±0.0‡
	Co-transfection	36±1.0	51±0.9	35±2.0	112±2.9	72±2.6‡	1.0±0.1‡
p.Arg1374His	Single transfection	43±2.5	61±2.8	25±1.4	80±2.3	68±3.0‡	1.7±0.1‡
	Co-transfection	60±1.2	85±2.4	35±3.8	111±6.4	95±4.8	1.7±0.1‡
p.Arg1583Trp	Single transfection	69±1.9	97±3.5	27±1.4	84±2.1	95±3.1	2.6±0.1
	Co-transfection	72±3.7	102±4.3	31±2.9	97±3.8	103±2.5	2.4±0.3
p.Tyr1584Cys	Single transfection	72±5.2	101±4.0	27±2.8	87±9.0	99±8.1	2.6±0.1
	Co-transfection	71±3.9	101±4.2	32±2.1	102±2.2	104±6.0	2.2±0.0
p.Val1822Gly	Single transfection	36±2.0	51±3.2	21±2.2	50±8.7	57±4.1‡	1.8±0.1‡
	Co-transfection	52±1.5	73±0.6	33±2.7	105±5.7	85±4.2‡	1.6±0.1‡

*VWF:Ag (mU) was produced by about 7×10^5 cells in the medium or lysate. VWF:Ag is also expressed as percentage relative to the amount of VWF:Ag in WT-VWF transfected cells. Each value represents the mean ± SEM of three independent experiments performed in duplicate.

†Ratio of the absolute amount of VWF:Ag in Medium to VWF:Ag in Lysate.

‡Compared with WT-VWF $p<0.05$ (student's-*t* test).

Basal and regulated secretion of VWF

To investigate the effect of VWF mutants on the secretion of VWF, both basal and regulated secretion were studied. Basal secretion was defined as the amount of VWF:Ag in the conditioned medium 72 hours post transfection (Table 2 and Figure 4A). p.Ser1285Pro, p.Arg1583Trp and p.Tyr1584Cys variants show normal basal secretion of VWF compared to WT-VWF (Figure 4A). Furthermore, the total VWF protein production (VWF in both medium and lysate) was similar for WT-VWF and these mutants (Table 2). Effects on WPB formation (Figure 1) could therefore not be explained by reduced expression levels. In contrast, the p.Leu1307Pro,

p.Arg1374His and p.Val1822Gly mutations have considerable reduction of basal secretion compared to WT-VWF. This was apparent in both the amount of VWF secreted into the medium as well as the reduced ratio between the absolute amount of VWF protein produced in medium and lysate (Table 2). Also, the absolute amount of total VWF production was significantly reduced (60-70 mU VWF for the variants versus ± 100mU VWF for WT-VWF).

To mimic the heterozygous state of the patients, co-transfections of the variants with WT-VWF were performed. After co-transfection the basal secretion defect was restored for the p.Arg1374His mutation to almost normal (85% ± 2.4 relative to WT-VWF). The absolute amount of VWF protein produced by this mutant was also restored to normal levels (95mU ± 4.8 versus 102mU ± 3.7 for WT-VWF). The secretion defect observed for the variants p.Leu1307Pro and p.Val1822Gly was partly restored upon co-transfection with WT-VWF (51% ± 0.9 and 73% ± 0.6 respectively), but total protein production remained reduced (72mU ± 2.6, and 85mU ± 4.2, respectively, compared to 102mU ± 3.7 for WT-VWF).

The regulated secretion pathway of VWF was analyzed using PMA, which has been shown to efficiently induce exocytosis of WPB from transfected HEK293 cells and HUVECs [11,18]. After stimulation with PMA for 60 minutes, secretion of WT-VWF was increased almost 3-fold (9% of total VWF was secreted by cells stimulated with PMA versus 3% by control cells, Figure 4B). Compared to WT-VWF transfected cells, p.Arg1583Trp and p.Tyr1584Cys transfected cells showed comparable response to stimulation in both single and co-transfections (Figure 4B-C). This is in line with the adequate response found in patients carrying these mutations after DDAVP infusion (2.7-fold and 1.8-fold increase of VWF:RCo) [3]. Cells transfected with p.Ser1285Pro and p.Arg1374His gave an intermediate response to PMA stimulation, increasing the secretion only 1.5- and 1.6-fold. When co-transfected with WT-VWF p.Arg1374His showed a similar response (2.5-fold increase) as WT-VWF transfected cells, however, in absolute terms the VWF level was slightly lower. Co-transfection of p.Ser1285Pro did not improve the response. In the single transfections both p.Leu1307Pro and p.Val1822Gly showed little response to PMA (Figure 4B). Upon co-transfection the response remained minimal but increased for the p.Val1822Gly to 1.3-fold, consistent with the partial DDAVP response found in patients [3]. The response for p.Leu1307Pro remained the same upon co-transfection. DDAVP response obtained from a patient in our clinic would define this patient as a partial responder (unpublished data) as the VWF:RCo is lower than 50 IU/dl after desmopressin (6 to 22 IU/dl after infusion).

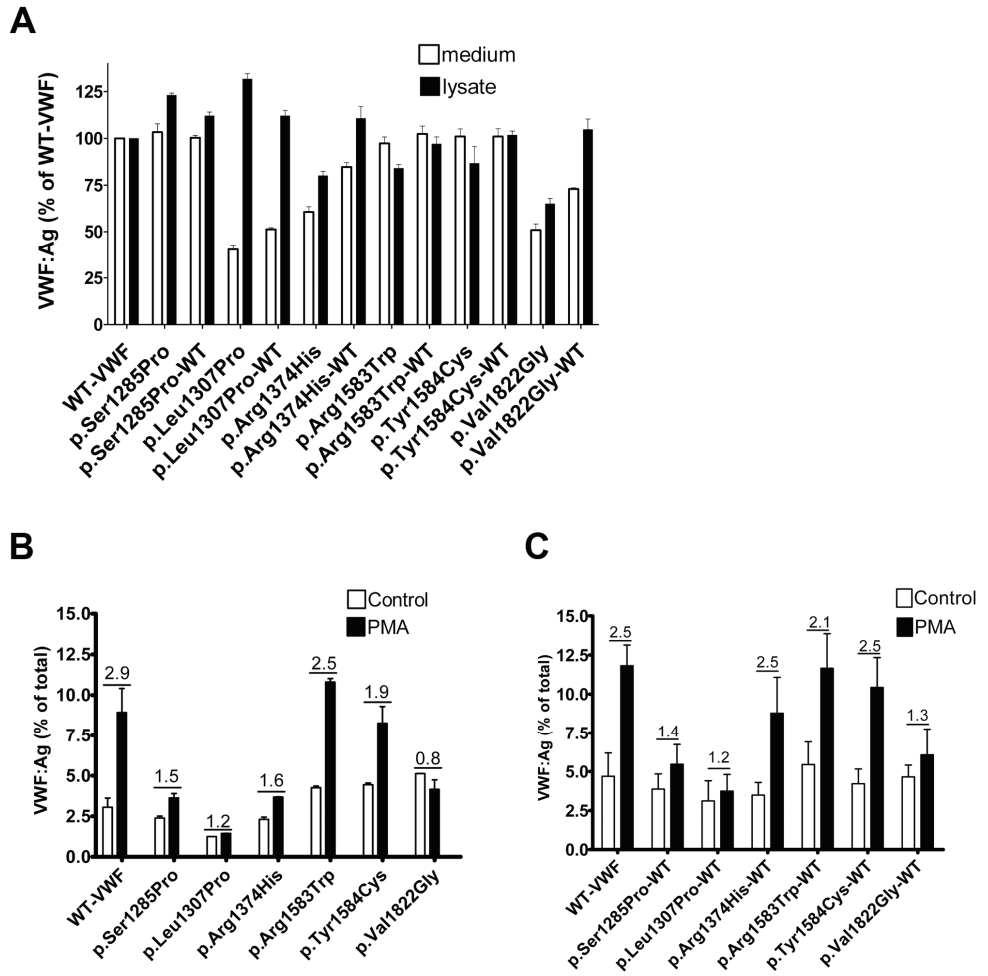

Figure 4. Basal and regulated secretion of VWF. HEK293 cells were transiently transfected with WT-VWF or mutants, or the mutants were co-transfected with WT-VWF at 1:1 ratio. (A) Seventy-two hours after transfection conditioned media and cell lysates were collected to determine basal secretion of VWF. WT-VWF is set at 100%. (B, C) Regulated secretion of VWF was analyzed as follows: seventy-two hours post transfection HEK293 cells were rinsed and incubated at 37°C for 60 minutes in release medium with DMSO (Control) or with 160 nM PMA (PMA). Panel B shows regulated secretion of single transfections, panel C shows secretion of co-transfections. Each bar represents VWF secreted into the medium as a fraction of total VWF (secreted plus intracellular). Mean and SEM are based on three independent experiments in duplicate. The numbers above the bars indicate the fold increase of secreted VWF upon stimulation of the cells.

Unfortunately no data is available on the DDAVP response in patients carrying the p.Ser1285Pro mutation. However, one might speculate that patients will show a poor to partial response to DDAVP infusion based on these in vitro data.

Multimeric structure of VWF

Compared to WT-VWF all variants, except p.Leu1307Pro, showed a similar and normal VWF multimer distribution in the conditioned medium (Figure 5A) and lysate (Figure 5B) in both single- and co-transfections. The p.Leu1307Pro mutation, however, led to a relative loss of the larger multimers in both medium and lysate. Upon co-transfection this loss was restored to a near normal distribution in medium as well as lysate. However, the multimer patterns showed relatively more monomers and dimers compared to WT-VWF.

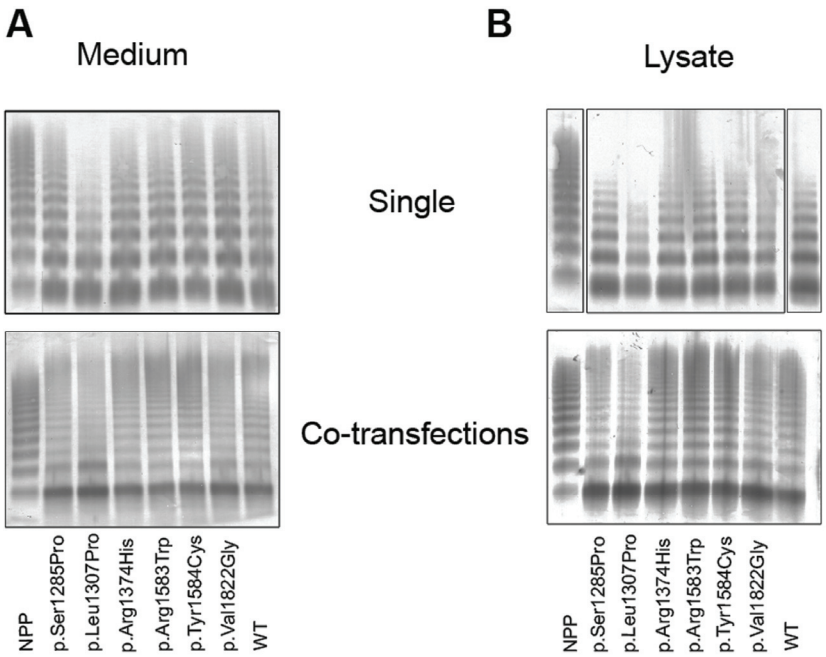

Figure 5. Multimeric analysis of recombinant VWF. Multimer patterns were analysed in conditioned media (A) and cell lysates (B). Multimers are shown for single transfections and co-transfections. The multimer patterns were analyzed by SDS-agarose gel electrophoresis and Western blot under non-reducing conditions. The separate lanes in panel B are from the same blot, but normal pool plasma and wt-VWF were switched.

Discussion

In this study we have expressed six VWF variants located within the A domains of VWF that were previously identified in patients diagnosed as having type 1 VWD [6-9]. The p.Ser1285Pro, p.Leu1307Pro and p.Arg1374His mutations are located within the A1 domain, the p.Arg1583Trp and p.Tyr1584Cys are located in the A2 domain and the p.Val1822Gly mutation is located in the A3 domain of VWF. None of these mutations have been studied before regarding the biogenesis and exocytosis of WPB.

Of the mutations located in the A1 domain, only p.Ser1285Pro showed a normal basal secretion. This variant led to the formation of only round pseudo-WPB and showed ER retention, which was supported by the relative increase of unprocessed, uncleaved proVWF. This seems contradictory to the normal basal secretion of p.Ser1285Pro. However, the regulated secretion is impaired suggesting that this variant passes basal secretion pathway but not the regulated secretion pathway. All the A domains of VWF (VWA) contain a conserved aspartic acid in a DXS motif that appears to be buried within the protein and is required to maintain the VWA fold by electrostatic interactions [19,20]. The serine at position 1285 is part of a DXS motif. The substitution of a serine by proline disrupts this motif and this might subsequently change the folding of the A1 domain. Indeed, it was found that substituting aspartic acid at position 1283 by alanine attenuated binding of the protein to glycoprotein Ib, probably by relaxation of the VWA fold [19]. Whether relaxation of the protein causes the defective regulated secretion of this variant remains unclear.

Patients with the p.Leu1307Pro and p.Arg1374His mutations have previously been included among both type 2A and type 1 VWD [6,7,21]. Indeed in single transfections the p.Leu1307Pro mutation resulted in the loss of the largest multimers, characteristic of type 2A VWD. However, upon co-transfection the VWF multimers were normalized. Both mutations showed round pseudo-WPB and a decreased basal secretion. In addition p.Leu1307Pro showed severe ER retention. The leucine at position 1307 is located directly after the α1 helix of the A1 domain. Proline has a poor helix forming propensity because it cannot donate an amide hydrogen bond and because its side chain interferes sterically with the backbone of the preceding turn [22]. Prediction software predicts that the substitution to a proline will lead to loss of stability of the protein, and this could therefore be the cause of ER retention of this VWF variant.

The p.Arg1374His variant showed a good response to stimulation with PMA in the

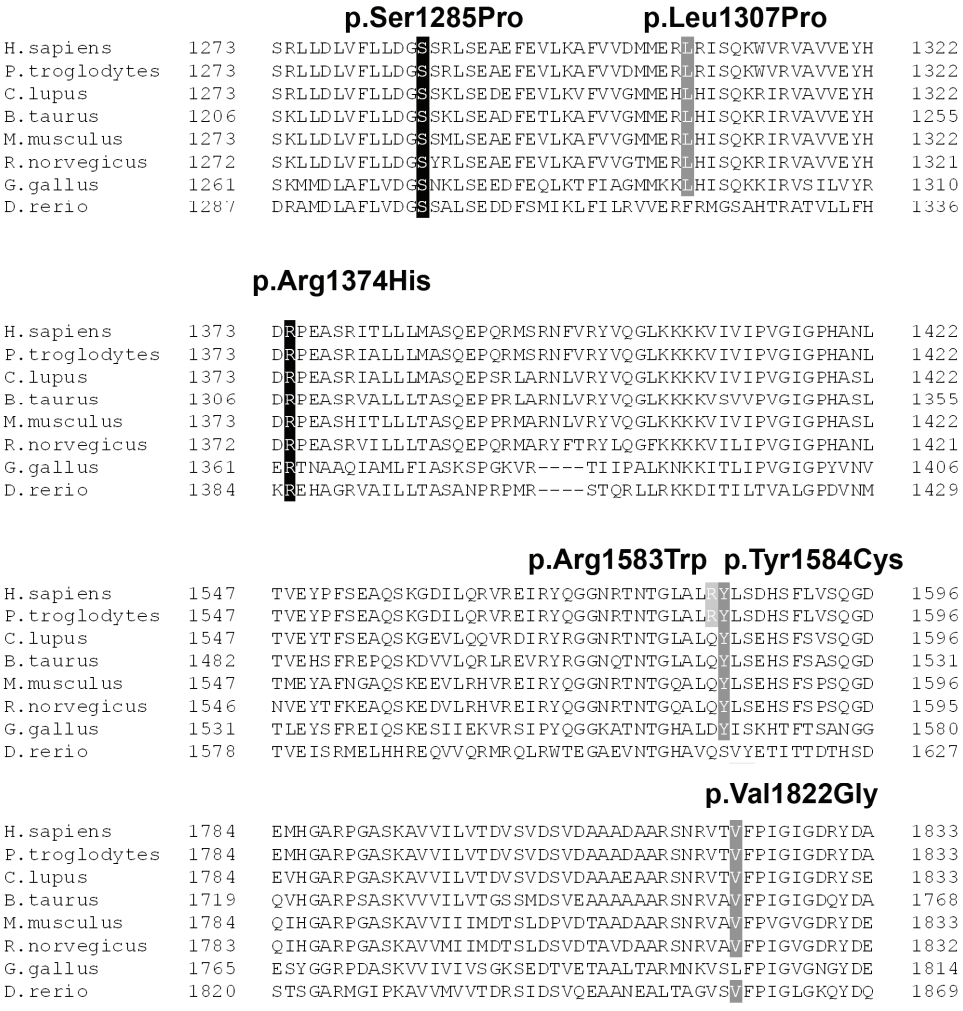

Figure 6. Conservation of amino acids in different vertebrate species. The VWF gene was aligned using the HomoloGene database. Alignments of regions surrounding the studied mutations with corresponding VWF sequences from different vertebrate species are shown in detail. Residues highlighted in black indicate fully conserved amino acids, whereas grey residues are highly conserved.

co-transfection experiment. The data on response to DDAVP infusion in patients heterozygous for this mutation are conflicting, ranging from non-response to good response [3,21]. One out of 4 patients responded in a study by Federici et al [21]

and previously we have shown a good response to DDAVP, raising VWF:Ag levels more than 3-fold in three family members with this mutation [7]. Such a variation in response among patients carrying the same mutation suggests that the response to DDAVP is not only influenced by the mutation in the *VWF* gene. The arginine at position 1374 is part of a salt bridge network that stabilizes Asp1277 at the bottom of the strand βA [23]. Within the crystal structure of the A1 domain the arginine is buried and this mutation will probably lead to destabilization of the folded structure [20].

The p.Arg1583Trp mutation was the only variant that showed normal basal and stimulated secretion of VWF as well as the formation of elongated pseudo-WPB. In line with this observation, patients carrying this mutation have a mild phenotype and respond well to DDAVP infusion [3]. Alignment of the VWF amino acid sequence (Figure 6) shows that the arginine at position 1583 is poorly conserved among different vertebrate species, suggesting that the amino acid at this position is not critical and that substitution by tryptophan does not materially influence the structure or function of the protein. Since the expression data of p.Arg1583Trp are indistinguishable from WT-VWF, one might doubt that this amino acid change is really pathogenic.

The p.Tyr1584Cys variant has been described as a polymorphism and as a mutation [24-26]. This variant was found frequently in index cases in both the Canadian type 1 VWD study as well as in the European MCMDM-1VWD study, and often in association with blood group O, a known modifier of VWF plasma levels [6,9,27]. The p.Tyr1584Cys has been expressed before, where the total protein expression reached levels of 80% and 85% compared to WT-VWF transfected cells [9,28]. This is slightly less than our observations (table 1), but variable expression levels between different cell types have been reported for p.Tyr 1584Cys [28]. Despite the formation of mostly round pseudo-WPB, the number of storage organelles as well as the basal and stimulated secretion is normal. This might explain why patients with this mutation have a mild phenotype with low penetrance of bleeding and respond well to DDAVP infusions [3,29,30]. The amino acid substitution by cysteine does not lead to a significant conformational change of the protein. However, a new solvent-exposed reactive thiol group is apparent [9]. This free thiol group might lead to an instable protein by interacting with VWF or with other proteins, thereby impairing the elongation of WPB.

The p.Val1822Gly substitution is located in the A3 domain of VWF. Although not necessary for the formation of elongated WPB [4,5], our data suggests that

mutations in the A3 domain can affect elongation of the pseudo-WPB. This variant showed in co-transfection with WT-VWF partial response to stimulation with PMA in accordance with results obtained from DDAVP infusions in patients. The number of pseudo-WPB was also decreased in the single transfections, but this could be due to the lower expression of total VWF compared to WT-VWF.

In summary, we have shown that naturally occurring VWD variants that are located within the A domains of VWF can cause defects in both WPB formation and regulated secretion of VWF. This is in contrast with previous data which suggested that for normal WPB formation only the propeptide and D1'-A1 domain of VWF are essential [5] In our study however, we found that two mutations located within the A2 and A3 domain (p.Tyr1584Cys and p.Val1822Gly) also interfere with the formation of elongated WPB as evidenced by round storage organelles containing (disorganized) VWF tubules. As visualized by EM, we showed that the round structures that stained positive for VWF under the confocal microscope are indeed pseudo-WPB, suggesting that variants outside the D1-A1 domains can indeed affect VWF assembly into organized tubules. Missense mutations found in patients with a reduced response to DDAVP were almost all located within the A1-A3 domain of VWF [3]. We showed that mutations located in A1 and A3, but not A2, showed a poor response to stimulation. Interestingly, two variants (p.Arg1374His and p.Tyr1584Cys) which produced only round pseudo-WPB did respond well to stimulation, suggesting that elongation of WPB is not necessary for an adequate response. How mutations, which effect WPB formation, therefore influence the response to stimulation remains unclear.

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References

1. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem* 1998; **67**:395-424.
2. Keeney S, Cumming AM. The molecular biology of von Willebrand disease. *Clin Lab Haematol* 2001; **23**:209-230.
3. Castaman G, Lethagen S, Federici AB, Tosetto A, Goodeve A, Budde U, Batlle J, Meyer D, Mazurier C, Fressinaud E, Goudemand J, Eikenboom J, Schneppenheim R, Ingerslev J, Vorlova Z, Habart D, Holmberg L, Pasi J, Hill F, Peake I, Rodeghiero F. Response to desmopressin is influenced by the genotype and phenotype in type 1 von Willebrand disease

(VWD): results from the European Study MCMDM-1VWD. *Blood* 2008; **111**:3531-3539.
4. Huang RH, Wang Y, Roth R, Yu X, Purvis AR, Heuser JE, Egelman EH, Sadler JE. Assembly of Weibel-Palade body-like tubules from N-terminal domains of von Willebrand factor. *Proc Natl Acad Sci U S A* 2008; **105**:482-487.
5. Michaux G, Abbitt KB, Collinson LM, Haberichter SL, Norman KE, Cutler DF. The physiological function of von Willebrand's factor depends on its tubular storage in endothelial Weibel-Palade bodies. *Dev Cell* 2006; **10**:223-232.
6. Goodeve A, Eikenboom J, Castaman G, Rodeghiero F, Federici AB, Batlle J, Meyer D, Mazurier C, Goudemand J, Schneppenheim R, Budde U, Ingerslev J, Habart D, Vorlova Z, Holmberg L, Lethagen S, Pasi J, Hill F, Hashemi SM, Baronciani L, Hallden C, Guilliatt A, Lester W, Peake I. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood* 2007; **109**:112-121.
7. Castaman G, Eikenboom JC, Rodeghiero F, Briet E, Reitsma PH. A novel candidate mutation (Arg611-->His) in type I 'platelet discordant' von Willebrand's disease with desmopressin-induced thrombocytopenia. *Br J Haematol* 1995; **89**:656-658.
8. Hilbert L, Gaucher C, Mazurier C. Effects of different amino-acid substitutions in the leucine 694-proline 708 segment of recombinant von Willebrand factor. *Br J Haematol* 1995; **91**:983-990.
9. O'Brien LA, James PD, Othman M, Berber E, Cameron C, Notley CR, Hegadorn CA, Sutherland JJ, Hough C, Rivard GE, O'Shaunessey D, Lillicrap D. Founder von Willebrand factor haplotype associated with type 1 von Willebrand disease. *Blood* 2003; **102**:549-557.
10. Budde U, Schneppenheim R, Eikenboom J, Goodeve A, Will K, Drewke E, Castaman G, Rodeghiero F, Federici AB, Batlle J, Perez A, Meyer D, Mazurier C, Goudemand J, Ingerslev J, Habart D, Vorlova Z, Holmberg L, Lethagen S, Pasi J, Hill F, Peake I. Detailed von Willebrand factor multimer analysis in patients with von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 von Willebrand disease (MCMDM-1VWD). *J Thromb Haemost* 2008; **6**:762-771.
11. Wang JW, Valentijn KM, de Boer HC, Dirven RJ, van Zonneveld AJ, Koster AJ, Voorberg J, Reitsma PH, Eikenboom J. Intracellular storage and regulated secretion of von Willebrand factor in quantitative von Willebrand disease. *J Biol Chem* 2011; **286**:24180-24188.
12. Wang JW, Groeneveld DJ, Cosemans G, Dirven RJ, Valentijn KM, Voorberg J, Reitsma PH, Eikenboom J. Biogenesis of Weibel-Palade bodies in von Willebrand disease variants with impaired von Willebrand factor intrachain or interchain disulfide bond formation. *Haematologica* 2012; **97**:859-866.
13. Tjernberg P, Vos HL, Castaman G, Bertina RM, Eikenboom JC. Dimerization and multimerization defects of von Willebrand factor due to mutated cysteine residues. *J Thromb Haemost* 2004; **2**:257-265.
14. Zenner HL, Collinson LM, Michaux G, Cutler DF. High-pressure freezing provides insights into Weibel-Palade body biogenesis. *J Cell Sci* 2007; **120**:2117-2125.
15. Haberichter SL, Jacobi P, Montgomery RR. Critical independent regions in the VWF propeptide and mature VWF that enable normal VWF storage. *Blood* 2003; **101**:1384-1391.
16. Rehemtulla A, Kaufman RJ. Preferred sequence requirements for cleavage of pro-von Willebrand factor by propeptide-processing enzymes. *Blood* 1992; **79**:2349-2355.

17. Shapiro J, Sciaky N, Lee J, Bosshart H, Angeletti RH, Bonifacino JS. Localization of endogenous furin in cultured cell lines. *J Histochem Cytochem* 1997; **45**:3-12.
18. Valentijn KM, van Driel LF, Mourik MJ, Hendriks GJ, Arends TJ, Koster AJ, Valentijn JA. Multigranular exocytosis of Weibel Palade bodies in vascular endothelial cells. *Blood* 2010; **116**:1807-1816.
19. Matsushita T, Sadler JE. Identification of amino acid residues essential for von Willebrand factor binding to platelet glycoprotein Ib. Charged-to-alanine scanning mutagenesis of the A1 domain of human von Willebrand factor. *J Biol Chem* 1995; **270**:13406-13414.
20. Emsley J, Cruz M, Handin R, Liddington R. Crystal structure of the von Willebrand Factor A1 domain and implications for the binding of platelet glycoprotein Ib. *J Biol Chem* 1998; **273**:10396-10401.
21. Federici AB, Mazurier C, Berntorp E, Lee CA, Scharrer I, Goudemand J, Lethagen S, Nitu I, Ludwig G, Hilbert L, Mannucci PM. Biologic response to desmopressin in patients with severe type 1 and type 2 von Willebrand disease: results of a multicenter European study. *Blood* 2004; **103**:2032-2038.
22. Blaber M, Zhang XJ, Matthews BW. Structural basis of amino acid alpha helix propensity. *Science* 1993; **260**:1637-1640.
23. Jenkins PV, Pasi KJ, Perkins SJ. Molecular modeling of ligand and mutation sites of the type A domains of human von Willebrand factor and their relevance to von Willebrand's disease. *Blood* 1998; **91**:2032-2044.
24. Bowen DJ, Collins PW. An amino acid polymorphism in von Willebrand factor correlates with increased susceptibility to proteolysis by ADAMTS13. *Blood* 2004; **103**:941-947.
25. Keeney S, Grundy P, Collins PW, Bowen DJ. C1584 in von Willebrand factor is necessary for enhanced proteolysis by ADAMTS13 in vitro. *Haemophilia* 2007; **13**:405-408.
26. Bowen DJ, Collins PW, Lester W, Cumming AM, Keeney S, Grundy P, Enayat SM, Bolton-Maggs PH, Keeling DM, Khair K, Tait RC, Wilde JT, Pasi KJ, Hill FG. The prevalence of the cysteine1584 variant of von Willebrand factor is increased in type 1 von Willebrand disease: co-segregation with increased susceptibility to ADAMTS13 proteolysis but not clinical phenotype. *Br J Haematol* 2005; **128**:830-836.
27. James PD, Notley C, Hegadorn C, Leggo J, Tuttle A, Tinlin S, Brown C, Andrews C, Labelle A, Chirinian Y, O'Brien L, Othman M, Rivard G, Rapson D, Hough C, Lillicrap D. The mutational spectrum of type 1 von Willebrand disease: Results from a Canadian cohort study. *Blood* 2007; **109**:145-154.
28. Pruss CM, Golder M, Bryant A, Hegadorn CA, Burnett E, Laverty K, Sponagle K, Dhala A, Notley C, Haberichter S, Lillicrap D. Pathologic mechanisms of type 1 VWD mutations R1205H and Y1584C through in vitro and in vivo mouse models. *Blood* 2011; **117**:4358-4366.
29. Davies JA, Collins PW, Hathaway LS, Bowen DJ. Effect of von Willebrand factor Y/C1584 on in vivo protein level and function and interaction with ABO blood group. *Blood* 2007; **109**:2840-2846.
30. Davies JA, Collins PW, Hathaway LS, Bowen DJ. von Willebrand factor: evidence for variable clearance in vivo according to Y/C1584 phenotype and ABO blood group. *J Thromb Haemost* 2008; **6**:97-103.