Pathophysiology of von Willebrand factor in bleeding and thrombosis

Pagliari, M.T.

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.

Haemophilia. 2022;28(2):292-300.

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.

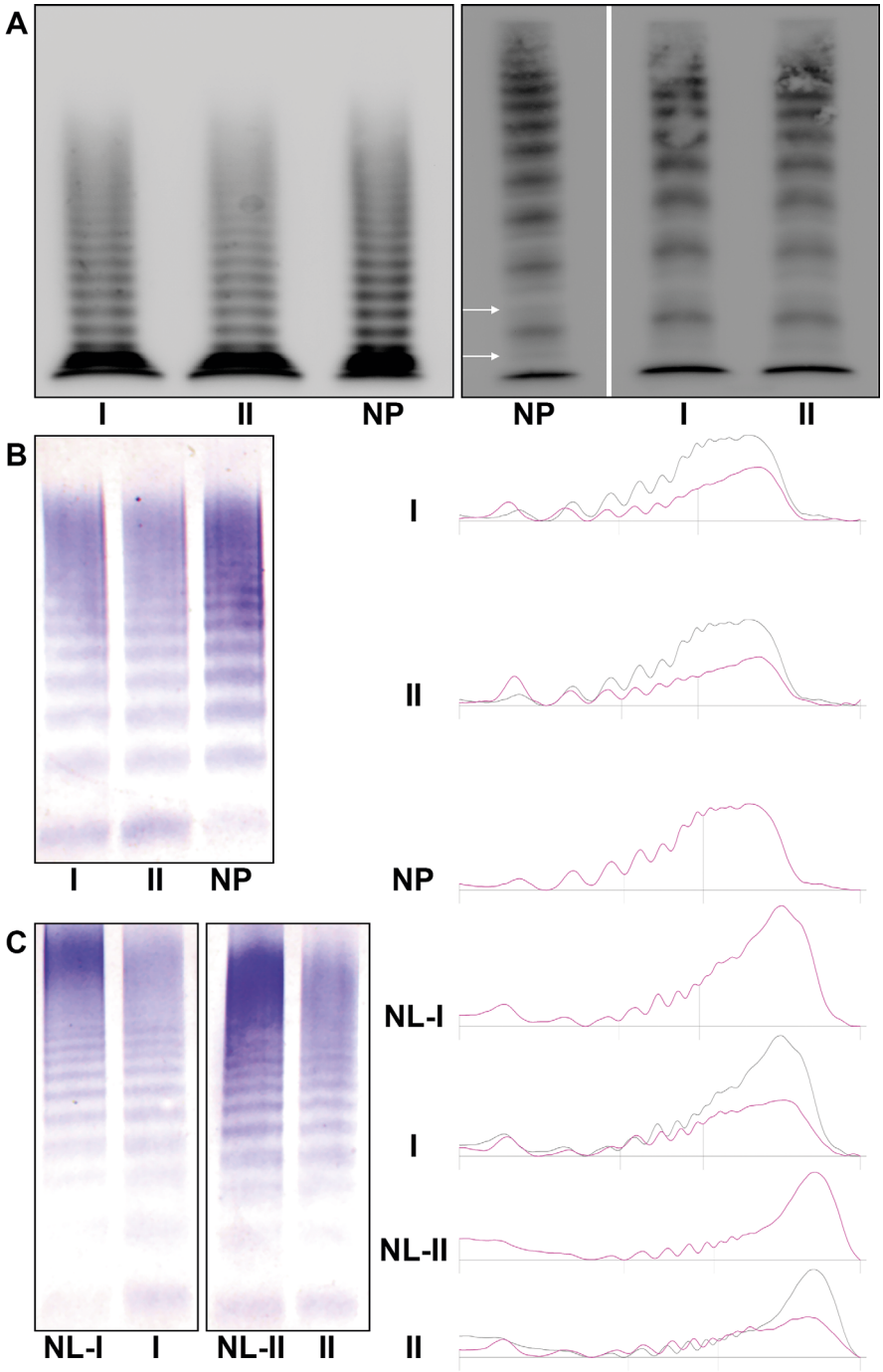
A
I
II
NP
NP
I
II
B
I
II
NP
I
II
NP
C
NL-I
I
NL-II
II
NL-I
I
NL-II
II

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.

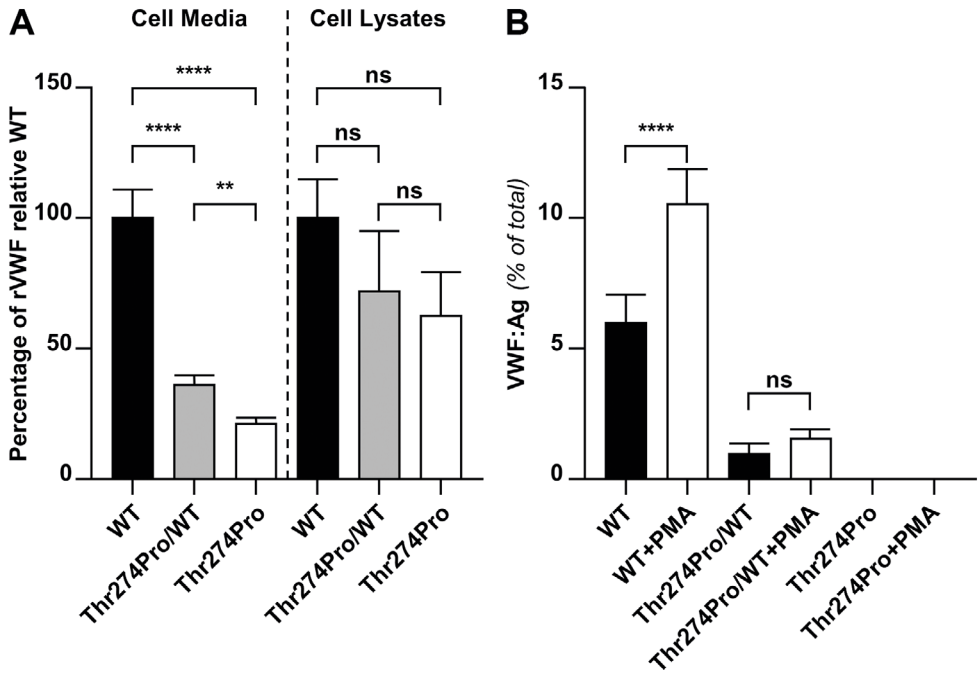

Figure 2. Basal and stimulated secretion of wild-type, hybrid and mutant recombinant von Willebrand factor. **(A)** basal secretion of wild-type (WT), hybrid (Thr274Pro/WT) and mutant (Thr274Pro) recombinant von Willebrand factor (rVWF) was measured using an ELISA. The amount of Thr274Pro/WT and Thr274Pro-rVWF 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.

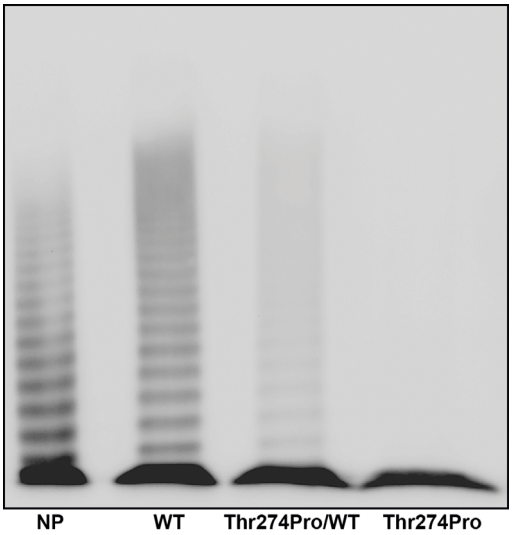

Figure 3. Multimeric analysis of rVWF. Multimeric structure of wild-type (WT), hybrid (Thr274Pro/WT) and mutant (Thr274Pro) recombinant von Willebrand factor (rVWF) were visualized in a non-reducing low-resolution gel (1.2% HGT agarose/0.1% SDS). NP, normal plasma was used as reference. Hybrid Thr274Pro/WT 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

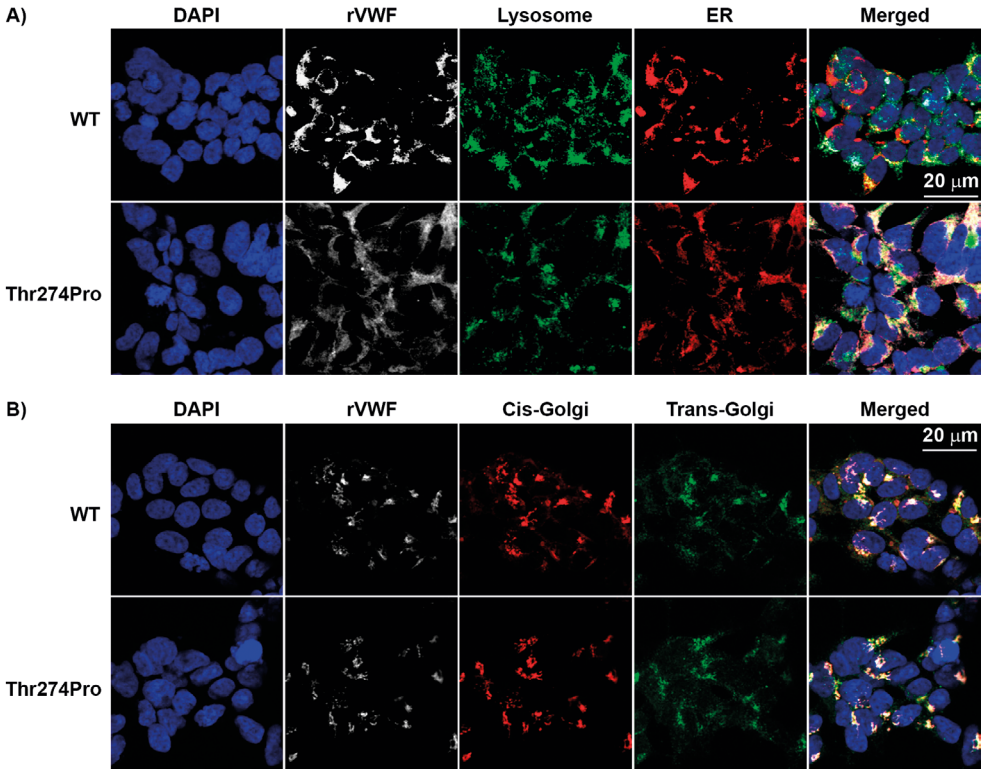

Figure 4. Intracellular localization of recombinant von Willebrand factor (VWF) in HEK293 cells using confocal microscopy. (A) HEK293 transiently transfected with wild-type (WT) or mutant Thr274Pro expression vectors were stained for VWF, lysosomes and endoplasmic reticulum (ER). Nuclei were stained 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

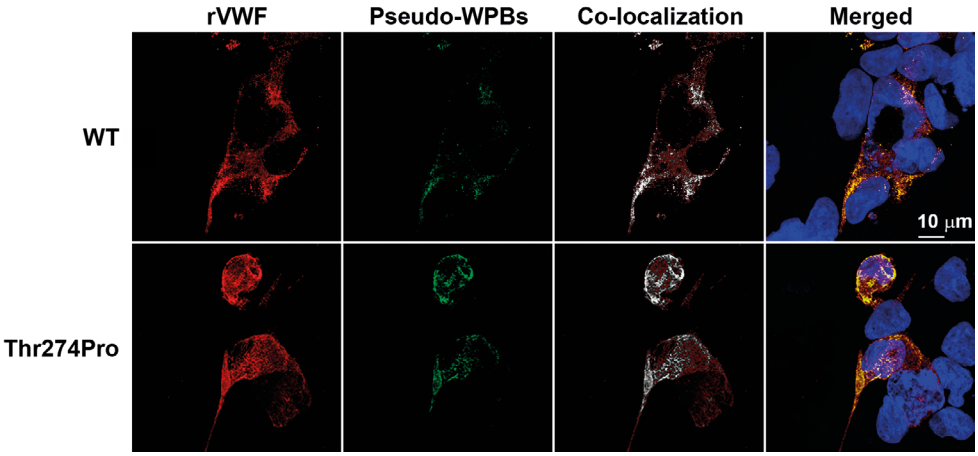

Figure 5. Pseudo Weibel Palade bodies immunofluorescence staining. HEK293 cells were transiently transfected with wild-type (WT) or mutant Thr274Pro expression vectors. Then, cells were stained for von Willebrand factor (VWF; red) and pseudo-Weibel Palade bodies (pseudo-WPBs; green) and nuclei (DAPI, 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.

ACKNOWLEDGEMENT

This work was partially supported by the Italian Ministry of Health-Bando Ricerca Corrente. The authors acknowledge L. F. Ghilardini for the illustration work and Prof. P.M. Mannucci for critical advice. Open Access Funding provided by Universita degli Studi di Milano within the CRUI-CARE Agreement.

REFERENCES

1. Sadler JE, Budde U, Eikenboom JC, Favaloro EJ, Hill FG, Holmberg L, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. J Thromb Haemost. 2006; 4:2103-2114.

2. Baronciani L, Peyvandi F. How we make an accurate diagnosis of von Willebrand disease. Thromb Res 2020; 196: 579-589.

3. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. J Thromb Haemost. 2003; 1:1335-1342.

4. Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. J Clin Invest. 1973; 52:2757-2764.

5. Vischer UM, Wagner DD. von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. Blood. 1994; 83:3536-3544.

6. Wagner DD, Mayadas T, Marder VJ. Initial glycosylation and acidic pH in the Golgi apparatus are required for multimerization of von Willebrand factor. J Cell Biol. 1986; 102:1320-1324.

7. Wagner DD, Olmsted JB, Marder VJ. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. J Cell Biol. 1982; 95:355-360.

8. Allen S, Abuzenadah AM, Hinks J, Blagg JL, Gursel T, Ingerslev J, et al. A novel von Willebrand disease-causing mutation (Arg273Trp) in the von Willebrand factor propeptide that results in defective multimerization and secretion. Blood. 2000; 96:560-568.

9. Baronciani L, Federici AB, Cozzi G, La Marca S, Punzo M, Rubini V, et al. Expression studies of missense mutations p.D141Y, p.C275S located in the propeptide of von Willebrand factor in patients with type 3 von Willebrand disease. Haemophilia. 2008; 14:549-555.

10. Cumming A, Grundy P, Keeney S, Lester W, Enayat S, Guilliatt A, et al. An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. Thromb Haemost. 2006; 96:630-641.

11. Eikenboom J, Hilbert L, Ribba AS, Hommais A, Habart D, Messenger S, et al. Expression et al. Expression of 14 von Willebrand factor mutations identified in patients with type 1 von Willebrand disease from the MCMDM-1VWD study. J Thromb Haemost. 2009; 7:1304-1312.

12. Goodeve A, Eikenboom J, Castaman G, Rodeghiero F, Federici AB, Batlle J, et al. 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.

13. James PD, Notley C, Hegadorn C, Leggo J, Tuttle A, Tinlin S, et al. The mutational spectrum of type 1 von Willebrand disease: Results from a Canadian cohort study. Blood. 2007; 109:145-154.

14. Obser T, Ledford-Kraemer M, Oyen F, Brehm MA, Denis CV, Marschalek R, et al. Identification and characterization of the elusive mutation causing the historical von Willebrand Disease type IIC Miami. J Thromb Haemost. 2016; 14:1725-1735.

15. Rosenberg JB, Haberichter SL, Jozwiak MA, Vokac EA, Kroner PA, Fahs SA, et al. The role of the D1 domain of the von Willebrand factor propeptide in multimerization of VWF. Blood. 2002; 100:1699-1706.

16. Rodeghiero F, Tosetto A, Abshire T, Arnold DM, Coller B, James P, et al. ISTH/SSC bleeding

assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. J Thromb Haemost. 2010; 8:2063-2065.

17. Federici AB, Canciani MT, Forza I, Mannucci PM, Marchese P, Ware J, et al. A sensitive ristocetin co-factor activity assay with recombinant glycoprotein Ibalpha for the diagnosis of patients with low von Willebrand factor levels. Haematologica. 2004; 89:77-85.

18. Rodeghiero F, Castaman GC, Tosetto A, Lattuada A, Mannucci PM. Platelet von Willebrand factor assay: results using two methods for platelet lysis. Thromb Res. 1990; 59:259-267.

19. Budde U, Scharf RE, Franke P, Hartmann-Budde K, Dent J, Ruggeri ZM. Elevated platelet count as a cause of abnormal von Willebrand factor multimer distribution in plasma. Blood. 1993; 82:1749-1757.

20. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015; 17:405-424.

21. Baronciani L, Federici AB, Punzo M, Solimando M, Cozzi G, La Marca S, et al. Type 2A (IIH) von Willebrand disease is due to mutations that affect von Willebrand factor multimerization. J Thromb Haemost. 2009; 7:1114-1122.

22. Baronciani L, Federici AB, Beretta M, Cozzi G, Canciani MT, Mannucci PM. Expression studies on a novel type 2B variant of the von Willebrand factor gene (R1308L) characterized by defective collagen binding. J Thromb Haemost. 2005; 3:2689-2694.

23. Pagliari MT, Baronciani L, Stufano F, Garcia-Oya I, Cozzi G, Franchi F, et al. von Willebrand disease type 1 mutation p.Arg1379Cys and the variant p.Ala1377Val synergistically determine a 2M phenotype in four Italian patients. Haemophilia. 2016; 22:e502-e511.

24. Baronciani L, Federici AB, Cozzi G, Canciani MT, Mannucci PM. Biochemical characterization of a recombinant von Willebrand factor (VWF) with combined type 2B and type 1 defects in the VWF gene in two patients with a type 2A phenotype of von Willebrand disease. J Thromb Haemost. 2007; 5:282-288.

25. Eikenboom J, Federici AB, Dirven RJ, Castaman G, Rodeghiero F, Budde U, et al. VWF propeptide and ratios between VWF, VWF propeptide, and FVIII in the characterization of type 1 von Willebrand disease. Blood. 2013; 121:2336-2339.

26. Haberichter SL, Castaman G, Budde U, Peake I, Goodeve A, Rodeghiero F, et al. Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: molecular and clinical markers for the diagnosis and management of type 1 VWD (MCMDM-1VWD). Blood. 2008; 111:4979-4985.

27. Wang JW, Bouwens EA, Pintao MC, Voorberg J, Safdar H, Valentijn KM, et al. Analysis of the storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from patients with von Willebrand disease. Blood. 2013; 121:2762-2772.

28. James PD, Connell NT, Ameer B, Di Paola J, Eikenboom J, Giraud N, et al. ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. Blood Adv. 2021; 5:280-300.

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