haematologica
Journal of the Ferrata Storti Foundation

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Received: May 7, 2025.
Accepted: August 8, 2025.

Citation: Bas Calcoen, Irina Portier, Inge Pareyn, Aline Vandenbulcke, Anna-Elina Lehtinen, Riitta Lassila, Claudia Tersteeg, Karen Vanhoorelbeke, Simon F. De Meyer and Timea Szanto. A novel p.C1130S variant in a Finnish family with a complex phenotype of von Willebrand disease.
Haematologica. 2025 Aug 28. doi: 10.3324/haematol.2025.287900 [Epub ahead of print]

A novel p.C1130S mutation in a Finnish family with a complex phenotype of von Willebrand disease

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Running heads:

A p.C1130S variant causes a complex VWD phenotype

Data-sharing statement:

Original data and protocols can be obtained upon request via the corresponding author.

Funding

This research was supported by a research grant (G062023N) from FWO Flanders (Fonds voor Wetenschappelijk Onderzoek Vlaanderen) to S.F.D.M. and by a grant from the Helsinki University Hospital competitive research fund (TYH2020318) to T.S. and R.L.

Author contributions

Conceptualization, B.C., I.P.1 (Irina Portier), T.S., K.V. and S.F.D.M.; methodology, B.C., I.P.1, T.S. I.P.2 (Inge Pareyn), A.V., A.E.L., R.L., K.V. and S.F.D.M., formal analysis, B.C., I.P.1, T.S., C.T., K.V. and S.F.D.M.; writing—original draft preparation, B.C., I.P.1, T.S., A.E.L., R.L. and S.F.D.M.; writing—review and editing, B.C., I.P.1, T.S., I.P.2, A.V., A.E.L., R.L, C.T., K.V. and S.F.D.M, visualization, B.C., I.P.1, T.S. and S.F.D.M.; supervision, T.S., C.T., K.V. and S.F.D.M.; funding acquisition, T.S., and S.F.D.M. All authors have read and agreed to this written manuscript.

Disclosure information

All authors have nothing to disclose.

Key words

von Willebrand disease, von Willebrand factor, Blood Coagulation and Fibrinolysis, Disorders of Coagulation and Fibrinolysis

Letter to editor

Von Willebrand disease (VWD) is the most common inherited bleeding disorder caused by mutations that lead to either a deficient or dysfunctional von Willebrand factor (VWF). VWF is crucial for normal hemostasis as it mediates platelet adhesion and stabilizes coagulation factor VIII (FVIII). VWD patients typically suffer from mucocutaneous bleeds. Their disease is classified into type 1 or type 3, characterized by reduced or absent levels of VWF, respectively or into type 2, which includes four subtypes and involves abnormal VWF function.[1,2] Although correct classification is important for optimal patient management, allocation of individual patients into this classification system is not always straightforward due to overlapping clinical and/or laboratory phenotypes of the different (sub)types.[2] The multiple protein and glycan interactions of VWF underlie the heterogeneous molecular basis of VWD. In this letter, we present a family with a complex VWD laboratory phenotype and share our clinical and experimental insights of a novel VWF variant (i.e., p.C1130S), which was identified in all family members. All patients gave a written informed consent, and this study was approved by the local ethical committee (HUS/1395/2018).

The family members (Figure 1) were recalled as part of a comprehensive re-evaluation of historical VWD diagnoses made in the Helsinki University Hospital Comprehensive Cancer Center.[3] The index patient, a 52-year old male, was initially diagnosed with type 2A VWD after experiencing prolonged bleeding following an adenoidectomy. He was treated on-demand, first with cryoprecipitates and later with plasma-derived VWF:FVIII or desmopressin (DDAVP) with good efficacy. Currently, he only reports minor bleeding episodes, which are managed using tranexamic acid. His four children (three daughters and one son) were also diagnosed with VWD during their early childhood due to mucocutaneous bleeds and were treated on-demand by either plasma-derived VWF:FVIII, DDAVP and/or tranexamic acid. We performed the following laboratory tests during the re-evaluation: platelet function analysis (PFA), FVIII activity (FVIII:C), VWF antigen (VWF:Ag), VWF platelet binding (VWF:GPIbM), VWF collagen binding (VWF:CB), FVIII binding of VWF (VWF:FVIIIB), VWF multimers and genetic sequencing of exons 2-52 of the *VWF* gene (Table 1). Briefly, the index patient showed a low VWF:Ag with a normal multimer pattern (Figure S1), and a normal VWF:Ag/GPIbM ratio, suggesting a type 1 VWD. However, the severely reduced FVIII:C and VWF:FVIIIB laboratory phenotype resembled VWD type 2N rather than type 1. Additionally, VWF:CB and VWF:CB/Ag ratio were low. Similar observations were noted in all four children, but with higher FVIII:C and borderline-to-normal VWF:FVIIIB, resulting in a high VWF:FVIIIB/Ag ratio. Based on the results, the phenotype of the children most likely resembled a collagen-binding-dominant type 2M VWD, particularly given that both VWF:GPIbM/Ag ratio and multimer pattern were normal in combination with a low VWF:CB/Ag ratio.

Genetic analysis of the index patient revealed compound heterozygous variants in the *VWF* gene. The first variant was c.2561G>A (p.R854Q), located in exon 20 (VWF D'-domain), and is observed in up to 73% of all VWD type 2N patients. The change from an arginine to a glutamine at position 2561 results in a loss of positive charge, which is thought to be responsible for the loss of FVIII binding to VWF.[4] The second variant was c.3388T>A (p.C1130S), located in exon 26 (VWF D3-domain), and represents a novel mutation. Interestingly, all four children were heterozygous for the novel c.3388T>A (p.C1130S) variant, while none of them carried the c.2561G>A (p.R854Q) variant.

Since all patients had low VWF:Ag, we evaluated *in vitro* VWF synthesis and secretion of both identified variants via transient transfection experiments in cultured Chinese Hamster Ovary (CHO) K1 cells. Site-directed mutagenesis was used to introduce each of the two variants into full-length human wild type (WT) VWF, using the pNUT-VWF-WT expression vector.[5] The primers used for each variant are given in Table S1. The resulting plasmids pNUT-VWFR854Q and pNUT-VWFC1130S were used in transfection experiments. To mimic the heterozygous conditions in this family, we performed co-transfection experiments using a combination of pNUT-VWFR854Q and pNUT-VWFC1130S (the index patient) and a combination of pNUT-VWF-WT and pNUT-VWFC1130S (the children). For completeness, we also included co-transfections with pNUT-VWF-WT and pNUT-VWFR854Q and homozygous transfections with either pNUT-VWFR854Q or pNUT-VWFC1130S. As a negative control, mock transfections were performed without plasmids (mock control). Forty-eight hours

after transfection, VWF:Ag levels in the collected medium were determined via an in-house enzyme-linked immunosorbent assay (ELISA). Each transfection condition was performed five times and the mean VWF levels, corrected for transfection efficiency (Figure S2), are depicted in Figure 2. Compared to WT *VWF* (set to 100%), homozygous expression of both the *VWF* p.R854Q and the *VWF* p.C1130S variant was significantly lower (45.7 ± 9.3 % and 35.7 ± 8.6 %, respectively, $p < 0.0001$). Accordingly, co-transfection with p-NUT-VWFR854Q and p-NUT-VWFC1130S plasmids led to similar low VWF levels in the expression medium (45.9 ± 13.2 %). When the *VWF* p.R854Q or *VWF* p.C1130S variant was co-expressed with WT *VWF*, expression was partly recovered, resulting in VWF levels of 69.0 ± 14.1 % and 75.3 ± 8.3 %, respectively.

Remarkably, despite being heterozygous for the p.R854Q substitution, the index patient had undetectable VWF:FVIIIB levels. The recessive nature of VWD type 2N implies that the presence of either a homozygous 2N mutation or a compound heterozygous with another mutation (i.e., type 2N or null allele) is necessary to cause a 2N phenotype. The p.C1130S substitution in the VWF D3-domain may also reduce FVIII binding to VWF, as this interaction relies on contact points within the D3-region.[6] The variant disrupts Cys^{1130}, which normally forms a disulfide bond with Cys^{1126} or Cys^{1149}, potentially altering the domain's structure and impairing FVIII binding.[7] Along the same lines, substitution of Cys^{1130} with a phenylalanine or arginine has been previously reported to result in impaired FVIII binding to VWF.[8,9] The loss of this cysteine could also explain the borderline VWF:FVIIIB values and lowered FVIII:C levels of the children in our study, due to their heterozygosity for the p.C1130S substitution. One limitation of our study was the inability to reliably determine VWF:FVIIIB and assess VWF multimer distribution, owing to low VWF concentrations (i.e., low transfection efficacy) in the media. Besides the known effect of p.R854Q on FVIII binding, our data surprisingly suggest that this mutation may also impair *in vitro* VWF production or secretion. This finding aligns with other studies of reduced secretion of the p.R854Q variant after transient transfection in multiple cell lines.[10,11] Moreover, similar to our findings, homozygous expression of the recombinant variant p.R854W in HEK293 cells severely decreased the secretion of this variant into the medium, whereas co-transfection with WT *VWF* and p.R854W *VWF* yielded intermediate results.[11] In contrast, patients with type 2N VWD carrying the p.R854Q variant typically exhibit normal VWF:Ag levels, which may be due to heterozygosity permitting normal VWF production – unless when there is a null allele – and the variant's specific impact on FVIII binding rather than on VWF production or secretion.

As mentioned, the novel p.C1130S variant, which was identified in all family members, leads to a loss in an intrachain disulphide bond, possibly locally distorting its secondary structure.[7] Such misfolding may decrease the production or secretion, as observed in our experiments. Our finding of the novel variant impairing VWF production or secretion is corroborated by other studies showing intracellular retention of VWF variants in which the cysteines Cys^{1130} or Cys^{1149} are lost.[12,13] Although we and Tjernberg et al[14] did not observe a dominant-negative effect of the loss of Cys^{1130} on WT *VWF* when co-expressed, Eikenboom et al[12] noticed that the p.C1149R variant inhibited the secretion of WT *VWF* subunits up to 35 %. In all family members, we noticed much lower *ex vivo* VWF:Ag levels than we experimentally observed *in vitro*. We hypothesize that this discordance may be due to increased *in vivo* clearance of the VWF variants. Indeed, a DDAVP trial within the index patient induced a strong initial response followed by a rapid decline in VWF:Ag levels four hours post-DDAVP (data not shown). Such a reduced *in vivo* survival of VWF post-DDAVP administration has also been observed in patients carrying either the p.C1130F or p.C1149R variant.[8,15] Collectively, our findings support and extend existing evidence that the D3 domain constitutes a hotspot for VWF variants linked to increased clearance.

Altogether, we highlighted the effects of a novel p.C1130S variant identified in a family with a complex VWD phenotype and showed that it causes reduced VWF production or secretion *in vitro*. Alone, or together with the p.R854Q variant, this novel variant most likely explains the low VWF:Ag and perhaps even the low VWF:FVIIIB levels observed in this family. As the D3-domain contributes to overall conformation, the p.C1130S variant may alter spatial arrangement of distal domains, possibly explaining the collagen binding defect observed in the children. Future studies should focus on further unravel the pathophysiological mechanisms by which both p.R854Q and p.C1130S variants impact VWF production, secretion and clearance by, for example, measuring intracellular VWF:Ag, analyzing VWF mRNA expression and/or pre- and post-

DDAVP levels of VWF propeptide. Especially the latter is of importance since these results may impact therapeutic guidance on DDAVP use in patients carrying the novel p.C1130S variant.

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Tables and Figure Legends

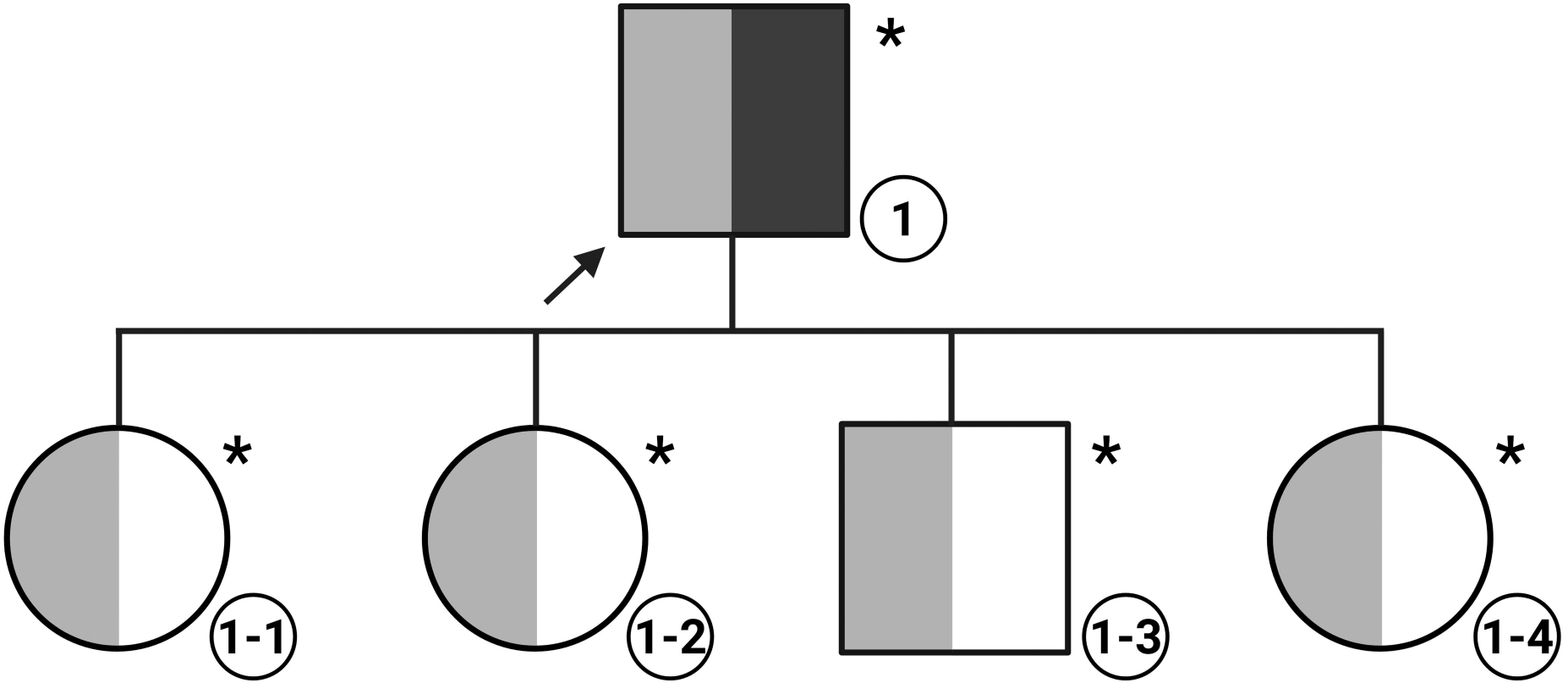
Table 1. Laboratory test results and genetic analysis of the index patient and his four children.

Laboratory test	Patient ID 1	Patient ID 1-1	Patient ID 1-2	Patient ID 1-3	Patient ID 1-4
Age at recall (years) *	52	21	19	17	15
Sex (F/M)	M	F	F	M	F
Blood group (A/B/AB/O^{RhD})	A^-	A^+	A^+	O^+	A^+
PFA-200 (sec) [C/EPI: 82 – 150 sec] [C/ADP: 62 – 100 sec]	C/EPI > 300 C/ADP > 300	C/EPI > 300 C/ADP > 300	C/EPI > 300 C/ADP > 300	C/EPI > 300 C/ADP > 300	C/EPI > 300 C/ADP > 300
VWF:Ag (IU/dL) [50 – 190 IU/dL]	16	19	26	17	17
VWF:GPIbM (IU/dL) ** [50 – 190 IU/dL]	12	15	17	15	13
VWF:GPIbM/Ag ratio (#) [≥ 0.7]	0.75	0.79	0.65	0.88	0.76
VWF:CB (IU/dL) [50 – 172 IU/dL]	10	10	15	9	9
VWF:CB/Ag (#) [≥ 0.7]	0.63	0.53	0.58	0.53	0.53
FVIII:C (IU/dL) [52 – 148 IU/dL]	6	41	38	25	33
VWF:FVIIIB (IU/dL) [50 – 100 IU/dL]	< 10	63	67	49	51
VWF:FVIIIB/Ag ratio (#) [≥ 0.75]	NC	3.32	2.58	2.88	3.00
Genetic analysis ***	c.2561G>A c.3388T>A	c.3388T>A	c.3388T>A	c.3388T>A	c.3388T>a

* Recall in 2015. Fasting peripheral venous blood samples were collected via cubital venipuncture using a BD Vacutainer® system at a time remote from acute infection, inflammation, or surgery. Citrated whole blood samples were centrifuged at 2000 g for 10 min and plasma aliquots were separated and stored at -70 °C if testing could not be immediately performed. PFA was done on a PFA-200® analyser (Siemens Healthcare Diagnostics, Marburg, Germany). Both VWF:Ag and VWF:GPIbM were measured on a BCS XP analyser (Siemens, Marburg, Germany). A Standard Human plasma (ORKL 13, Siemens) was used as a reference for both assays. VWF:CB was assessed via a Technozym® kit (Technoclone, GmbH, Vienna, Austria) by EVOLIS analyser (Bio-Rad Laboratories, Berkely, CA, USA). VWF:FVIIIB was measured via the Asserachrom® VWF:FVIIIB assay (stago Diagnostica, Düsseldorf, Germany). The binding of FVIII to VWF was compared to a normal control (set at 100 IU/dL), a homozygous type 2N control and a heterozygous type 2N control (Department of Clinical Chemistry, University and Regional Laboratories, Region Skåne, Sweden). FVIII:C was assessed using a one-stage assay and a Pathromtin SL and Coagulation FVIII deficient plasma (OTXW 13, Siemens,) was used as a reference. ** The VWF:GPIbM assay measures the VWF binding to recombinant gain-of-function mutant GPIbα fragments. *** Genetic analysis included screening of exons 2-52 of the human *VWF* gene (East Genomic Laboratory Hub, Addenbrooke's Treatment Centre, Cambridge University Hospital NHS Foundation Trust, UK). Fluorescent sequencing analysis included Mutation Surveyor of exons 20 and 26 of the *VWF* gene. Reference values of each quantitative assay or ratio are given between square brackets. Abbreviations: VWF = von Willebrand factor, Ag = antigen, GPIbM = platelet binding, CB = collagen binding, FVIIIB = factor VIII binding, RhD: Rhesus D, PFA = platelet function analyser, C/EPI = collagen/epinephrine cartridge, C/ADP = collagen/adenosine diphosphate cartridge, FVIII:C = FVIII coagulant activity, NC = not calculated.

Figure 1. Family pedigree of the index patient. Males and females are indicated by squares and circles, respectively. The arrow points at the index patient of this report. All individuals were symptomatic and are indicated with an asterisk plus an ID label (1, 1-1, 1-2 ,1-3 and 1-4). Individuals carrying the c.3388T>A and c.2561G>A variant are filled in light gray and black, respectively. While the index patient carried both mutations on two different alleles, his four children carried the c.3388T>A variant on one allele in combination with a wild type allele (white half). This figure was created using *Biorender*, the online scientific image and illustration software (www.biorender.com).

Figure 2. VWF secretion of transiently transfected CHO K1 cells. Secretion levels of WT or variant *VWF* in the medium of transfected CHO K1 cells were determined via the VWF:Ag ELISA 48 hours after transfection. The VWF secretion levels were first corrected for the transfection efficacy of each condition before being expressed relative to the amount of secreted WT *VWF*. VWF secretion levels of an Fmock condition (pure jetPRIME® buffer) were measured as a negative control. The tested homozygous conditions included WT, C1130S and R854Q and heterozygous conditions included C1130S with R854Q, C1130S with WT and R854Q with WT. **** One-way ANOVA: $p < 0.0001$, *** One-way ANOVA: $p < 0.001$, ** One-way ANOVA: $p < 0.01$. Statistical analysis was compared to the WT condition. All experimental conditions were performed five times. Error bars represent mean ± SD. Abbreviations: VWF = von Willebrand factor, WT = wild type, CHO = Chinese hamster ovary, ELISA = enzyme-linked immunosorbent assay, SD = standard deviation. Statistical analysis was performed using GraphPad Prism (v9.0.0 for Windows, GraphPad Software, USA). A p-value < 0.05 was considered as statistically significant.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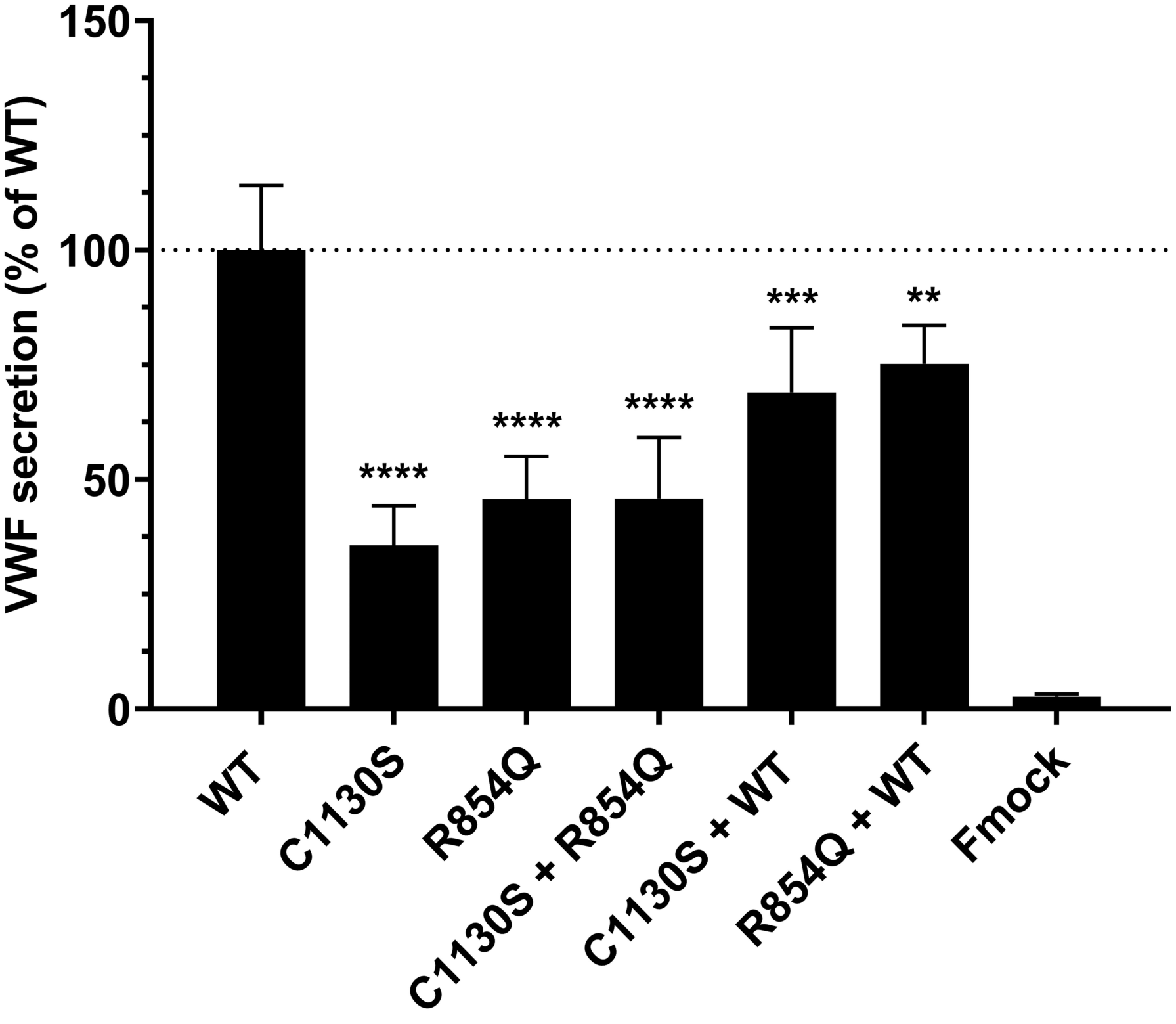
150
100
50
0
VWF secretion (% of WT)
****
****
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***
**
WT
C1130S
R854Q
C1130S + R854Q
C1130S + WT
R854Q + WT
Fmock

A novel p.C1130S mutation in a Finnish family with a complex phenotype of von Willebrand disease

1. *Supplementary Figures and Tables*

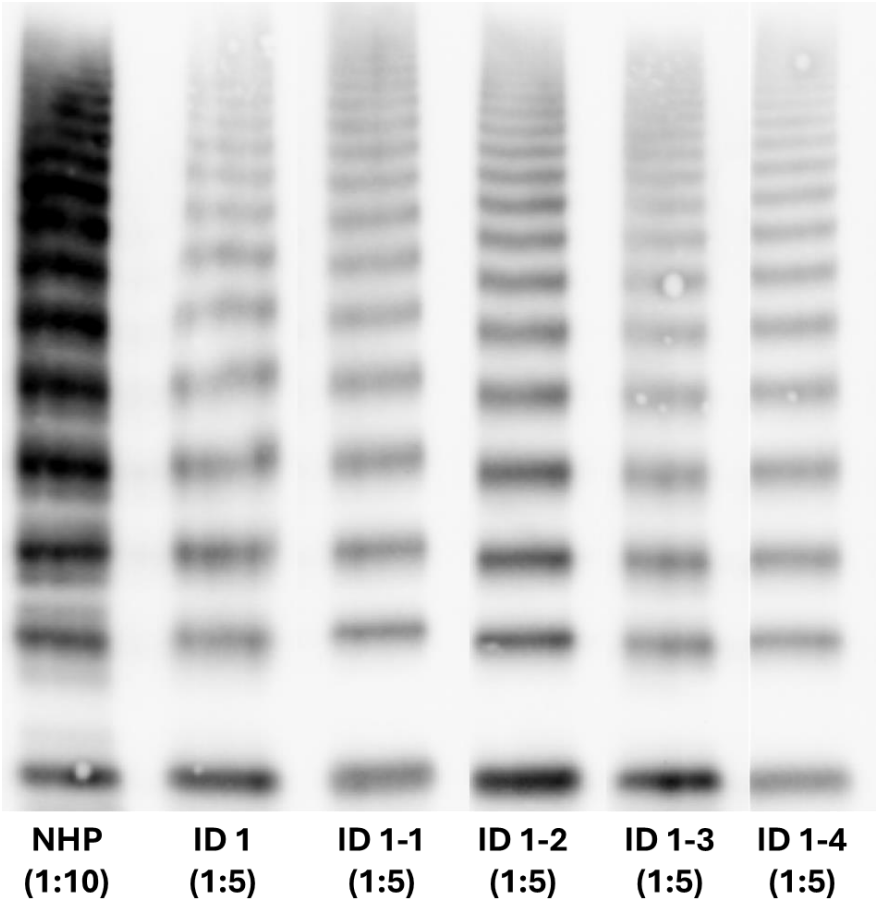


Figure S1. VWF multimer analysis of all family members. VWF multimer analysis was carried out via a 1.5% sodium dodecyl sulphate (SDS) agarose gel electrophoresis, followed by Western blotting and luminescent visualization using polyclonal anti-VWF antibodies labelled with alkaline phosphatase, which was recorded by photo-imaging. For loading the gel, sample dilution of both normal pooled human plasma (i.e;, NHP, normal control), and patient plasmas were performed following the standard procedure described by Budde et al.[1] NHP was loaded onto the gel at a 1:10 dilution, whereas all patient samples were loaded at a 1:5 dilution, as all had VWF:Ag levels below 50 IU/dL. The lanes represent, from left to right, the NHP, the index patient (ID 1), his son (ID 1-3) and his three daughters (ID 1-1, ID 1-2 and ID 1-4). No clearly visible abnormalities in VWF multimers were seen in any of the patients, but a possible subtle loss of the largest VWF multimers might be missed due to limited analytical sensitivity of the assay. Therefore, a densitometric analysis was performed (see Addendum Figure S1 on page 4 of this supplementary file) to assess the number of peaks on the multimer curves. Abbreviations: VWF = von Willebrand factor, SDS = sodium dodecyl sulfate, NHP = normal human plasma pool.

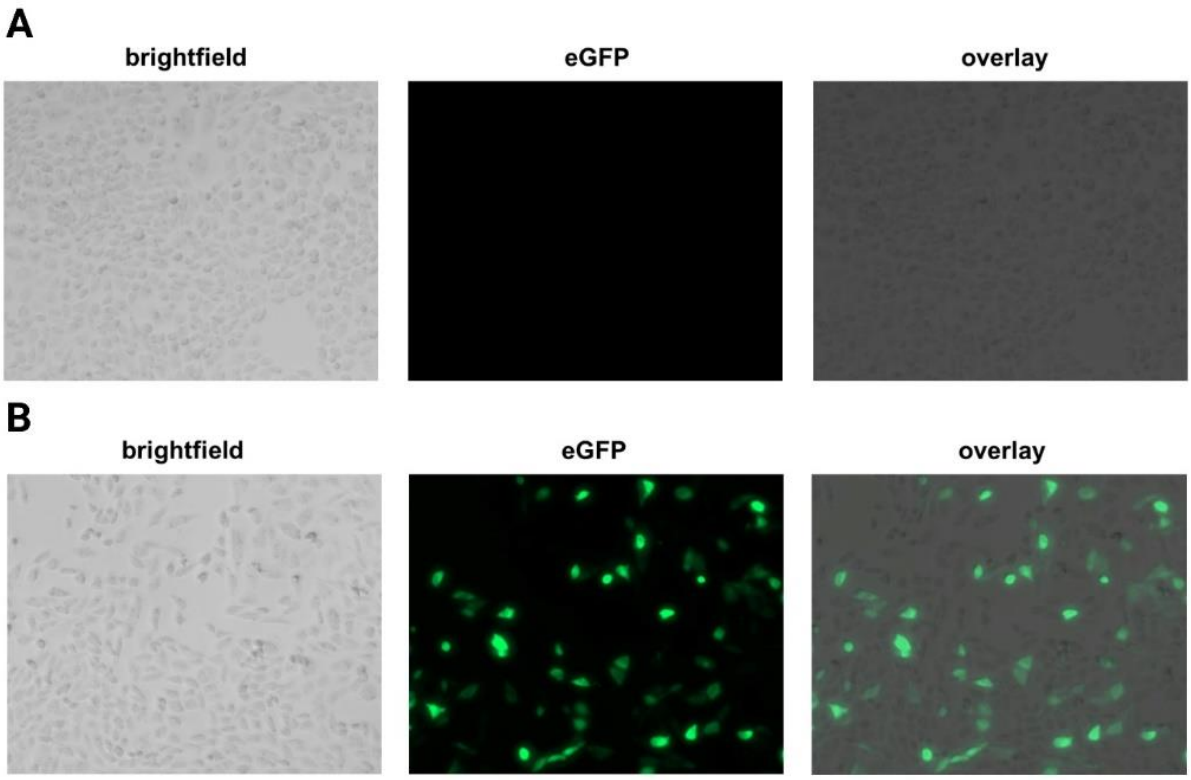
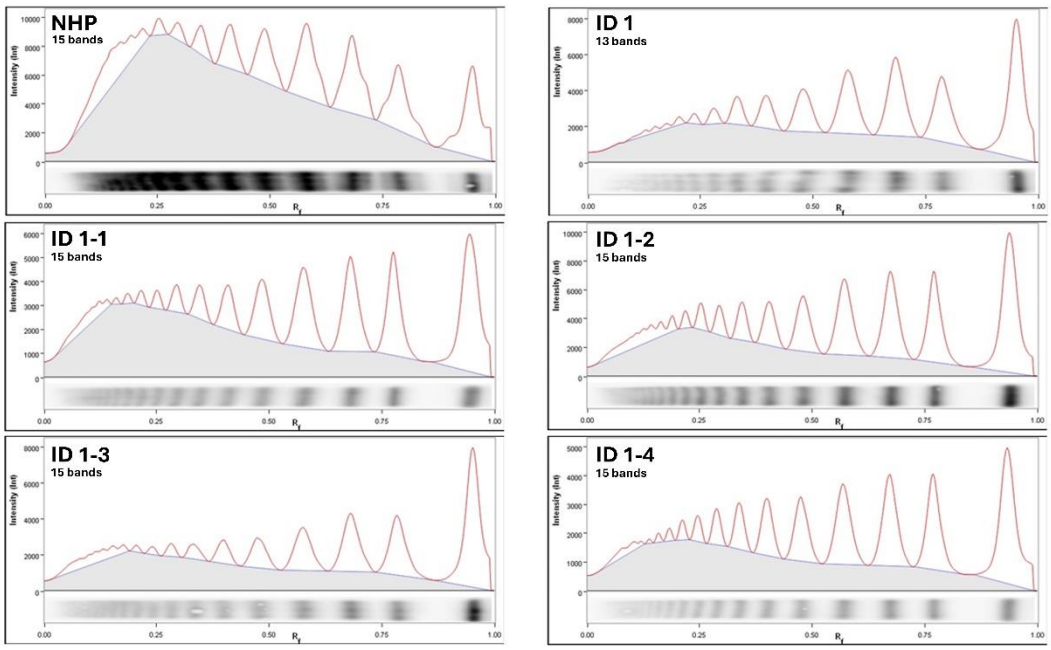


Figure S2. Determination of transfection efficacy in the CHO K1 cells. CHO K1 cells were cultured at 37 °C with 5 % CO_2, in Kaighn's modification of Ham's F12 medium (ref: 21127-022, Invitrogen, Carlsbad, CA) supplemented with 1 % antibiotic-antimycotic (ref: 15240-062, Invitrogen, Carlsbad, CA) and 10 % fetal calf serum (ref: 10500-064, Invitrogen, Carlsbad, CA). Transfection was performed using the jetPRIME® Transfection Reagent (Polyplus transfection, Illkirch, France) according to the manufacturer's instructions. For each transfection, a 3:1 jetPRIME® to DNA ratio was used, with 6 µg of DNA per transfection. When testing heterozygous conditions, 3 µg of both expression plasmids were used. To determine transfection efficiency, 0.6 µg of a Green Fluorescent Protein (GFP)-expressing plasmid (i.e. pMax-GFP; Lonza, Basel, Switzerland) was additionally co-transfected. As a negative control, an Fmock condition containing pure jetPRIME® buffer was studied. Visualization of GFP expression was done via fluorescence microscopy (Zeiss Axio observer fluorescence microscope, Carl Zeiss AG, Oberkochen, Germany). For each flask, both the amount of GFP-expressing CHO K1 cells and total amount of CHO K1 cells were counted on three different fields 48 h after transfection. **A)** Brightfield, eGFP and overlay pictures of the negative Fmock control condition. **B)** Brightfield, eGFP and overlay pictures under an arbitrarily selected test condition to exemplify a condition with a transfection efficacy of 31.02 %. Abbreviations: CHO = Chinese hamster ovary, GFP = green fluorescence protein.



Addendum Figure S1. Densitometry analyses of the VWF multimers of all family members. The results showed that all family members had at least 13 detectable bands, with the father (i.e., ID 1) exhibiting the fewest. In comparison, the normal human plasma pool (NHP) showed 15 bands. After thorough discussion among experts, it was concluded that all family members had a normal VWF multimer pattern, as the presence of at least 13 out of 15 bands was not considered indicative for a pathological multimer distribution. Abbreviations: VWF = von Willebrand factor, NHP = normal human plasma pool.

Table S1. Primers used for site-directed mutagenesis. Site directed mutagenesis was done using the QuikChange® II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and was performed according to the manufacturer's instructions.

Primer	Sequence	Plasmid
VWF_mutR854Q_FP	GTGTCTGTCGGGACCAGAAGTGGAACTGCAC	pNUT-VWFR854Q
VWF_mutR854Q_RP	GTGCAGTTCCACTTCTGGTCCCGACAGACAC	pNUT-VWFR854Q
VWF_mutC1130S_FP	GCCCCCAGAGCAGCGAGGAGAGG	pNUT-VWFC1130S
VWF_mutC1130S_RP	CCTCTCCTCGCTGCTCTGGGGGC	pNUT-VWFC1130S

2. *References supplementary data*

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