

# The common *VWF* single nucleotide variants c.2365A>G and c.2385T>C modify *VWF* biosynthesis and clearance

Ahmad H. Mufti,<sup>1,\*</sup> Kenichi Ogiwara,<sup>2,\*</sup> Laura L. Swystun,<sup>2</sup> Jeroen C. J. Eikenboom,<sup>3</sup> Ulrich Budde,<sup>4</sup> Wilma M. Hopman,<sup>5</sup> Christer Halldén,<sup>6</sup> Jenny Goudemand,<sup>7</sup> Ian R. Peake,<sup>1</sup> Anne C. Goodeve,<sup>1</sup> David Lillicrap,<sup>2</sup> and Daniel J. Hampshire,<sup>1</sup> on behalf of the European Group on von Willebrand disease (EU-VWD) and Zimmerman Program for the Molecular and Clinical Biology of von Willebrand disease (ZPMCB-VWD) Study Groups

<sup>1</sup>Haemostasis Research Group, Department of Infection, Immunity & Cardiovascular Disease, University of Sheffield, Sheffield, United Kingdom; <sup>2</sup>Richardson Laboratory, Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada; <sup>3</sup>Eindhoven Laboratory for Vascular and Regenerative Medicine, Department of Thrombosis and Hemostasis, Leiden University Medical Center, Leiden, The Netherlands; <sup>4</sup>Hämostaseologie, Medilys Laborgesellschaft mbH, Hamburg, Germany; <sup>5</sup>Department of Public Health Sciences, Queen's University, Kingston, ON, Canada; <sup>6</sup>Biomedicine, Kristianstad University, Kristianstad, Sweden; and <sup>7</sup>Centre de Référence Maladie de Willebrand, Hématologie, Hôpital Cardiologique, Lille, France

## Key Points

- *VWF* variants c.2365A>G and c.2385T>C independently influence *VWF* biosynthesis and clearance, increasing *VWF* plasma levels.
- Commonly inherited *VWF* variants can directly influence the protein and may contribute to hemostatic and thrombotic disease risk/severity.

Plasma levels of von Willebrand factor (*VWF*) vary considerably in the general population and this variation has been linked to several genetic and environmental factors. Genetic factors include 2 common single nucleotide variants (SNVs) located in *VWF*, rs1063856 (c.2365A>G) and rs1063857 (c.2385T>C), although to date the mechanistic basis for their association with *VWF* level is unknown. Using genotypic/phenotypic information from a European healthy control population, *in vitro* analyses of recombinant *VWF* expressing both SNVs, and *in vivo* murine models, this study determined the precise nature of their association with *VWF* level and investigated the mechanism(s) involved. Possession of either SNV corresponded with a significant increase in plasma *VWF* in healthy controls ( $P < .0001$ ). *In vitro* expression confirmed this observation and highlighted an independent effect for each SNV ( $P < .0001$  and  $P < .01$ , respectively), despite close proximity and strong linkage disequilibrium between them both. The influence of c.2365A>G on *VWF* levels was also confirmed *in vivo*. This increase in *VWF* protein corresponded to an increase in *VWF* messenger RNA (mRNA) resulting, in part, from prolonged mRNA half-life. In addition, coinheritance of both SNVs was associated with a lower *VWF* propeptide-to-*VWF* antigen ratio in healthy controls ( $P < .05$ ) and a longer *VWF* half-life in *VWF* knockout mice ( $P < .0001$ ). Both SNVs therefore directly increase *VWF* plasma levels through a combined influence on *VWF* biosynthesis and clearance, and may have an impact on disease phenotype in both hemostatic and thrombotic disorders.

## Introduction

von Willebrand factor (*VWF*) is an important plasma glycoprotein that performs 2 major hemostatic functions. It recruits platelets to sites of vascular injury and carries coagulation factor VIII (FVIII), protecting FVIII from degradation while conveying it to sites of vascular injury.<sup>1</sup> There is considerable natural variation in *VWF* plasma levels; between 50% and 200% of average normal levels are seen in 95% of the general population.<sup>2</sup>

In humans, there is strong evidence of a genetic influence on *VWF* levels. In pedigree analyses, the attributable genetic influence is ~30%,<sup>3</sup> whereas twin studies have shown that it is ~70%.<sup>4</sup> It is well

known that the ABO blood group locus accounts for ~30% of this heritable influence on VWF levels,<sup>5,6</sup> but recent association studies have also highlighted the influence of genetic variation at the *VWF* locus. The Cohorts for Heart and Aging Research in Genome Epidemiology study indicated that the common *VWF* single nucleotide variant (SNV) rs1063857 was significantly associated with interindividual variation in plasma VWF levels ( $P = 1.7 \times 10^{-32}$ ).<sup>7</sup> Furthermore, a study of African Americans identified several *VWF* SNV influencing levels of the protein including the frequently inherited rs1063856, found to result in a significant increase in VWF levels ( $1.05 \times 10^{-9}$ ).<sup>8</sup> Other studies have reported similar observations for rs1063856,<sup>9-11</sup> rs1063857,<sup>12-15</sup> or both.<sup>16</sup>

rs1063856 (c.2365A>G; p.T789A) and rs1063857 (c.2385T>C; p.Y795=) are reported to be in strong linkage disequilibrium (LD;  $r^2 = 0.980-1.000$ )<sup>17</sup> and have a minor allele frequency of ~33% (ranging from 9% in east Asian populations to 64% in African populations).<sup>17,18</sup> They are located in *VWF* exon 18, which encodes part of the D'D3 assembly, functionally important for binding of VWF to FVIII (VWF:FVIII). Notably, both rs1063856<sup>7,8</sup> and rs1063857<sup>19</sup> have also been found to have a significant influence on FVIII procoagulant activity (FVIII:C). However, despite their significant association with VWF and FVIII levels, no studies have investigated the mechanistic basis for this influence. The aim of this study was therefore to investigate the mechanism(s) by which these SNVs are able to influence VWF and FVIII levels using a combined in vitro and in vivo approach.

## Methods

### Study population and genotypic/phenotypic analysis

European (predominantly white) healthy control individuals ( $n = 1166$ ; Table 1) were recruited as part of the Molecular and Clinical Markers for the Management and Diagnosis of Type 1 von Willebrand Disease (MCMDM-1VWD) study. Available phenotypic data for VWF antigen (VWF:Ag), ristocetin cofactor activity (VWF:RCo), collagen binding (VWF:CB), propeptide (VWFpp), FVIII:C, and VWF:FVIII were measured as previously described.<sup>20-24</sup> Genotypic analysis of SNV c.2365A>G and c.2385T>C was performed using the MassARRAY matrix-assisted laser desorption ionization–time of flight system (Sequenom Inc, San Diego, CA) as described.<sup>25</sup> Experiments involving healthy control individuals were performed with the approval of the Sheffield Research Ethics Committee. In accordance with the Declaration of Helsinki, informed consent was obtained from all individuals at recruitment by signing a consent form in the individual's own language.

### Generation of human and murine VWF expression plasmids

A pcDNA3.1/Hygro<sup>(-)</sup> mammalian expression plasmid (Thermo Fisher Scientific, Paisley, United Kingdom) containing full-length human *VWF* complementary DNA (cDNA; vWF-pcDNA3.1) was generated as previously described.<sup>26</sup> Plasmids expressing c.2365A>G (p.T789A), c.2385T>C (p.Y795=) or both SNVs in *cis* (c.[2365A>G;2385T>C]; p.[T789A;Y795=]) were generated using a QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies LDA UK Ltd, Stockport, United Kingdom). Successful mutagenesis was confirmed via Sanger sequence analysis.

Murine *Vwf* (mVWF) cDNA was cloned into pSC11 under the transthyretin promoter as previously described.<sup>27</sup> Amino acid alignment demonstrated that the human *VWF* (hVWF) variants were poorly conserved across species (see Figure 6A), with nucleotide alignment between mVWF and hVWF indicating that mVWF encodes the nonreference (NR) hVWF allele for each corresponding SNV (see Figure 6B). mVWF was “humanized” by site-directed mutagenesis using a QuikChange II XL kit (Stratagene, La Jolla, CA) to alter the sequence to c.[2365G>A;2367T>C] (p.A789T), c.2385C>T (p.Y795=) or c.[2365G>A;2367T>C;2385C>T] (p.[A789T;Y795=]).

### In vitro measurement of VWF:Ag

HEK293T cells were maintained in Dulbecco modified Eagle medium containing GlutaMAX supplemented with 10% vol/vol fetal bovine serum (Thermo Fisher Scientific). Cell transfections were conducted in 9.6 cm<sup>2</sup> wells using Xfect (Takara Bio Europe S.A.S., Saint-Germain-en-Laye, France). To summarize, 2.5 μg of wild-type (WT) or variant VWF expression plasmid (or 1.25 μg of WT and variant VWF for 50:50 cotransfections) was transfected and left to incubate at 37°C/5% CO<sub>2</sub> for 24 hours before replacement of the culture media. Following a further 48-hour incubation, culture media was collected and the cells lysed in 300 μL of 1× passive lysis buffer (Promega UK Ltd, Southampton, United Kingdom). Measurement of VWF:Ag in collected media and lysates was performed using a matched-pair antibody human VWF enzyme-linked immunosorbent assay (ELISA; Enzyme Research Laboratories Ltd, Swansea, United Kingdom) according to the manufacturer's specifications.

### mRNA analysis

HEK293T cells were cultured and transfected as stated and harvested cells were centrifuged at 4000g for 5 minutes. Messenger RNA (mRNA) was extracted from pelleted cells using an EZ-RNA total RNA isolation kit (GeneFlow Ltd, Lichfield, United Kingdom) and reverse transcribed using a QuantiTect reverse transcription kit (Qiagen Ltd, Manchester, United Kingdom) following manufacturer guidelines. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays measuring both the target (*VWF*; Hs01109446\_m1) and an endogenous control ( $\beta_2$  microglobulin [*B2M*]; Hs00984230\_m1) in tandem were performed on a 7900HT real-time PCR system using TaqMan gene expression master mix (Thermo Fisher Scientific) according to manufacturer guidelines. Following assay completion, calculation of  $\Delta\Delta$  cycle threshold<sup>28</sup> was used to indicate relative VWF RNA quantity.

mRNA half-life was measured using the same qRT-PCR protocol, but at 24 hours posttransfection, cells were treated with 5 μg/mL actinomycin D (Thermo Fisher Scientific). Cells were harvested at 0, 2, 3, and 4 hours posttreatment and half-life calculated as the time at which mRNA level was 50% of the level detected at 0 hours posttreatment.<sup>29</sup>

### Hydrodynamic injection

Expression of mVWF cDNA in vivo was induced by hydrodynamic tail vein injection in *VWF*/FVIII double-knockout (DKO) C57Bl/6 mice.<sup>30</sup> Plasma was collected via cardiac puncture 3 days postinjection. VWF:Ag was measured by ELISA using Dako (Glostrup, Denmark) anti-VWF antibodies for capture and detection

**Table 1. Population characteristics and regression analysis of VWF levels in the healthy control cohort**

Variable	Median (IQR)	c.2365A>G		c.2385T>C	
		Coefficient $\beta$ (95% CI)	P	Coefficient $\beta$ (95% CI)	P
VWF:Ag, n = 1156	97 IU/dL (75, 121)	7.184 (4.380 to 9.988)	<.001	6.986 (4.173 to 9.799)	<.001
VWF:RCo, n = 1155	87 IU/dL (65, 113)	2.215 (-1.721 to 6.151)	.270	1.744 (-2.173 to 5.661)	.382
FVIII:C, n = 1161	101 IU/dL (77, 127)	1.112 (-2.477 to 4.701)	.543	0.719 (-2.846 to 4.284)	.692
VWF:CB, n = 239	80 IU/dL (46, 119)	0.748 (-10.754 to 12.250)	.898	0.178 (-11.097 to 11.454)	.975
VWFpp, n = 389	118 IU/dL (104, 135)	1.172 (-2.674 to 5.018)	.549	0.897 (-2.922 to 4.716)	.644
VWFpp/VWF:Ag, n = 387*	1.24 (1.05, 1.48)	-0.060 (-0.114 to -0.006)	.030	-0.056 (-0.111 to -0.002)	.043
VWF:FVIIIb, n = 181†	1.09 (0.98, 1.20)	0.011 (-0.027 to 0.050)	.563	0.013 (-0.026 to 0.051)	.520

ABO blood group data were available for n = 1124 (n = 481 [42.8%] blood group O).

CI, confidence interval; IQR, interquartile range.

\*n = 387 (1 individual was excluded from analyses because he or she was classified as an outlier [mean + 3 standard deviations]).

†n = 181 of 1166 healthy control individuals (median age, 39 years [range, 4-100]; n = 497 [42.6%] male).

(A0082 and P0226). All animal experiments were performed with the approval of the Queen's University Animal Care Committee.

### VWF half-life evaluation and FVIII stabilization

VWF knockout (KO) mice received 200 U/kg WT or p.[A789T; Y795=] plasma-derived mVWF (FVIII-free; produced in VWF/FVIII DKO mice) by tail-vein injections. VWF:Ag was quantified prior to plasma pooling, and reconstituted for both WT and p.[A789T; Y795=] to equimolar concentrations (20 U/mL) using VWF/FVIII DKO mouse pooled plasma as required as previously described.<sup>31,32</sup> VWF:Ag levels were measured by Dako ELISA and endogenous murine FVIII:C was measured by chromogenic assay (Chromogenix Coatest SP Factor VIII; Diapharma Group Inc, West Chester, OH). VWF:Ag was measured relative to a 5-minute recovery (set to 100%) and half-life evaluated using a 1-phase exponential decay model in GraphPad Prism version 7.03 (GraphPad Software Inc, La Jolla, CA).

### Statistical and in silico analysis

Statistical analyses were performed using GraphPad. Comparisons of  $\geq 3$  groups were performed using either a 1-way/2-way analysis of variance (ANOVA; to compare mean values) or a Kruskal-Wallis test (to compare median values).<sup>33</sup> Multivariable linear regression analysis to test associations between variables was performed as previously described.<sup>34</sup> All models were adjusted for age and ABO blood group (and either SNV c.2365A>G or c.2385T>C; 14 models in total) using IBM SPSS v24.0 for Windows 2016 (Armonk, New York, NY).

In silico protein, RNA, and splicing predictions were performed using several online tools as specified in supplemental Table 1. In silico analysis to determine LD was performed using Haploview v4.2.<sup>35</sup>

## Results

### Association of c.2365A>G and c.2385T>C with VWF levels

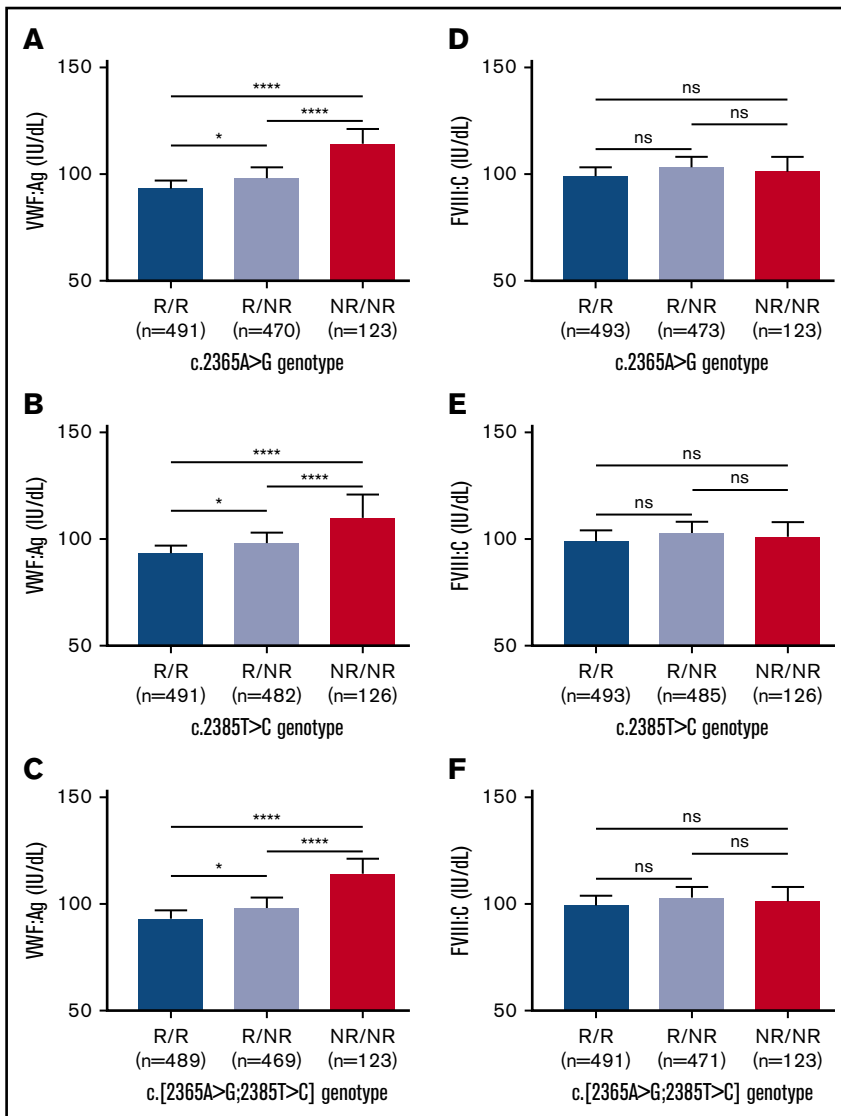
To confirm previous observations, that is, that c.2365A>G and c.2385T>C act as frequent small-scale genetic modifiers, the association between VWF SNV genotype and VWF levels was investigated in 1166 healthy control individuals (HCs). Analysis

confirmed that the NR allele for each SNV was associated with significantly higher VWF:Ag levels ( $P < .0001$ ; reference [R]/R, 93 and 93 IU/dL; R/NR, 98 and 98 IU/dL; NR/NR, 114 and 110 IU/dL c.2365A>G and c.2385T>C, respectively; Figure 1A-B) and when both SNV were combined ( $P < .0001$ ; R/R, 93 IU/dL; R/NR, 98 IU/dL; NR/NR, 114 IU/dL; Figure 1C). This association was also observed when accounting for ABO blood group (O,  $P = .0005$ ; non-O,  $P = .0009$ ; supplemental Figure 1A-B), which was confirmed by linear regression analysis (Table 1). However, analysis of FVIII:C levels showed no association with genotype (Figure 1D-F; Table 1), although, there was a trend toward increased FVIII:C levels associated with the SNV NR allele in the HCs with O blood group, but not in those with non-O blood group (supplemental Figure 1C-D). Likewise, SNV genotype was not associated with VWF:RCo or VWF:CB levels (Table 1; supplemental Figure 2A-B).

### In vitro expression of recombinant VWF protein and mRNA

In silico analysis of the HC genotype data confirmed that the SNV were in strong LD ( $r^2 = 0.991$ ) suggesting that the observed association with VWF:Ag levels could be attributable to only 1 of the 2 SNVs or to both. To investigate this possibility and to verify the observed association in HCs, VWF-expressing plasmids encoding each SNV independently or together in *cis* were generated and VWF expression compared against a WT plasmid. Expression of both SNVs independently and together in *cis* resulted in an increase in both retained and secreted VWF expression ( $P < .0001$ ,  $P < .01$ , and  $P < .0001$ , respectively; Figure 2). Despite the increased expression, no significant differences were observed in intracellular localization or pseudo-Weibel-Palade body formation between WT and variant VWF (supplemental Methods; supplemental Figure 3).

Given that both SNVs appeared to have an independent effect on VWF:Ag level, in silico and/or in vitro tools were used to predict whether nonsynonymous SNV c.2365A>G would have an impact on the VWF protein and also to predict whether either SNV would have an influence on mRNA processing or stability. c.2365A>G was not predicted to affect the protein structure/function and neither SNV was predicted to affect microRNA-binding sites nor influence splicing (supplemental Methods; supplemental Table 1; supplemental Figure 4). However, both were predicted to affect mRNA secondary structure (supplemental Table 1; supplemental



**Figure 1. Association between SNV genotype and median VWF:Ag and FVIII:C levels in HCs.** (A) SNV c.2365A>G and VWF:Ag levels. (B) SNV c.2385T>C and VWF:Ag levels. (C) Both SNVs in *cis* and VWF:Ag levels. (D) SNV c.2365A>G and FVIII:C levels. (E) SNV c.2385T>C and FVIII:C levels. (F) Both SNVs in *cis* and FVIII:C levels. Genotypes compared using a Mann-Whitney *U* test (\**P* < .05; \*\*\*\**P* < .0001). Bars indicate 95% CI. NR, nonreference allele; ns, not significant; R, reference allele.

Figure 5), which is known to influence mRNA stability and translation efficiency.<sup>36</sup> Analysis of cDNA isolated from cells expressing each SNV independently showed an increase in VWF mRNA expression (*P* < .01 and *P* < .05, respectively; Figure 3A-B), with an additive increase observed when both SNV were expressed in *cis* (*P* < .0001; Figure 3C).

### Determination of mRNA half-life

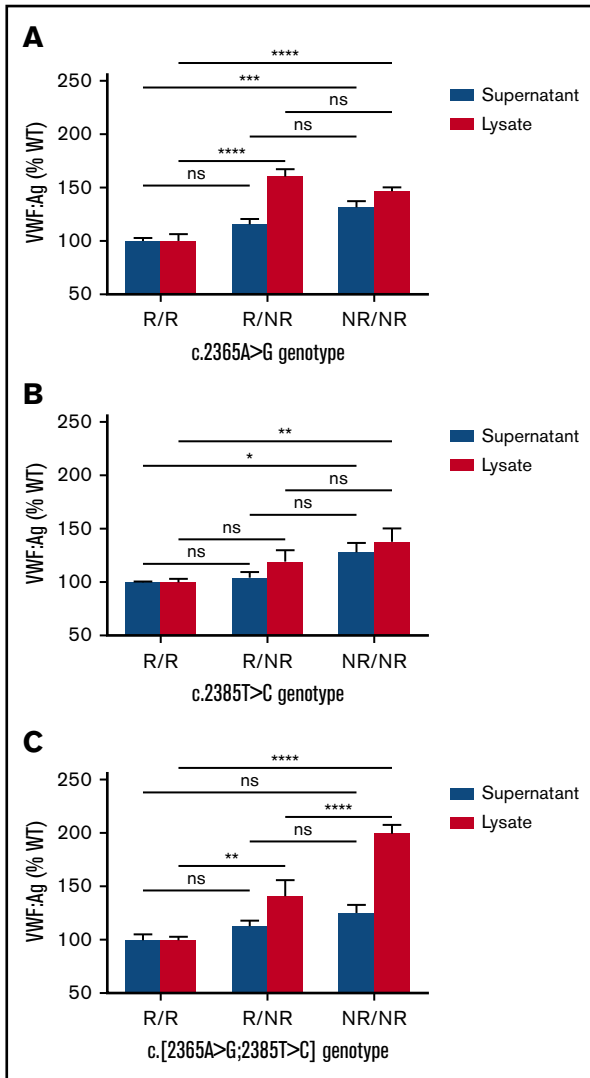
Based on the observation that both SNVs result in increased mRNA expression and a predicted effect on mRNA secondary structure, the decision was taken to compare the half-life of the mRNA generated by the WT expression plasmid against the half-life of mRNAs generated by variant expression plasmids. The data indicated that compared with the WT, both SNVs independently prolong the half-life of the expressed VWF mRNA (Figure 4A-B). In addition, the presence of both SNV in *cis* demonstrated a cumulative increase in mRNA half-life (Figure 4C-D), highlighting a direct correlation between prolonged mRNA half-life and the observed increase in VWF mRNA and protein expression.

### Association of c.2365A>G and c.2385T>C with VWF activity

There was no observed difference in the multimer profiles of the expressed recombinant VWF (supplemental Figure 6). In addition, and in contrast to the previously reported type 2N mutation c.2365A>C (p.T789P),<sup>37</sup> neither SNV had a significant effect on VWF:FVIII:B when investigated in HCs (Table 1; supplemental Figure 2C) or when investigated in vitro (supplemental Methods; supplemental Table 2). However, analysis did highlight that the combined variant haplotype, p.[T789A;Y795=] VWF, had a slightly decreased binding affinity to FVIII compared with WT VWF which was likely due to a faster dissociation rate in spite of a similar association rate (supplemental Methods; supplemental Table 2).

### Association of c.2365A>G and c.2385T>C with VWFpp and VWFpp/VWF:Ag ratio

Similar to the observed association between SNV genotype and VWF:Ag levels, the NR allele was also linked to higher VWFpp

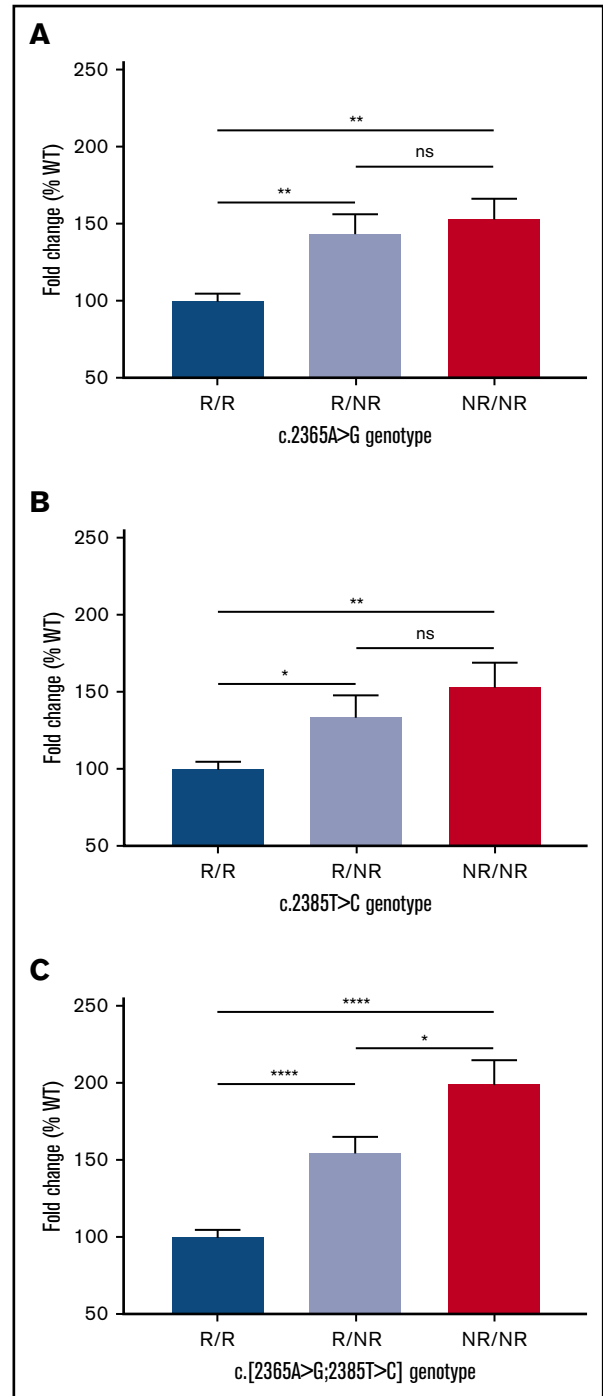


**Figure 2. In vitro expression of recombinant VWF protein.** (A) SNV c.2365A>G. (B) SNV c.2385T>C. (C) Both SNVs in *cis*. Genotypes compared using a 2-way ANOVA with the Tukey multiple comparisons test (\* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ ; \*\*\*\* $P < .0001$ ). Mean values for  $n = 3$  triplicate measurements are shown (bars indicate standard error of the mean).

levels in HCs (R/R, 117 IU/dL; R/NR, 119 IU/dL; NR/NR, 122 IU/dL), although this increase was not significant (Table 1; supplemental Figure 2D). However, analysis did highlight a significant association between the NR allele and reduced VWFp/VWF:Ag ratio ( $P < .05$ ; Figure 5), which was confirmed independently for both c.2365A>G ( $P = .03$ ) and c.2385T>C ( $P = .043$ ) using regression analysis (Table 1).

### Characterization of VWF expression and half-life in vivo

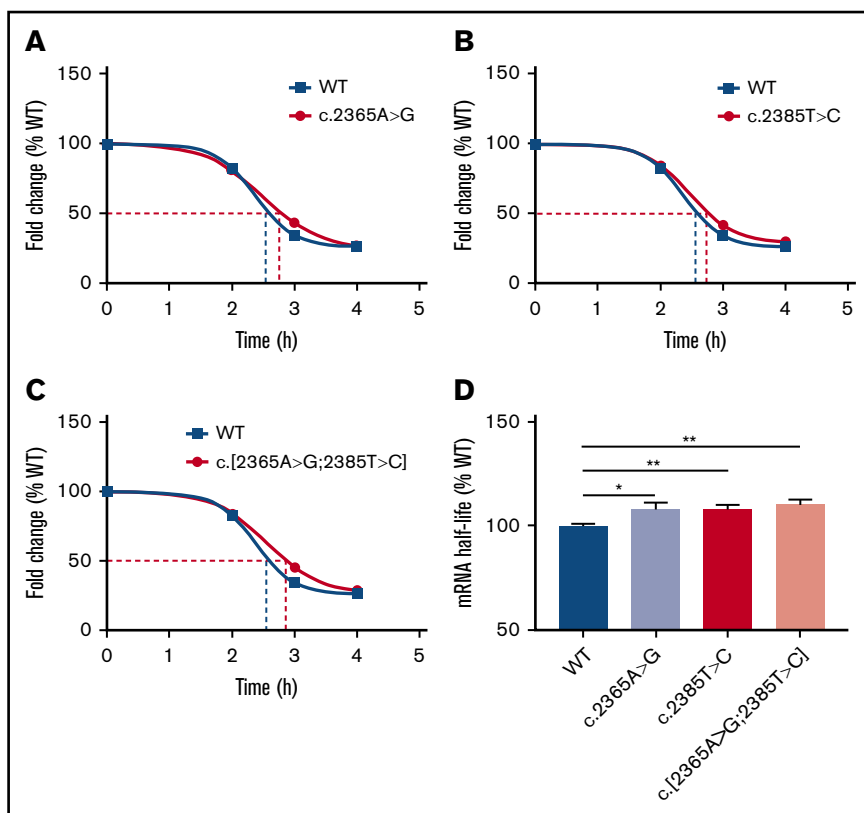
The influence of the c.[2365A>G;2385T>C] genotype on VWF was also investigated using an in vivo mVWF model to complement the in vitro findings. Expression of the single “humanized” mVWF variants (c.[2365G>A;2367T>C]; p.A789T and c.2385C>T; p.Y795=) or the combined in *cis* variant haplotype (c.[2365G>A;



**Figure 3. In vitro expression of recombinant VWF mRNA.** (A) SNV c.2365A>G. (B) SNV c.2385T>C. (C) Both SNVs in *cis*. Genotypes compared using an unpaired Student *t* test (\* $P < .05$ ; \*\* $P < .01$ ; \*\*\*\* $P < .0001$ ). Mean values for  $n = 3$  triplicate measurements are shown (bars indicate standard error of the mean).

2367T>C;2385C>T]; p.[A789T;Y795=]) was assessed 3 days posthydrodynamic tail-vein injection of mVWF cDNA into VWF/FVIII DKO mice. c.[2365G>A;2367T>C] ( $P < .0001$ ) and both mVWF variants in *cis* ( $P < .0001$ ) had decreased expression compared with WT, whereas c.2385C>T expression alone did not ( $P = .773$ )





**Figure 4. mRNA half-life of recombinant VWF.** WT VWF (half-life [ $t_{1/2}$ ] = 2:36 hours) compared against (A) SNV c.2365A>G ( $t_{1/2}$  = 2:51 hours; 8.1% increase); (B) SNV c.2385T>C ( $t_{1/2}$  = 2:49 hours; 7.7% increase). (C) Both SNVs in *cis* ( $t_{1/2}$  = 2:54 hours; 10.0% increase). (D) Comparison of fold change in mRNA half-life between genotypes. Genotypes compared using an unpaired Student *t* test (\* $P$  < .05; \*\* $P$  < .01). Bars indicate standard error of the mean.

(Figure 6C). The influence of the combined in *cis* variant haplotype on plasma VWF:Ag levels was compared with WT over a 4-week period. For all time points, the humanized mVWF demonstrated lower plasma expression when compared with WT mVWF (Figure 6D).

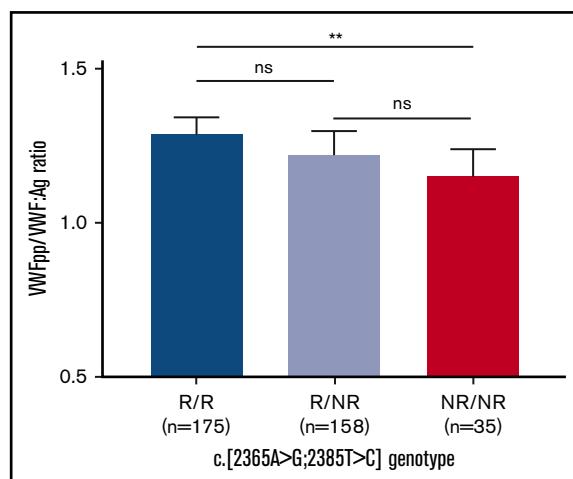
Plasma-derived mVWF that was FVIII-free was generated by hydrodynamic injection of the mVWF cDNA into VWF/FVIII DKO mice. Pooled plasma was then infused by tail vein injection into VWF KO mice and VWF half-life and endogenous FVIII:C stabilization were measured. Both SNV in *cis* had a shorter half-life than WT (2.53 vs 5.98 hours;  $P$  < .0001) (Figure 6E) and impaired stabilization of endogenous FVIII:C (area under the curve > 80% decrease;  $P$  < .001) (Figure 6F).

## Discussion

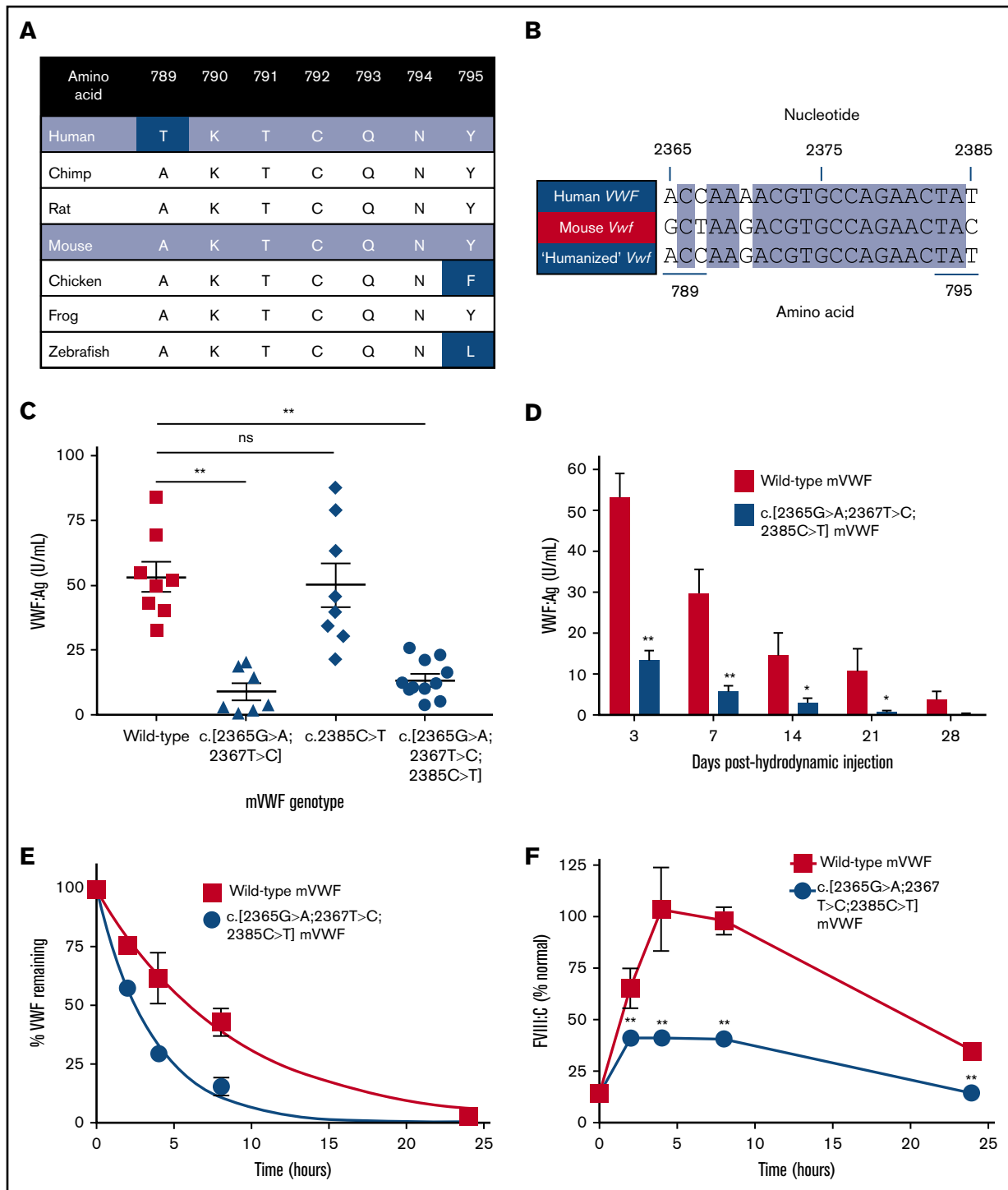
This study used both in vitro and in vivo analyses with the aim of determining how VWF SNV c.2365A>G and c.2385T>C influence VWF and FVIII levels. Previous studies had linked both SNV to variation in both VWF plasma and FVIII activity levels. Analysis in a European HC population has confirmed that both act as frequent small-scale genetic modifiers of VWF, showing that inheritance of the NR allele of each SNV is linked to a significant increase in VWF:Ag.

However, analysis in this HC population notably did not show any significant correlation with FVIII:C. Plasma VWF is known to circulate in excess to plasma FVIII under normal conditions.<sup>38</sup> Consequently, increases in VWF levels will have a limited impact on FVIII levels, which is confirmed by recent data highlighting that for every 1% change in VWF:Ag there is only a corresponding 0.54% change in FVIII:C levels.<sup>39</sup> This may suggest that a larger sample

cohort than the HC population used in this study would be required to observe an impact of these SNV on FVIII. The fact that previous studies investigating these SNV consistently reported lower levels of significance for FVIII:C associations ( $P = 3.6 \times 10^{-9}$ ,  $P = 1.24 \times 10^{-6}$  and  $P = .02$ , respectively)<sup>7,8,10</sup> compared with VWF:Ag ( $P = 1.7 \times 10^{-32}$ ,  $P = 1.05 \times 10^{-9}$  and  $P = .006$ , respectively)<sup>7,8,10</sup> supports this hypothesis.



**Figure 5. Association between SNV c.[2365A>G;2385T>C] genotype and median VWFpp/VWF:Ag ratio in healthy controls.** Genotypes compared using a Mann-Whitney *U* test (\*\* $P$  < .01). Bars indicate 95% CI.



**Figure 6. Influence of SNV on VWF expression and half-life in a murine model.** (A) Alignment of VWF amino acids p.789-795 across species. (B) Alignment of VWF nucleotides c.2365-2385 between human and mouse. (C) Expression of murine c.[2365G>A;2367T>C], c.2385C>T and c.[2365G>A;2367T>C;2385C>T] in vivo.  $**P < .0001$ . (D) Expression of wild-type and c.[2365G>A;2367T>C;2385C>T] murine VWF 4 weeks posthydrodynamic injection. (E) Half-life of plasma-derived murine VWF in VWF knockout mice. (F) Stabilization of endogenous FVIII:C in VWF knockout mice.

In vitro expression of recombinant VWF not only verified that the NR alleles resulted in higher VWF:Ag levels, but also highlighted that both SNV independently increased VWF levels. Despite an overall increase in total VWF expression, under all expressed conditions

the NR allele corresponded to a greater increase in retained VWF. In vivo, VWF undergoes extensive posttranslational modification and complex storage/secretion processes,<sup>40</sup> but this regulated secretory pathway is not present in HEK293T cells.<sup>41</sup> Given that the

mean levels of VWF secreted remained similar regardless of whether c.2365A>G and c.2385T>C were expressed independently or together in *cis* (R/NR: 115, 104, 113; NR/NR: 131, 128, 125), it seems plausible that the HEK293T cells were only able to process and release the produced VWF at a similar rate, giving rise to the greater increase in retained VWF observed.

Despite both SNV resulting in increased VWF levels, neither had a direct effect on VWF protein structure/function or any effect on mRNA splicing (supplemental Methods; supplemental Figure 4). This is not unexpected because any major impact on protein function or mRNA splicing would likely result in a more profound (disease-causing) phenotype which would be observed in a substantial number of individuals (given a general population frequency of ~33% for both SNV). Instead, the increased levels of VWF correspond to an increase in mRNA resulting, at least in part, from both SNV prolonging the mRNA half-life. The reason for this increased mRNA half-life is likely due to the influence of both SNV on mRNA secondary structure as this is known to influence RNA stability, which in turn impacts mRNA half-life.<sup>36</sup> The fact that replacement of an A or T nucleotide by either a C or G (which is the case for both SNV) is reported to increase mRNA stability,<sup>42</sup> supports this argument.

Plasma levels of VWF can be regulated by both biosynthetic and clearance-related mechanisms.<sup>40</sup> Support for the influence of c.2365A>G and c.2385T>C genotype on clearance was observed in a recent association study which demonstrated that while the NR allele for c.2365A>G was linked to an increase in both VWF:Ag and VWFpp levels, the effect size observed was smaller for VWFpp, which the authors attributed to a clearance-dependent mechanism.<sup>43</sup> Data on VWFpp levels (although not significant, likely due to data only being available for n = 389 HCs) in this current study confirm the effect size observation, whereas the VWFpp/VWF:Ag ratios support the clearance-dependent mechanism hypothesis as the NR allele was associated with a lower VWFpp/VWF:Ag ratio.

Finally, VWF/FVIII DKO mice expressing the humanized reference allele demonstrated decreased VWF:Ag levels compared with WT mVWF, consistent with human plasma VWF:Ag results. mVWF expressing both SNVs also had a shorter half-life than WT, consistent with the VWFpp and VWFpp/VWF:Ag ratio observations in the HCs and as previously reported.<sup>43</sup> Importantly, accelerated clearance of mVWF expressing both SNVs impaired stabilization of endogenous FVIII:C, which may account in part for the association of these SNVs with plasma FVIII:C in normal individuals. Although the influence of the SNVs was more pronounced for humanized mVWF than observed in HCs, this may relate to differences in the physicochemical influence of these SNVs on hVWF vs mVWF, and recognition by their respective clearance receptors. Alterations in the VWF amino acid sequence, glycosylation pattern and 3D conformation may influence VWF clearance by cells in the liver or spleen.<sup>40</sup> Although the specific mechanistic basis by which these SNV modify VWF half-life is not defined, it is plausible that the changes alter the affinity of mVWF for 1 or more VWF clearance receptors.

There is increasing evidence that frequently observed SNVs can affect protein function, expression, and clearance. Increased understanding of the genetic factors influencing VWF levels has important implications given that variation in VWF levels is associated

with susceptibility to both bleeding and thrombosis. Nonfunctional or reduced levels of VWF can result in von Willebrand disease (VWD).<sup>44</sup> Coinheritance of c.2365A>G and/or c.2385T>C with a known pathogenic VWF mutation causing VWD could potentially influence patient phenotype, for example, increasing VWF levels resulting in a less severe phenotype, and may contribute to reduced penetrance/variable expressivity observed in type 1 VWD.<sup>44</sup>

High VWF levels have been associated with increased risk of venous thrombosis (VT), myocardial infarction and ischemic stroke.<sup>45-47</sup> Inheritance of c.2365A>G has already been associated with an increased risk of VT in female patients<sup>48</sup> and as this SNV and c.2385T>C result in increased VWF levels, they could both confer an increased disease risk in other thrombotic disorders. Likewise, African Americans have a higher risk of thrombosis compared with other racial groups.<sup>49</sup> Given that the reported minor allele frequency for these SNVs is 57% in African Americans, compared with ~33% in Caucasians and 9% in East Asians,<sup>17</sup> it is plausible that these SNVs may contribute to this increased risk.

In conclusion, both c.2365A>G and c.2385T>C independently cause an increase in VWF plasma levels. This is due to an influence on VWF biosynthesis (similar to the c.7970G>A [p.R2657Q] gain-of-function variant previously reported in mice)<sup>50</sup> resulting from increased mRNA production of variant VWF and to secreted variant VWF protein having a greater half-life in the circulation. Inheritance of either or both SNVs could have a significant impact on the risk/severity of hemostatic and/or thrombotic disorders. In addition, these findings highlight that common SNVs can have a direct influence on protein levels and care should be taken before classifying common SNVs as “neutral” or “benign.”

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## Authorship

Contribution: I.R.P., A.C.G., D.L., and D.J.H. initiated the study; A.C.G., D.L. and D.J.H. coordinated the study; A.H.M., K.O., L.L.S., D.L., and D.J.H. designed the study and interpreted the data; A.H.M., K.O., L.L.S., J.C.J.E., U.B., C.H., and J.G. generated the data; A.H.M., K.O., L.L.S., W.M.H., and D.J.H. analyzed the data; A.H.M., K.O., L.L.S., and D.J.H. wrote the paper; L.L.S., A.C.G., D.L., and D.J.H. revised the paper; and all authors reviewed and approved the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Complete lists of the members of the EU-VWD and ZPMCB-VWD Study Groups appear in “Appendix.”

ORCID profiles: L.L.S., 0000-0001-9620-8401; J.C.J.E., 0000-0002-3268-5759; C.H., 0000-0002-9355-3901; A.C.G., 0000-0002-6177-7062; D.J.H., 0000-0002-1387-8926.



Correspondence: David Lillicrap, Richardson Laboratory, Department of Pathology and Molecular Medicine, Queen's University, 88 Stuart St, Kingston, ON K7L 3N6, Canada; e-mail: david.lillicrap@queensu.ca; and Daniel J. Hampshire, Haemostasis Research Group, Department of Infection, Immunity and Cardiovascular Disease, Faculty of Medicine, Dentistry and Health, University of Sheffield, Beech Hill Rd, Sheffield S10 2RX, United Kingdom; e-mail: d.hampshire@shef.ac.uk.

## Appendix: study group members

The members of the EU-VWD Study Group are: J. Batlle (A Coruña, Spain), E. Berntorp (Malmö, Sweden), I. Bodó (Budapest, Hungary),

U.B. (Hamburg, Germany), G. Castaman (Florence, Italy), J.C.J.E. (Leiden, The Netherlands), A. B. Federici (Milan, Italy), A. Gadisseur (Antwerp, Belgium), A.C.G. (Sheffield, United Kingdom), J.G. (Lille, France), M. Laffan (London, United Kingdom), F. Leebeek (Rotterdam, The Netherlands), P. M. Mannucci (Milan, Italy), I.R.P. (Sheffield, United Kingdom), F. Peyvandi (Milan, Italy), F. Rodeghiero (Vicenza, Italy), R. Schneppenheim (Hamburg, Germany), A. Tosetto (Vicenza, Italy), and A. Veyradier (Paris, France). The members of the ZPMCB-VWD Study Group are: R. Montgomery and S. Haberichter (Milwaukee, WI), D.L. and P. James (Kingston, ON, Canada), and A.C.G., I.R.P., and D.J.H. (Sheffield, United Kingdom).

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## Supplemental Data

### Methods

#### Immunofluorescence confocal microscopy

Immunofluorescence confocal microscopy was used to assess the influence of c.[2365A>G;2385T>C] genotype on intracellular localization and pseudo-Weibel-Palade body formation. Briefly, HEK293 cells were transiently transfected with equimolar concentrations of wild-type or c.[2365A>G;2385T>C] VWF expression plasmid using Lipofectamine™ (Thermo Fisher Scientific, Carlsbad, CA, USA) according to manufacturer's protocols. Post-transfection (24 h) cells were fixed using BD Cytofix/Cytoperm™ (BD Biosciences, Mississauga, ON, Canada), permeabilized using 0.1% Triton X-100 and blocked using Protein Block (Dako, Glostrup, Denmark). Cells were stained with an anti-VWF antibody (A0082; Dako), DAPI and Alexa Fluor® 647 phalloidin (Thermo Fisher). Slides were imaged using a Leica SP8 laser scanning confocal microscope using a 63X oil immersion objective (Leica Microsystems Inc., Concord, ON, Canada).

#### RNA splicing assays

Two different *in vitro* minigene splicing assays were utilized to investigate RNA splicing. The pET01 Exontrap plasmid (MoBiTec GmbH, Goettingen, Germany) was used to investigate any influence on acceptor / donor splice sites. Wild-type (WT) VWF DNA for exon 18 and ~300 bp flanking intronic sequence was inserted into pET01 via restriction enzyme-mediated cloning. Site-directed mutagenesis was used to generate pET01 plasmids encoding c.2365A>G, c.2385T>C and c.[2365A>G;2385T>C]. An exonic splice enhancer (ESE)-dependent splicing assay<sup>1</sup> utilizing the pcDNA-Dup (SF2-ASF3x) plasmid (kindly provided by Dr. Pascaline Gaildrat, University of Rouen, France) was used to investigate any influence on ESE motifs. Oligonucleotides containing SNV alleles of interest (reference and non-reference) and 15 bp flanking DNA sequence (details available on request) were inserted within the middle exon of pcDNA-Dup via restriction enzyme-mediated cloning. Plasmids containing a functional SF2/ASF ESE or a region of intronic DNA with no predicted ESE motif were used as positive and negative controls respectively. Generated pET01 or pcDNA-Dup plasmids were transfected into HEK293T cells, mRNA was isolated and reverse transcribed to cDNA. Agarose gel electrophoresis was used to separate specific cDNA products.

### **VWF:FVIII analysis**

The ability of recombinant VWF secreted by HEK293T cells to bind FVIII was evaluated using an ELISA as previously described, but with minor modifications.<sup>2,3</sup> Briefly, 10 mU/well of recombinant VWF concentrated by centrifugation (Centricon Plus-70 Centrifugal Filter, 100 kDa pore size; Millipore (Canada) Ltd., Etobicoke, ON) was captured on a 96 well plate coated with an anti-VWF antibody (A0082; Dako, Glostrup, Denmark) and 0.06-2.0 IU/mL recombinant FVIII (ADVATE, Baxter, Deerfield, IL, USA) and incubated for 1 h at 37°C in 10 mM calcium-containing buffer. Bound FVIII was measured with a HRP-conjugated anti-human FVIII antibody (F8C-EIA-D; Affinity Biologicals Inc., Ancaster, ON, Canada). Binding plots were fitted with a one-site binding model (hyperbola) and apparent dissociation rate constant values ( $K_d^{app}$ ) were obtained. The best-fit  $K_d^{app}$  values of VWF SNV were statistically compared to that of WT VWF using an *F*-test.

In order to evaluate precise binding kinetics, WT and p.[T789A;Y795=] VWF were purified with immunoaffinity chromatography using monoclonal antibody CLB-RAg20 and a CNBr sepharose 4B column (performed by Jesse Lai with support from Dr. Jan Voorberg, Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands).<sup>4</sup> A surface plasmon resonance (SPR) assay (using a Biacore 3000; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) was then performed on purified VWF as previously described.<sup>5</sup> Briefly, purified VWF was covalently immobilized on a C1 sensor chip at a coupling density of 2.0 ng/mm<sup>3</sup> and FVIII (0.313-10 nM; ADVATE) was injected at a rate of 10 µl/min for 4 min (association phase) and flown-out with buffer for 4 min (dissociation phase). The chip surface was regenerated with 4 M MgCl<sub>2</sub>, 1 M NaCl solution. Binding to the surface of the control uncoated flow cell was subtracted from binding to VWF-coated flow cells. Measurements were performed at 37°C. BIAevaluation v2.1 (GE Healthcare) was used to estimate kinetic rate constants and to perform nonlinear regression analysis to determine the rate constants for association ( $k_a$ ) and dissociation ( $k_d$ ). Dissociation constants (KD) were calculated as  $k_d/k_a$ .

**Table S1. Predicted *in silico* effect of SNV on protein and RNA**

Prediction tool		c.2365A>G	c.2385T>C
<b>Protein predictions</b>	Mutation Taster <sup>6</sup>	Polymorphism	NA
	PolyPhen-2 <sup>7</sup>	Benign	NA
	PON-P2 <sup>8</sup>	Neutral	NA
	PROVEAN <sup>9</sup>	Neutral	NA
	SIFT <sup>10</sup>	Tolerated	NA
<b>RNA predictions</b>	CentroidFold <sup>11</sup>	Alters secondary structure	Alters secondary structure
	miRBase <sup>12,13</sup>	No effect	No effect
<b>Splicing predictions</b>	ESEfinder <sup>*,14</sup>	No effect	Creates SRp55 motif, weakens SC35 motif
	HSF <sup>15</sup>	Creates ESS motif, cryptic donor activation	No effect
	NetGene2 <sup>†,16</sup>	Cryptic donor activation	No effect
	Neural Network <sup>†,17</sup>	No effect	No effect
	RESCUE-ESE <sup>*,18</sup>	No effect	Creates ESE motif
	SplicePort <sup>†,19</sup>	Cryptic donor activation, weakens existing donor	Cryptic acceptor activation, cryptic donor activation, weakens existing donor

ESE, exonic splice enhancer; ESS, exonic splice silencer; NA, not applicable. \*ESE / ESS predictions only; †acceptor / donor predictions only.

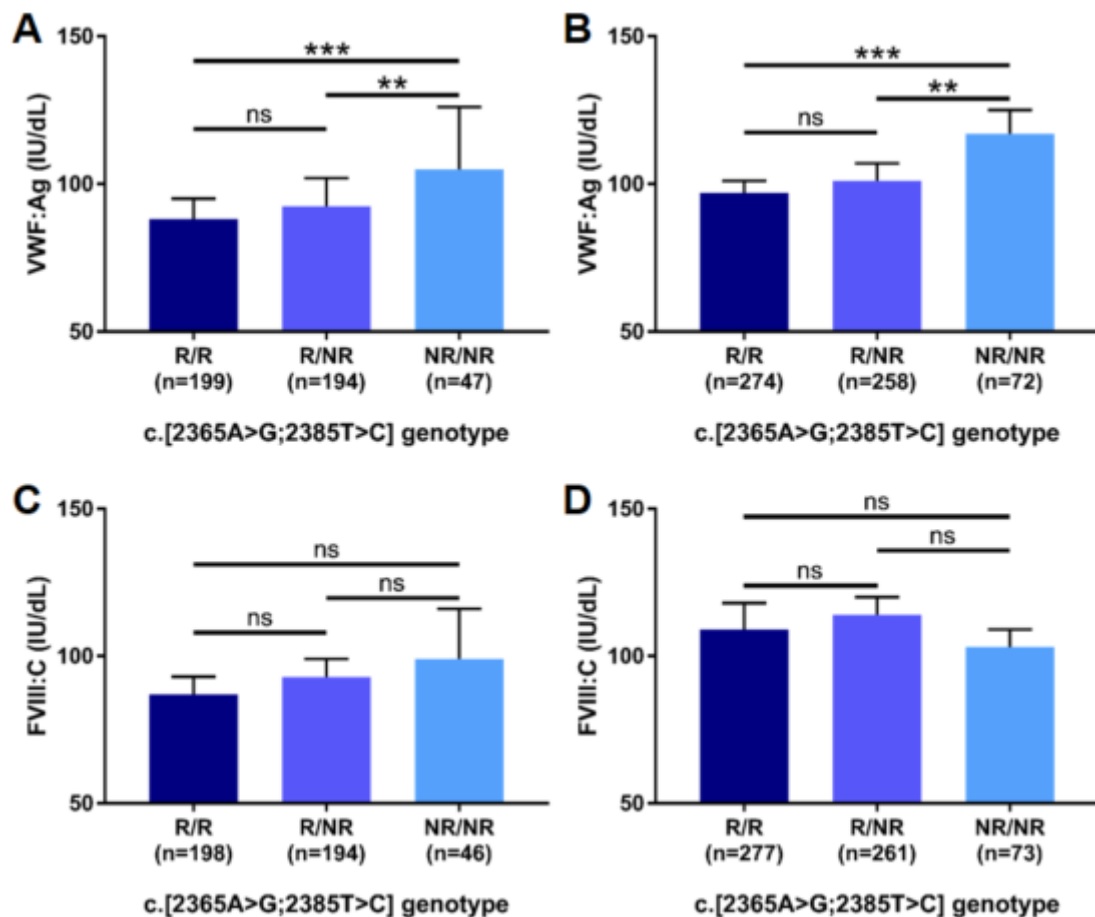


**Table S2. Influence of SNV on VWF:FVIIIIB**

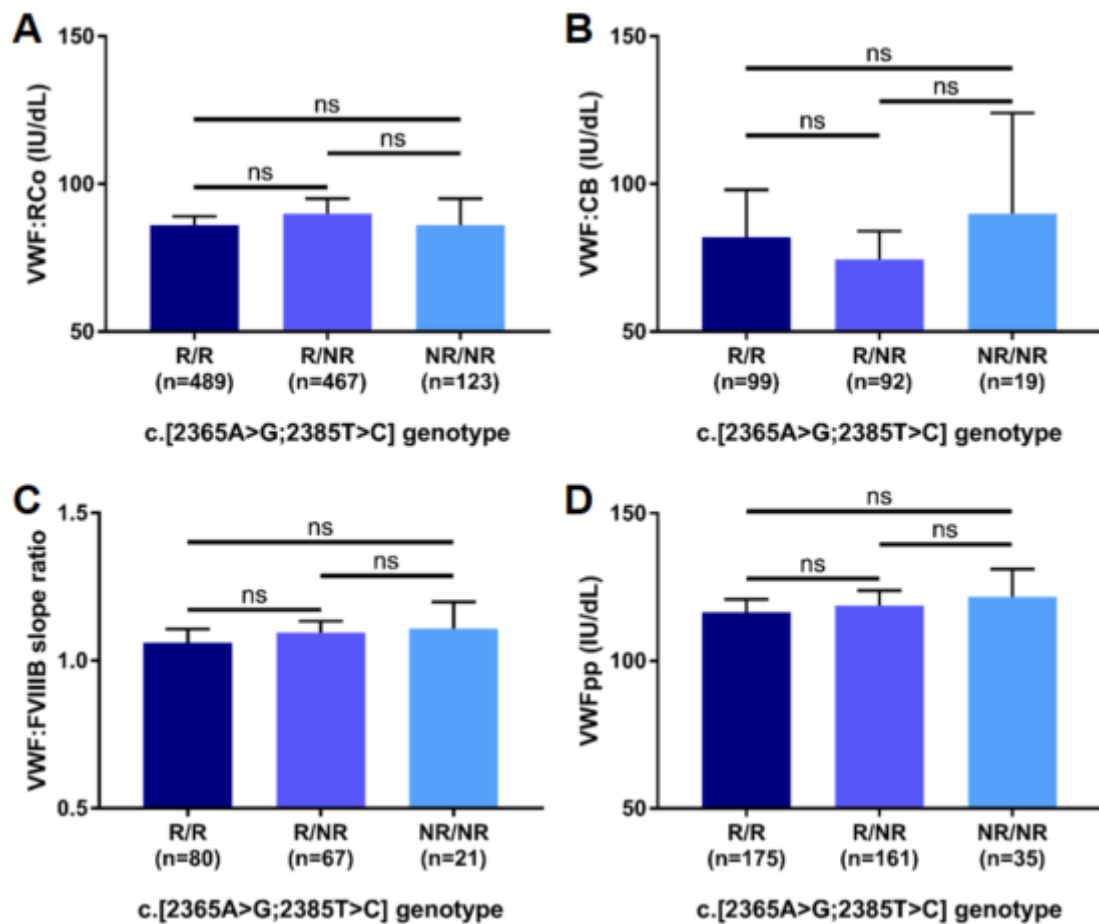
Expressed VWF	VWF:FVIIIIB*		VWF:FVIIIIB†	
	Kd <sup>app</sup> (nM)	ka x 10 <sup>6</sup>	kd x 10 <sup>-3</sup>	KD (nM)
Wild-type	0.10 ± 0.01	2.98 ± 0.015	1.63 ± 0.019	0.545
p.T789A	0.12 ± 0.01	nd	nd	nd
p.Y795=	0.11 ± 0.01	nd	nd	nd
p.[T789A;Y795=]	0.12 ± 0.01	2.98 ± 0.022	2.32 ± 0.030	0.777
p.T789P	No binding	nd	nd	nd

ka, association rate constant; kd, dissociation rate constant; KD, equilibrium dissociation constant; Kd<sup>app</sup>, apparent dissociation rate constant; nd, not determined.

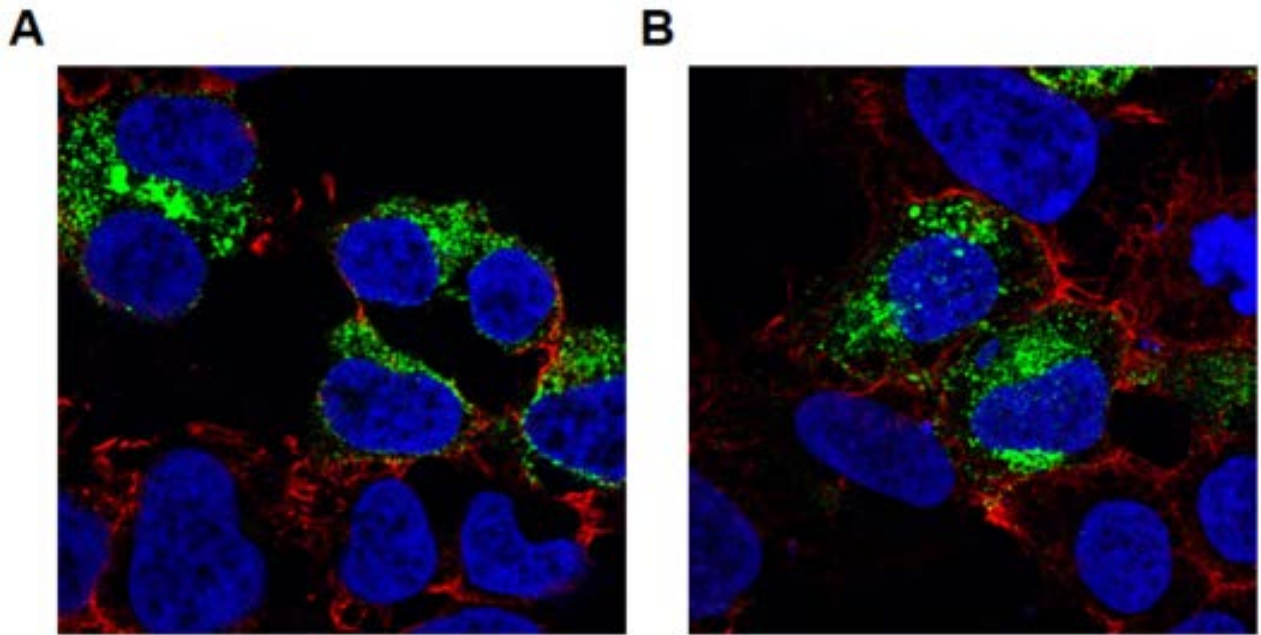
\*VWF:FVIIIIB determined by ELISA; †VWF:FVIIIIB determined by SPR.



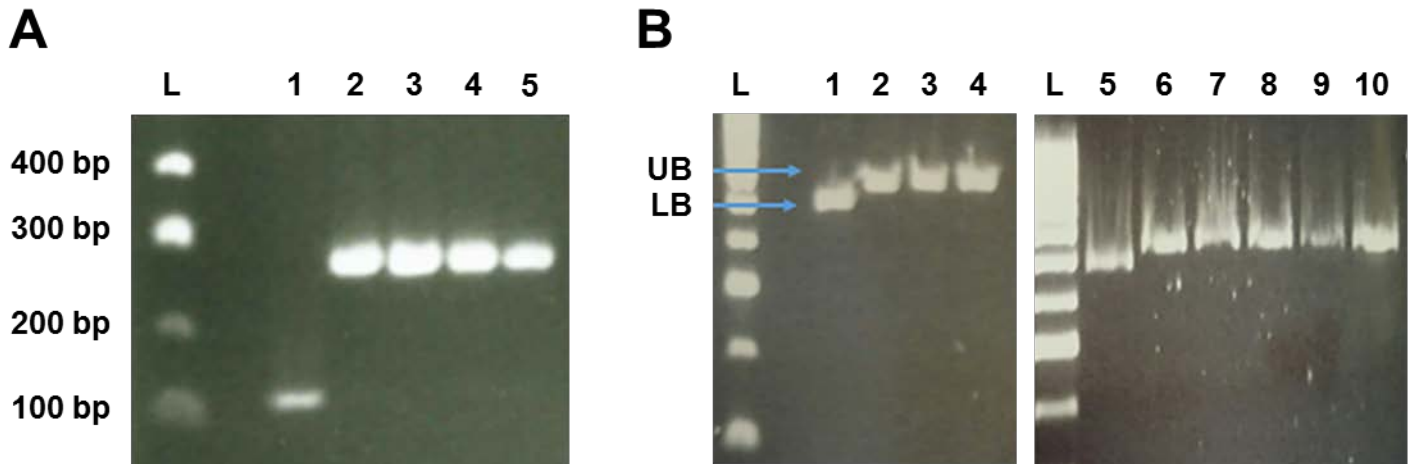
**Figure S1. Association between SNV c.[2365A>G;2385T>C] genotype and median VWF:Ag and FVIII:C levels accounting for ABO blood group. (A) VWF:Ag in O blood group individuals. (B) VWF:Ag in non-O blood group individuals. (C) FVIII:C in O blood group individuals. (D) FVIII:C in non-O blood group individuals. NR, non-reference allele; R, reference allele. Genotypes compared using a Mann-Whitney test (\*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; ns, not significant). Bars indicate 95% confidence interval.**



**Figure S2. Association between SNV c.[2365A>G;2385T>C] genotype and VWF activity / propeptide levels in healthy controls.** (A) Median VWF:RCo levels. (B) Median VWF:CB levels. (C) Median VWF:FVIII B slope ratio. (D) Median VWFpp levels. NR, non-reference allele; R, reference allele. Genotypes compared using a Mann-Whitney test (ns, not significant). Bars indicate 95% confidence interval.



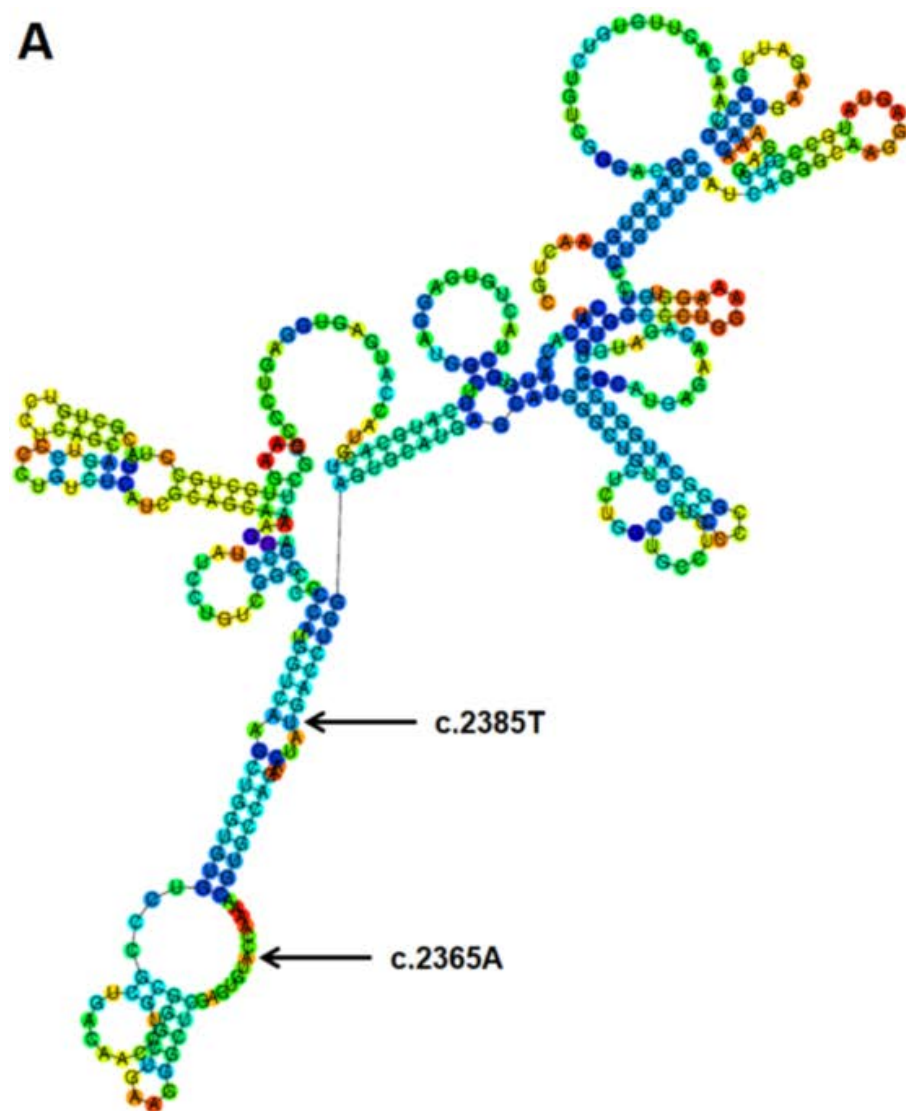
**Figure S3. Intracellular localization and pseudo-Weibel-Palade body formation for wild-type (A) and c.[2365A>G;2385T>C] (B) VWF. Blue, nucleus; green, VWF; red, F-actin.**



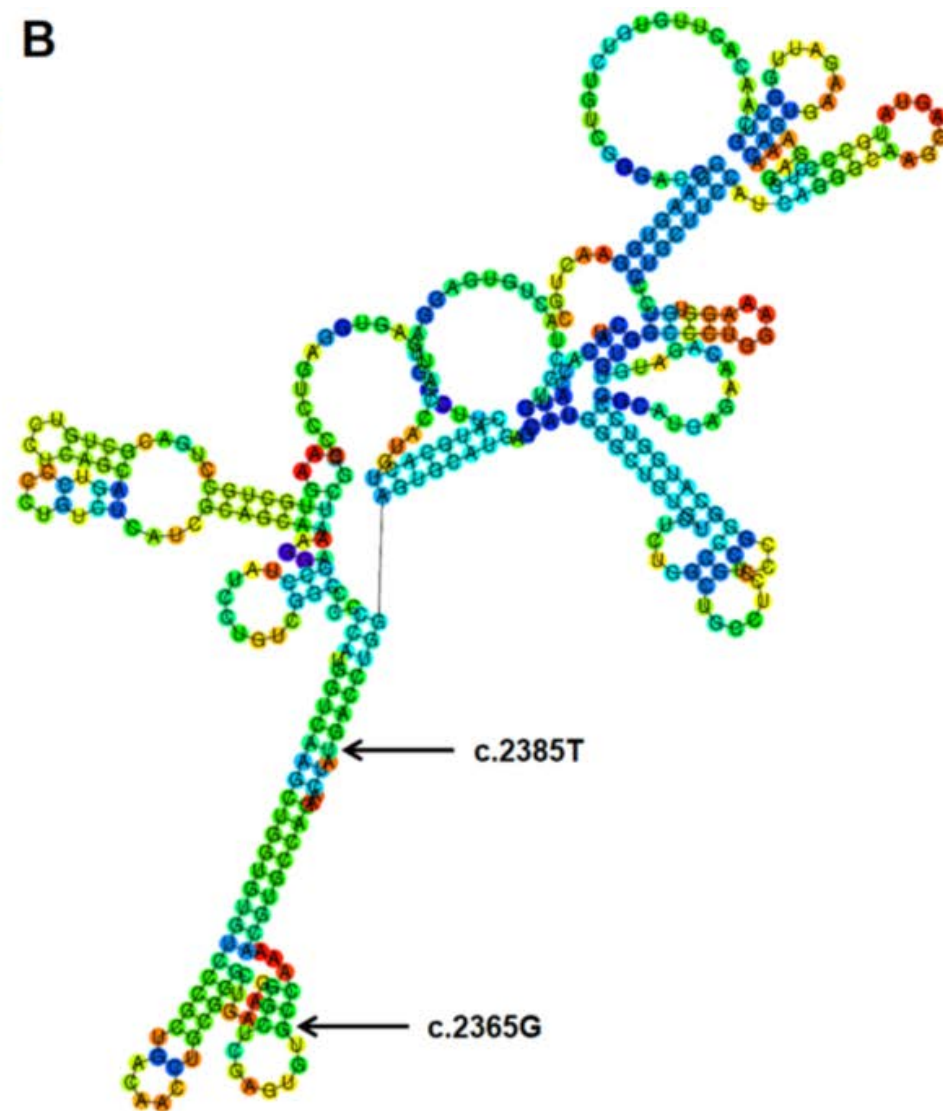
**Figure S4. *In vitro* analysis of SNV effect on splicing.** (A) Influence on acceptor / donor splice sites (lane 1: negative control; lane 2: WT; lane 3: c.2385T>C; lane 4: c.2365A>G; lane 5: c.[2365A>G;2385T>C]). (B) Influence on exonic splice enhancer motifs (lanes 1 and 5: negative control; lanes 2 and 6: positive control; lanes 3, 7 and 9: WT; lane 4: c.2385T>C; lane 8: c.2365A>G; lane 10: c.[2365A>G;2385T>C]). L, HyperLadder 100bp; LB, lower band (i.e. 2 exons); UB, upper band (i.e. 3 exons).

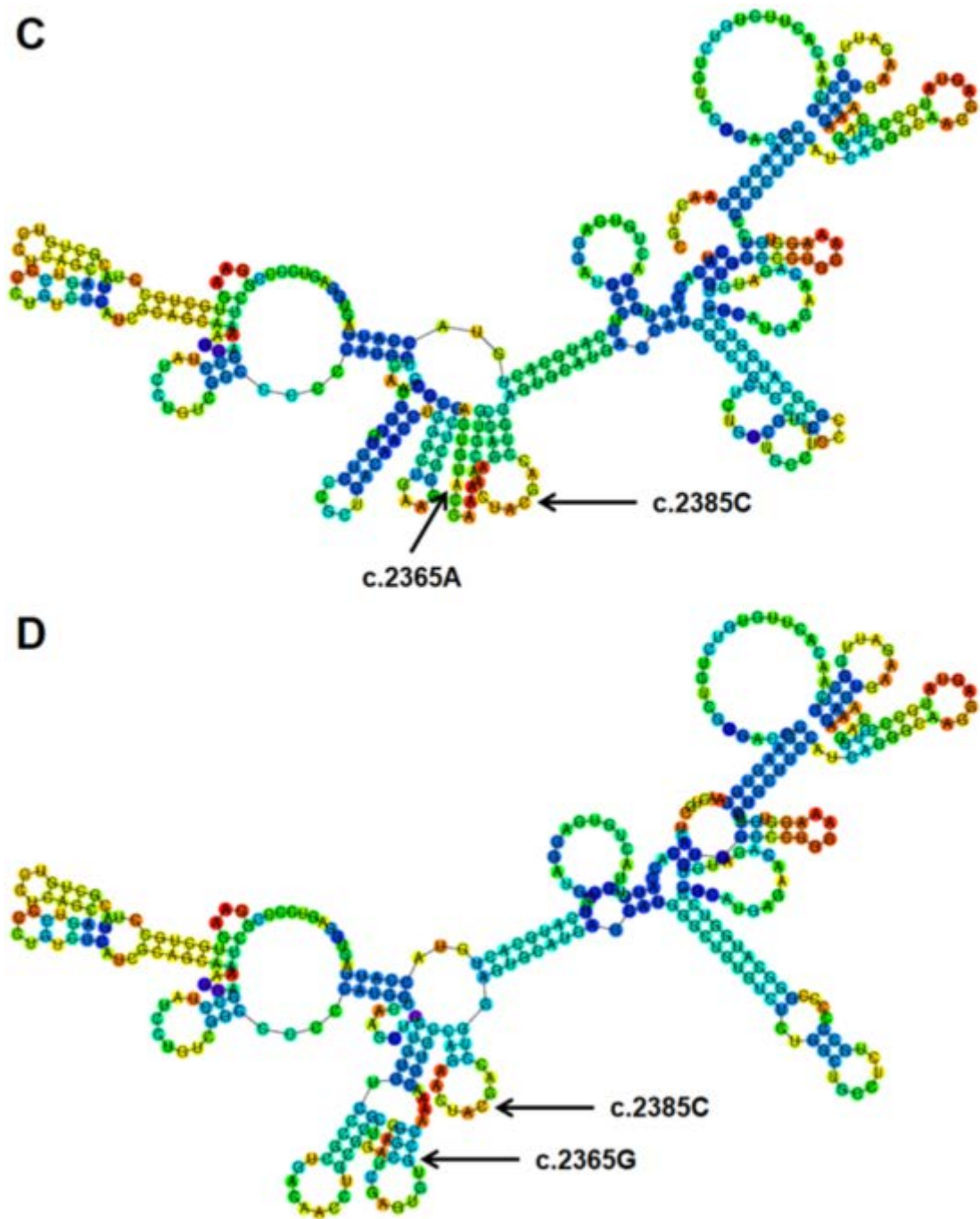


**A**

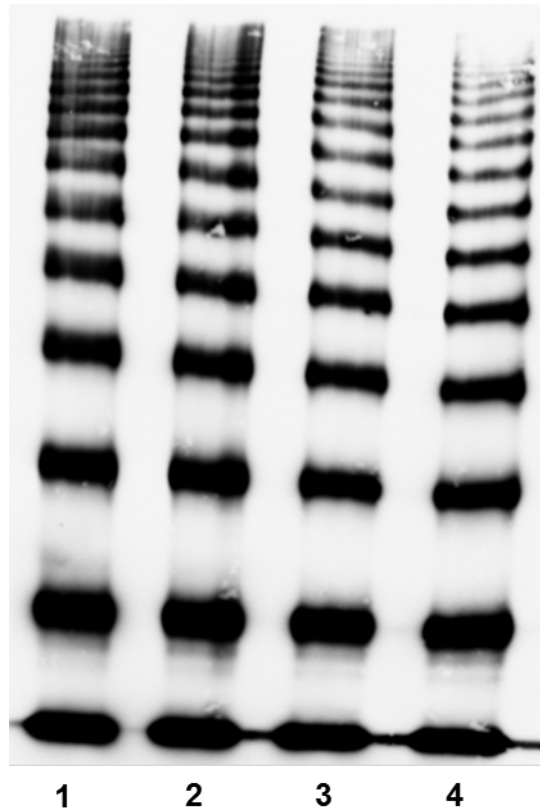


**B**





**Figure S5. RNA secondary structure predictions.** (A) Wild-type. (B) c.2365A>G. (C) c.2385T>C. (D) c.[2365A>G;2385T>C]. Data generated with CentroidFold<sup>11</sup> using default settings.



**Figure S6. Multimers profiles of expressed recombinant VWF.** Multimer analysis of secreted VWF was performed via electrophoresis on a 1.6% (w/v) SDS-agarose gel (lane 1: wild-type; lane 2: c.2365A>G; lane 3: c.2385T>C; lane 4: c.[2365A>G;2385T>C]).

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