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Characterization of *VWF* gene conversions causing von Willebrand disease

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Summary

We previously reported that von Willebrand Factor gene (*VWF*) conversions are a relatively frequent cause of von Willebrand disease (VWD), however, their molecular pathomechanisms resulting in variant phenotypes is largely unknown. Here, we characterized *VWF* conversions harbouring missense and synonymous mutations, through generating a series of mutant constructs followed by transient expression in 293 cells, and qualitative and quantitative analysis of recombinant VWF (rVWF). The characterization of mutant rVWF showed the critical roles of synonymous variants in the pathogenicity of *VWF* conversions. The gene conversion variants p.Val1229Gly, p.Asn1231Thr, p.Asn1231Ser and p.Ala1464Pro in the absence of synonymous p.Ser1263= and p.Gln1449= showed minimal effect on rVWF synthesis and activity. Interestingly, a construct including the synonymous variants displayed significantly low rVWF expression and activity. The variant p.Pro1266Leu showed gain of rVWF function toward glycoprotein Ibα; surprisingly, this function was significantly abolished in the presence of gene conversion variants p.Val1229Gly-p.Asn1231Thr. Taken together, our expression studies suggest that synonymous variants in the combination of other gene conversion variants suppress the protein expression, possibly due to defective primary mRNA structure or processing. The variants p.Val1229Gly-p.Asn1231Thr affected the VWF gain of function caused by variant p.Pro1266Leu, probably due to conformational changes in VWF.

Keywords: von Willebrand disease, synonymous variants, gene conversion mutation, site directed mutagenesis, *in vitro* expression.

Von Willebrand disease (VWD) is an inherited, relatively common but heterogeneous bleeding disorder, characterized by quantitative and/or qualitative defects of von Willebrand factor (VWF) (Baronciani *et al*, 2017). VWF is a large multimeric plasma protein containing several structural and functional domains. It is essential for platelet-dependent haemostasis at the injured vessel wall, where it binds to exposed collagen under conditions of high shear forces. This activates VWF A1 domain binding sites for platelet glycoprotein (GP)Ibα to capture circulating platelets (Schneppenheim & Budde, 2011), which leads to activation of platelet GPIIb/IIIa, an additional binding partner of VWF in a high shear environment (De Marco *et al*, 1986).

The heterogeneity of VWD is due to the multifunctional properties of VWF with its corresponding different domains. The mutations causing quantitative deficiencies in VWF correspond to VWD type 1 or the most severe form, VWD type 3, whereas missense mutations in different VWF domains may affect only a particular functional property of VWF in type 2 VWD (Sadler *et al*, 2006).

Gene conversions are rare non-homologous recombination events that lead to fusions between functional genes and pseudogenes (Chen *et al*, 2007). Although gene conversions are considered a relatively infrequent cause of VWD worldwide, they are relatively common in the Indian population (Gupta *et al*, 2005; Ahmad *et al*, 2013a,b). All gene conversions of *VWF* identified to date are located in the VWF A1 domain (Eikenboom *et al*, 1994; Kanaji *et al*, 1996; Gupta *et al*, 2005; Ahmad *et al*, 2013b). To date, most of the mutations identified in the VWF A1 domain are gain of function

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(GOF) missense mutations causing VWD type 2B with enhanced affinity of mutant VWF to platelet GPIbα and loss of high molecular weight multimers (HMWM), and loss of function mutations causing VWD type 2M with apparently normal VWF HMWM.

A part of *VWF* including exon 28 was found to be highly homologous with its pseudogene on chromosome 22. *VWF* exon 28 is considered a hot spot for gene conversions and other types of mutations. The gene conversions in this area are believed to be facilitated by the presence of chi and chi-like sequences (CCTGGTGG and GCTGGTGG) in the vicinity (Eikenboom *et al*, 1994; Kanaji *et al*, 1996; Gupta *et al*, 2005). Such gene conversion leads to exchange of genetic material containing nonsense, missense and synonymous variants (Gupta *et al*, 2005; Ahmad *et al*, 2013b). The nonsense and missense mutations are well known to exert deleterious effect on protein synthesis, secretion and function, however, synonymous alteration or single nucleotide polymorphisms (SNPs) have long been believed to be a non-causative variant. Accumulating sets of data suggest that synonymous alteration in a gene may lead to mRNA instability, error in mRNA splicing and/or alteration in the secondary structure of mRNA (Czech *et al*, 2010; Yadegari *et al*, 2016; Chen *et al*, 2017). Studies have shown that synonymous variants may also exert post-transcriptional effects, including translation efficiency, protein confirmation and function (Komar *et al*, 1999; Edwards *et al*, 2012; Buhr *et al*, 2016; Simhadri *et al*, 2017).

The present study focused on naturally occurring synoymous and missense mutations in the VWF A1 domain, resulting from gene conversions between *VWF* and its pseudogene on chromosome 22. We characterized several combinations of *VWF* gene conversion variants (p.Val1229Gly-p.Asn1231Thr, p.Val1229Gly-p.Asn1231Ser, p.Val1229Gly-p.Asn1231Thr-Pro1266Leu and p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln) as well as individually to delineate their pathogenicity. Additionally, the synonymous variants, p.Ser1263= and p.Gln1449=, of gene conversion identified in patients, were characterized both individually and in the presence of other missense mutations of gene conversion.

Materials and methods

Expression studies

In vitro mutagenesis and expression studies were performed as described previously (Schneppenheim *et al*, 2001; Haberichter *et al*, 2010). Briefly, site-directed mutagenesis of full-length *VWF* cDNA was performed to generate mutants using the Quick Change kit (Stratagene, La Jolla, CA, USA).

Relatively long (40–45 bp) primer sets carrying the respective base exchange at a central position were used. The MACH1T1 supercompetent cells were transformed using expression vector pRC.CMV containing full-length mutant and wild-type (WT) *VWF* cDNA. Plasmids were purified by the Endofree Plasmid Maxi Kit (Qiagen, Hilden, Germany). A total of 4 μg of mutant or WT full-length *VWF* cDNA was used for transient transfection of 293 cells [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany] by liposomal transfer using Lipofectamine 2000 kit (Life Technologies, Paisley, UK). Cells were plated and grown for 24 h in complete medium (Dulbecco's modified Eagle medium with 10% fetal bovine serum and 1% antibiotic cocktail) and 48 h in serum-free medium (OPTIPRO-SFM; Thermo Fisher Scientific, Waltham, MA, USA). Secreted VWF in medium and VWF present in the cell lysate were both analysed by enzyme-linked immunosorbent assays (ELISAs) to measure VWF antigen (VWF:Ag), GPIbα binding and VWF collagen binding (VWF:CB) respectively. ELISA plates were coated with anti-VWF (DAKO, Hamburg, Germany), human type III Collagen (Southern Biotech, Brimingham, AL) and rGPIbα for VWF:Ag, VWF:CB and VWF:GPIb binding assays, respectively (Federici *et al*, 2004; Schneppenheim *et al*, 2010).

Multimer analysis

Multimer analysis of recombinant VWF from conditioned medium of transfected cells was performed by sodium dodecyl sulfate (SDS)–agarose gel electrophoresis, as described previously (Schneppenheim *et al*, 1988). Briefly, recombinant VWF in the collected medium of transfected 293 Epstein–Barr virus nuclear antigen (EBNA) cells was analysed by gel electrophoresis using 2% high-gelling temperature agarose resolving gel containing 1% SDS. The separated VWF was transferred on to Immobilin-FL (Millipore, Darmstadt, Germany) and the membrane was developed by incubation with primary antibody followed by incubation with horseradish peroxidase-labelled secondary antibodies and chemiluminescence.

Statistics

Differences between data groups were evaluated for significance using unpaired *t*-test with GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean ± standard error of the mean. For all tests, a $P < 0{\cdot}05$ was considered for statistically significant.

Results

Our previous reports on four patients with type 2 VWD carrying gene conversions in exon 28 of *VWF* presented with moderate bleeding manifestation including easy bruising, ecchymosis, epistaxis and gum bleeding (Ahmad *et al*, 2013b). In addition to these clinical manifestations, menorrhagia was reported in two female patients (Ahmad *et al*, 2013b).

Three of four patients carried *VWF* conversions with variants p.Val1229Gly-p.Asn1231Thr-c.3789G>A-p.Pro1266Gln,

and presented a decreased VWF function with normal VWF multimers. In addition, one patient had a *VWF* conversion (p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln-P.p.Ala1464Pro) with a decreased VWF function and loss of VWF HMWM (Ahmad *et al*, 2013b). We characterized these two and few previously reported gene conversions by *in vitro* mutagenesis and recombinant expression (Ahmad *et al*, 2013b). The type 2B VWD mutation p.Ala1461Asp was included as a positive control.

Role of gene conversion variants in differential phenotype of VWD:

The comparative expression studies for gene conversions p.Val1229Gly-p.Asn1231Thr-p.Pro1266Leu, p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln and p.Val1229Gly-p.Asn1231 Thr-p.Pro1266Gln-p.Ala1464Pro were performed by generating a series of mutant *VWF* constructs as described in Table I. We previously identified and characterized the variant p.Asn1231Ser in the vicinity of the gene conversion and reported that this mutant has minimal impact on VWD pathogenicity (Ahmad *et al*, 2013b). Hence, we included this mutant with p.Val1299Gly (p.Val1229Gly-p.Asn1231Ser) and compared with variants p.Val1229Gly-p.Asn1231Thr, identified in VWD patients. The characterization of rVWF reveals that even the combinations of these variants (p.Val1229Gly-p.Asn1231Thr and p.Val1229Gly-p.Asn1231Ser) have negligible effects on rVWF synthesis and/or secretion and function, as rVWF antigen (rVWF:Ag) levels, collagen binding (rVWF:CB) and GPIbα binding (rVWF:GPIbα) were comparable to WT rVWF (Table I).

Next, we generated and characterized the mutant constructs p.Pro1266Gln and p.Pro1266Leu, and expression data showed that p.Pro1266Leu induced GPIbα specific gain of rVWF function. However, no prominent defect was seen in rVWF for p.Pro1266Gln as all analysed qualitative and quantitative parameters of rVWF were comparable to WT (Table I). To study the gene conversion variant combinations, mutant p.Pro1266Gln and p.Pro1266Leu were inserted in the construct p.Val1229Gly-p.Asn1231Thr. Consistently, as observed in patients, mutant rVWF from the construct p.Val1229Gly-p.Asn1231Thr-p.Pro1266Leu displayed significantly increased rVWF:GPIbα binding in homozygous as well as in co-expression conditions. In stark contrast, opposing effect of variants p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln was observed and the combination of gene conversion variants diminishes the GPIbα-specific rVWF function, especially in the homozygous condition, as reflected by low VWF:GPIbα binding (Table I). The multimer analysis of rVWF from both variant combinations showed a normal multimer pattern of (Fig 1). Hence, these data suggest that the *VWF* conversion variants p.Val1229Gly-p.Asn1231Thr and p.Val1229Gly-p.Asn1231Ser have no significant impact on VWF expression or function, at least under *in vitro* conditions.

Role of mutation p.Ala1464Pro in the pathogenesis of VWD

The patient with *VWF* conversion p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln-p.Ala1464Pro presented decreased plasma VWF function with loss of HMWM. The mutation p.Ala1464Pro found to be located on the same allele and was not a part of the gene conversion event (Ahmad *et al*, 2013b). Hence, we sought to characterize and delineate the pathogenicity of the p.Ala1464Pro mutation. The characterization of mutant rVWF revealed a comparable rVWF function and multimer pattern to WT rVWF and the mutation was found to have no effect on VWF in *in vitro* conditions (Fig 1). To further characterize the role of this mutation in the presence of other gene conversion variants, mutant p.Ala1464Pro was inserted in to the construct p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln. The expression of this construct in homozygous as well as heterozygous conditions revealed no prominent defect, as rVWF synthesis, secretion and function was comparable to WT rVWF (Table I). Moreover, rVWF multimer analysis showed a normal pattern of mutant rVWF multimer (Fig 1). These data indicate that the p.Ala1464Pro mutation, individually or in combination with other missense gene conversion variants, has no impact on VWF synthesis, multimerization and secretion, and GPIbα- and collagen-dependent function in *in vitro*.

Synonymous variants of gene conversion suppress the VWF expression and cause functional defects

Given that no prominent defects of missense variants of gene conversion, individually as well as in combinations, were observed, we hypothesized that synonymous variants of gene conversions might have a role in the modulation of protein expression and/or function. In this series, we generated another set of constructs including synonymous and non-synonymous variants of gene conversions, and characterized them through *in vitro* expression and quantitative and qualitative analysis.

Interestingly, the synonymous mutations of *VWF* conversion p.Ser1263= or p.Gln1449= individually showed a significant effect on rVWF protein expression because low levels of VWF:Ag was observed in cell lysates. Consistently, a similar effect was seen in the VWF-collected medium (Fig 2A, B). Decreased GPIbα- and collagen-dependent functions of rVWF were observed in the collected medium, but these normalized with VWF:Ag levels (Fig 2C–F). The multimer pattern of mutant rVWF was comparable to WT rVWF (Fig 3).

Interestingly, when the synonymous variant p.Ser1263= was combined with other gene conversion variants in the construct p.Val1229Gly-p.Asn1231Thr-p.Ser1263= -p.Pro1266 Gln, a lower rVWF:Ag level was observed in both the cell lysates and collected medium. Moreover, a greater effect on GPIbα binding, specifically in the homozygous condition (Fig 2C, D), was observed, which was also reflected by the construct p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln without

Table I. Recombinant VWF characterization from different mutants.

Mutant	rVWF:Ag in medium (WT%)		rVWF:Ag in Lysates (WT%)		rVWF:CB in medium (WT%)		rVWF:GPIb in medium (WT%)		GPIba/rVWF:Ag ratio	CB/rVWF:Ag ratio
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
WT	100·00	2·07	100·00	5·29	100·00	7·53	100·00	9·77	1·00	1·00
p.Pro1266Gln	95·22	4·60	88·99	5·77	90·58	2·51	94·05	8·11	0·99	0·95
p.Pro1266Gln + WT	89·25	0·52	94·01	2·85	92·03	8·23	89·19	4·29	1·00	1·03
p.Pro1266Leu	100·60	4·93	139·50	8·03	84·78	3·77	191·89	14·17	1·91	0·84
p.Pro1266Leu + WT	83·58	3·62	112·90	6·33	72·46	4·53	148·65	6·75	1·78	0·87
p.Ala1464Pro	90·75	4·93	90·17	3·54	81·88	12·36	101·08	3·38	1·11	0·90
p.Ala1464Pro + WT	87·76	2·69	102·77	5·11	86·96	13·22	88·11	7·66	1·00	0·99
p.Val1229Gly-p.Asn1231Ser	107·16	5·4	103·4	6·14	96·38	4·53	91·89	3·38	0·86	0·9
p.Val1229Gly-p.Asn1231Thr	93·13	6·46	85·88	7·58	82·61	12·1	95·14	11·95	1·02	0·89
p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln	90·15	2·74	82·63	3·82	81·88	2·51	75·14	1·87	0·83	0·91
p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln + WT	80·90	8·13	87·40	10·95	81·16	3·32	81·62	3·38	1·01	1·00
p.Val1229Gly-p.Asn1231Thr-p.Pro1266Leu	92·54	3·62	94·53	10·37	83·33	10·27	141·62	11·04	1·53	0·90
p.Val1229Gly-p.Asn1231Thr-p.Pro1266Leu + WT	88·06	4·42	91·20	5·80	80·43	2·17	117·84	12·28	1·34	0·91
p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln- p.Ala1464Pro	112·92	1·69	89·89	1·83	125·00	5·09	104·67	9·85	0·93	1·11
p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln- p.Ala1464Pro + WT	122·47	6·38	94·36	0·63	122·06	2·55	118·69	12·64	0·97	1·00
p.Ala1461Asp	92·40	4·56	141·74	11·69	79·80	8·75	328·46	21·93	3·55	0·86

GPIb(α), glycoprotein Ib(α); rVWF:Ag, recombinant von Willebrand factor antigen; rVWF:CB, recombinant von Willebrand factor collagen binding; SD, standard deviation.

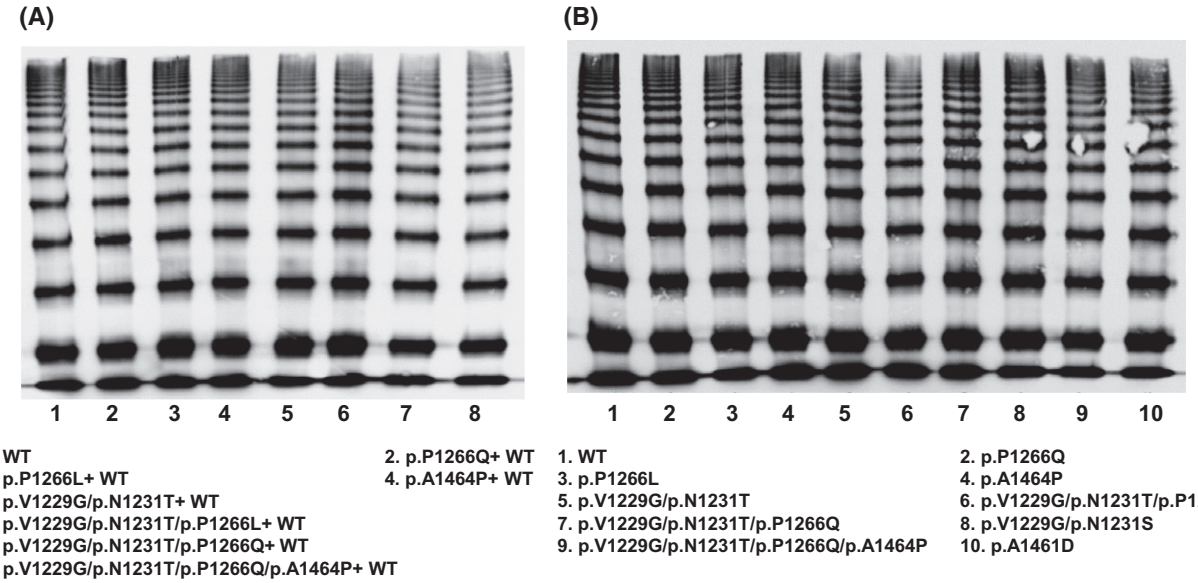

Fig. 1. Multimer pattern of mutants and wild type (WT) recombinant von Willebrand factor obtained from (A) heterozygous and (B) homozygous expression. WT, wild type.

synonymous p.Ser1263=. The platelet-dependent functional defect of rVWF was further demonstrated by a low GPIbα/VWF:Ag ratio (Fig 2C, D). These data suggest that synonymous p.Ser1263= has significant impact on either mRNA transcription or translation.

Furthermore, as no major effect of missense variants of gene conversion identified in a patient was observed, we hypothesized whether the synonymous variants p.Ser1263= and p.Gln1449= of gene conversion might play a role in the clinical presentation of patients. In this series, the synonymous variants were inserted in the construct p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln-p.Ala1464Pro and expression studies were performed. In contrast to phenotype observed from a construct containing only amino acid substitution variants (p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln-p.Ala1464Pro), the full length construct containing the synonymous variants displayed a profound effect, as significantly less VWF expression was demonstrated in cell lysate, (Fig 2A). Moreover, the secreted VWF in the medium showed functional defects and was found to have significantly low GPIbα and collagen binding (Fig 2C–F), without any apparent effect on multimer pattern (Fig 3).The GPIbα/VWF:Ag ratio for the mutant VWF was consistently low. These data suggest that the synonymous variants of gene conversion probably have significant impact on both mRNA transcription as well as protein folding, which lead to both quantitative and qualitative defects in rVWF.

Mutant p.Val1229Gly-p.Asn1231Thr normalizes the gain of VWF function caused by p.Pro1266Leu

Although our expression studies suggest that variant p.Val1229Gly-p.Asn1231Thr has no deleterious effect on rVWF, these variants were found to have significant impact on rVWF GOF caused by the p.Pro1266Leu mutation. Interestingly, we observed that the mutant construct harbouring variants p.Val1229Gly-p.Asn1231Thr-p.Pro1266Leu displayed a significant correction in GOF. This correction in rVWF GOF was found to correlate with gene dosage (Fig 4). The data suggest that p.Pro1266Leu induces an approximate 2-fold gain in GPIbα dependent function in the homozygous condition and an approximate 1·5-fold increase in the heterozygous. However, when this mutant was inserted with variant p.Val1229Gly-p.Asn1231Thr, the rVWF GOF reduced approximately 1·4-fold in the homozygous condition and almost normalized in the heterozygous condition.

Discussion

Gene conversion is a relatively frequent cause of VWD in patients from India and mostly occurs in the *VWF* exon 28 region (Eikenboom *et al*, 1994; Kanaji *et al*, 1996; Gupta *et al*, 2005; Ahmad *et al*, 2013b). The presence of a pseudogene on chromosome 22, which is homologous to *VWF* exon 23–34, is the main cause of *VWF* conversions. Several *VWF* conversions have been reported but only a few of them have been characterized so far.

In the current study, we have generated serial mutant *VWF* constructs that harbour different gene conversions and analysed their particular effects on VWF expression and function. The variable phenotypes of different gene conversion mutants have been observed through our expression studies. Consistent with a previous report (Gupta *et al*, 2005), our results suggest that *VWF* conversions harbouring the variants p.Val1229Gly/p.Asn1231Thr have no prominent

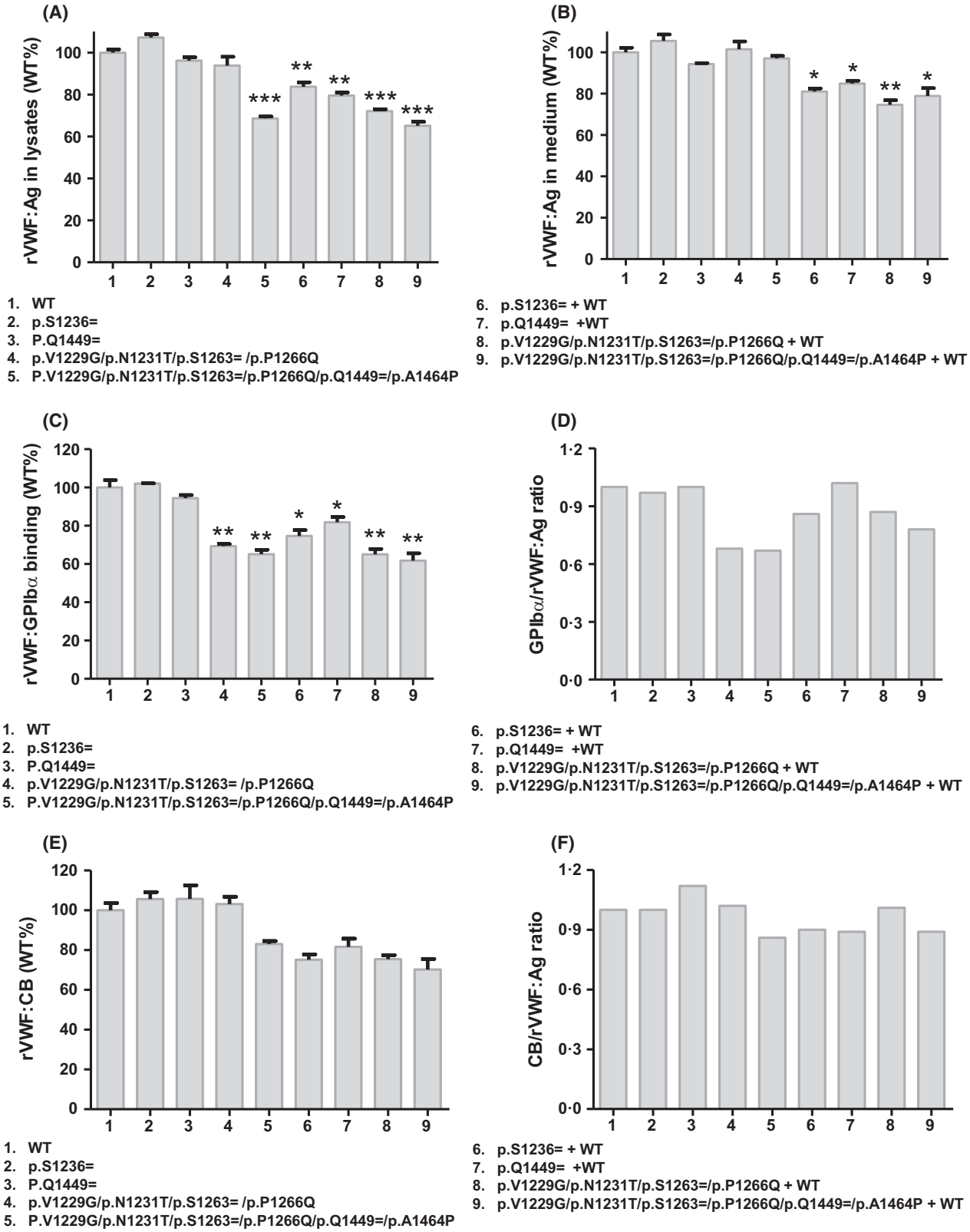

Fig. 2. Characterization of rVWF. Bar diagrams show (A) rVWF:Ag levels in cell lysate (B) rVWF:Ag levels in collected medium (C) rVWF GPIbα binding (D) GPIbα binding and VWF antigen (GPIbα/rVWF:Ag) ratios (E) rVWF:CB, and (E) Collagen binding and VWF antigen (CB/rVWF:Ag) ratios for wild type and mutant constructs. ***$P = 0{\cdot}0001$; **$P < 0{\cdot}005$; *$P < 0{\cdot}05$. GPIbα, glycoprotein Ibα; rVWF:Ag, recombinant von Willebrand factor antigen; rVWF:CB, recombinant von Willebrand factor collagen binding; WT, wild type.

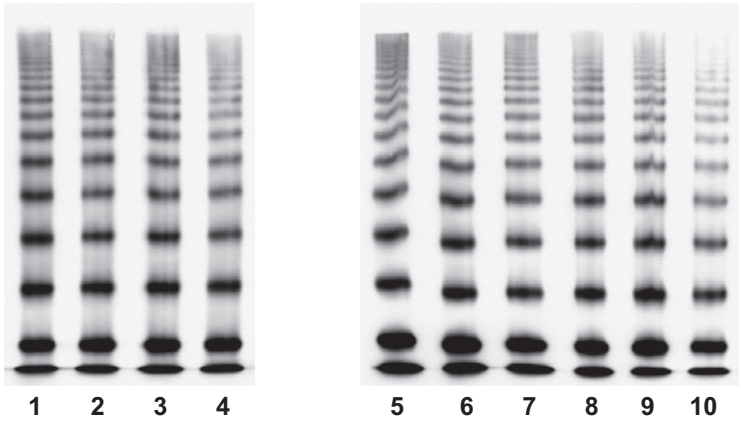

Fig. 3. Multimer pattern of recombinant von Willebrand factor obtained from homozygous and heterozygous expression of synonymous and non-synonymous gene conversion variants and wild type (WT).

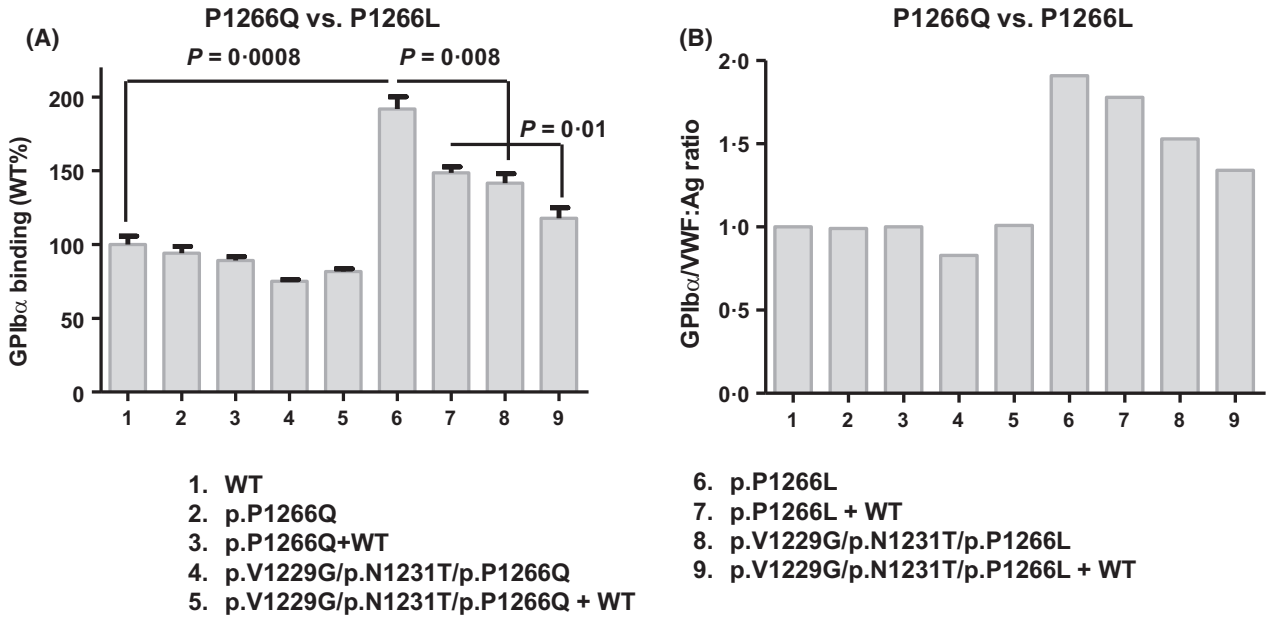

Fig. 4. Effect of mutations p.Pro1266Gln and p.Pro1266Leu on GPIbα binding; (A) Bar diagrams show the GPIbα binding of different mutant and wild type recombinant VWF (B) GPIbα/VWF:Ag ratio for different mutants rVWF. GPIbα, glycoprotein Ibα; rVWF:Ag, recombinant von Willebrand factor antigen; WT, wild type.

effect on rVWF quality and/or quantity. Similarly, the p.Asn1231Ser mutation was found to have minimal role in the pathogenicity of VWD. Further investigation of p.Val1229Gly-p.Asn1231Ser and side-by-side comparison with p.Val1229Gly-p.Asn1231Thr revealed that these variants have minimal effects on VWF function under static conditions. Hence, these variants might only have an impact on VWF under shear conditions that probably result in a VWF:GPIbα binding defect in the patient.

Our study of variants p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln and p.Val1229Gly/p.Asn1231Thr/p.Pro1266Leu resulted in different phenotypes. We observed a decreased

rVWF:GPIbα binding of mutant p.Val1229Gly-p.Asn1231Thr-p.Pro1266Gln, specifically in the homozygous condition, and enhanced VWF affinity towards GPIbα for the *VWF* conversion with p.Pro1266Leu, which suggests the crucial pathogenic role of residue 1266 in VWF. The differential impact of glutamine and leucine at the same residues of VWF could be due to their opposing impact on conformational changes in the GPIbα binding site of the VWFA1 domain.

Recombinant mutant p.Ala1464Pro has shown a normal rVWF multimer pattern with no effect on VWF function, at least under *in vitro* conditions. In contrast, patients' plasma VWF showed decreased VWF activity with loss of HMWM, suggesting a type 2A mutation. Although most of the mutations found in this vicinity are reported to cause type 2B VWD, only a few have also been reported for type 2A VWD (Penas *et al*, 2004). In this line, mutations that affect the p.Cys1272-Cys1458 disulfide loop (e.g., p.Cys1272Arg and p.Cys1458Tyr) destabilize the A1 domain, resulting in loss of GPIbα-dependent VWF function, whereas most of the A1 domain mutations induce conformational changes leading to VWF GOF (Cooney & Ginsburg, 1996; Ware, 2013).

Although splice site synonymous variants are well known to disrupt mRNA splicing and result in aberrant protein expression (Kannan *et al*, 2009; Hampshire & Goodeve, 2011; Yadegari *et al*, 2016), synonymous variants in the coding region are believed to be silent and play a minimal role in the protein expression process. However, accumulating sets of literature over the last decade clearly suggest that not only missense mutations but also synonymous variants in the coding region of a gene may affect the protein expression. Synonymous variants have been reported to negatively impact the transcription, translation and even protein folding, subsequently disrupting the protein function (Hamy *et al*, 1990; Hunt *et al*, 2009; Edwards *et al*, 2012; de Coulgeans *et al*, 2014). Consistent with these reports, the current study found deleterious effects of the synonymous variants p.Ser1263= and p.Gln1449= that affect VWF protein expression and function. These two synonymous variants were found to lie close to the carboxyl and amino terminal of the VWF A1 domain. Importantly, the loop structure of the A1 domain is known to play a crucial role in platelet GPIbα receptor binding (Blenner *et al*, 2014). The presence of these two synonymous variants in the vicinity could be a cause of defective primary mRNA structure and, subsequently, aberrant protein folding. This could possibly lead to a conformational change in the VWF A1 domain loop results-in loss of platelet GpIbα dependent function.

The impact of p.Val1229Gly-p.Asn1231Thr on the GOF variant p.Pro1266Leu is interesting. Although these mutations did not show any detrimental effect on rVWF properties, they might reverse the conformational change of p.Pro1266Leu VWF, thereby normalizing the VWF function.

Taken together, our studies suggest that different gene conversions lead to differential properties of rVWF. The synonymous variants in the gene conversions were found to play a significant role in modulating VWF expression and/or function. Individually, the *VWF* conversion variants p.Val1229Gly, p.Asn1231Thr, p.Asn1231Ser and p.Ala1464-Pro had no effect on VWF synthesis or secretion *in vitro*. Moreover, combinations of the said missense variants in the absence of the synonymous variants, p.Ser1263= and p.Gln1449=, have shown minimal pathogenicity. These synonymous variants, individually or in combination with other gene conversion variants, showed significant impact on protein synthesis or post-translational modification, probably through faulty primary mRNA processing.

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Author contributions

FA; performed experiments, analysed data and wrote the manuscript, MK; performed experiments, acquired and analysed data, TO and SS; performed experiments and acquired data, UB and RS; designed study and analysed data, RS; designed study, analysed data and wrote the manuscript.

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