

Impact of novel missense VWF gene variants on protein expression and secretion in type 3 von Willebrand disease

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Article

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Abstract

Type 3 von Willebrand disease (VWD) is an autosomal recessive bleeding disorder typically associated with homozygous or compound heterozygous mutations in the *VWF* gene. This study investigated the expression and secretion of two novel missense variants, p.Tyr357His and p.Asp1076Glu, identified in patients with Type 3 VWD in the Pakistani population. Site-directed mutagenesis was used to introduce the variants into the *pcDNA3.1-VWF* plasmid, and successful incorporation was confirmed by Sanger sequencing. Expression analysis in HEK293T cells demonstrated that both variants were expressed at levels comparable to wild-type VWF, indicating normal intracellular expression. In contrast, analysis of conditioned media revealed a significant reduction in the secretion of p.Tyr357His, whereas p.Asp1076Glu showed secretion comparable to wild-type VWF. Protein concentrations were 0.6 µg/mL for p.Tyr357His, 3.0 µg/mL for p.Asp1076Glu, and 3.3 µg/mL for wild-type VWF. These differences were statistically significant ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test. Collectively, these findings suggest that p.Tyr357His impairs VWF secretion and may contribute to the severe Type 3 VWD phenotype, whereas p.Asp1076Glu does not appear to disrupt VWF secretion. These findings provide insight into the molecular effects of VWF variants on protein processing and secretion.

Introduction

Inherited quantitative or qualitative abnormalities in *VWF* (von Willebrand factor) gene underlie the pathophysiology of von Willebrand disease (VWD), manifesting as mucocutaneous bleeding of variable severity¹. Epidemiological data indicate that VWD affects approximately 0.1% in Western populations, with the majority diagnosed with type 1 VWD, which is classified through partial quantitative deficiency of VWF that is functionally normal^{2,3,4}. In contrast, Type 1 VWD prevalence in Asian populations seems to be lower, with a relatively higher frequency of type 2 subtypes and type 3, which are attributed to qualitative deficiencies in VWF function or a complete absence of VWF, respectively^{5,6}. The clinical phenotype is predominantly characterized by elevated mucocutaneous haemorrhage, including severe menorrhagia, as well as haemophilia-like bleeding manifestations such as deep tissue hematomas and hemarthroses^{7,8}.

The genetic factors contributing to Type 3 VWD are well determined and mutations related to this distributed on entire *VWF* gene leading to markedly reduced or absent synthesis of von Willebrand factor (VWF), typically defined by VWF antigen levels (VWF: Ag) below 5 IU/dL⁹. Type 3 von Willebrand disease is inherited in an autosomal recessive manner and is primarily associated with either homozygous or compound heterozygous mutations in the *VWF* gene. Nevertheless, approximately 40–50% of affected individuals exhibit patterns suggestive of codominant inheritance, as evidenced by heterozygous family members displaying a mild type 1 VWD phenotype. Despite the near-total absence of circulating VWF in type 3 VWD patients, there is considerable interindividual variability in the clinical severity of bleeding manifestations^{10,11}.

Molecular genetic studies of type 3 von Willebrand disease (VWD) cohorts across diverse populations have revealed that biallelic pathogenic variants in the *VWF* gene either in homozygous or compound heterozygous configurations are identified in approximately 85% of affected individuals^{10,12}. Rest of approximately 15% of patients, either non-pathogenic variants are detected or only a single heterozygous *VWF* mutation is found, which is insufficient to account for the severe quantitative deficiency of *VWF* characteristic of the type 3 VWD phenotype^{13–15}.

In individuals with type 3 von Willebrand disease (VWD), the near-complete absence of von Willebrand factor (VWF) protein can lead to the development of neutralizing alloantibodies, primarily due to repeated exposure to VWF-containing concentrates during treatment¹⁶. The frequency of autoantibodies development in type 3 VWD patients is estimated to be between 5–10%, with the 3WINTERS-IPS study reporting an incidence of approximately 6%. Most cases involving *VWF* null alleles are associated with homozygous mutations^{17,18}. Emicizumab, a bispecific monoclonal antibody that mimics factor VIII activity, has been proposed as a potential therapeutic option to prevent bleeding episodes in type 3 VWD patients with inhibitory alloantibodies. Another management approach includes platelet transfusions, which may offer additional hemostatic benefits^{19,20}. This study focused on novel missense variants identified at the genetic level in the Pakistani population. However, further investigation at the expression level is needed to investigate the underlying molecular processes contributing to the severity of VWD. The aim of this study was to characterize the novel missense variants of the *VWF* gene identified in the Pakistani population by evaluating their impact on von Willebrand factor (VWF) expression and secretion. To achieve this, two novel missense variants of the *VWF* gene, identified from the local Pakistani population, were expressed to explore the effect of the introduced variants on VWF protein expression and secretion.

Material and Methods

Selection of novel variants

The research was conducted in compliance with institutional and national ethical guidelines. Ethical approval was obtained from the Institute of Microbiology and Molecular Genetics Research Ethics and Biosafety Committee, University of the Punjab, Pakistan (Approval No. D/27/3–20/MMG). In the present study, two novel missense variants in the *VWF* gene were identified in individuals from the Pakistani population through prior genetic screening but their characterization on von Willebrand factor (VWF) protein expression and their role in the pathogenesis of von Willebrand disease (VWD) had not been explored²¹. To address this gap, the present study focused on the *in vitro* characterization of protein expression associated with the following missense variants.: p.Tyr357His (c.1069T > C) located in exon 9, and p.Asp1076Glu (c.3228C > G) located in exon 25. Both variants were selected for experimental validation based on their potential pathogenicity and their location within functionally significant domains of the *VWF* protein.

Site directed mutagenesis

Site-directed mutagenesis was performed to introduce two missense mutations p.Tyr357His (c.1069T > C), and p.Asp1076Glu (c.3228C > A) into the wild-type *VWF* cDNA cloned into the pcDNA3.1(+) expression vector. Mutagenesis was carried out using the KOD Hot Start DNA Polymerase (71086) (Sigma-Aldrich) and a template concentration was 50 ng. Mutagenic primers were designed using standard guidelines to introduce the desired nucleotide substitutions and the primer sequences for the variants were provided in Table 1. Following PCR amplification, the parental methylated DNA was digested with DpnI restriction enzyme (1 µL, 1 hour at 37°C) to selectively remove the non-mutated template plasmid. Gel electrophoresis was then conducted to extract the DNA from the gel using the E.Z.N.A.® Gel Extraction Kit (V-spin) SKU: D2500-01 (Omega Bio-Tek), and the concentration of DNA was estimated using a Nanodrop.

Table 1
Mutagenic Primer sequences used to induce specific mutations within *VWF* Gene.

Primer name	Primer sequence (5'-3')	Product size
p.Tyr357His F	GTGCATTCCGGAAAGCGCCACCCTCCCGGCACCTCCCTC	10kb
p.Tyr357His R	GAGGGAGGTGCCGGGAGGGTGGCGCTTTCCGGAATGCAC	
p.Asp1076Glu F	GACTGCAACAAGCTGGTGGAGCCCGAGCCATATCTGG	
p.Asp1076Glu R	CCAGATATGGCTCGGGCTCCACCAGCTTGTTGCAGTC	

Cloning of Novel Variants

XL 10-Gold Ultracompetent *E. coli* cells (Catalog No.50-125-094) from Thermofisher scientific were used to transform the site-directed mutated DNA with specific variants into the competent cells. Plasmids (pcDNA3.1) were isolated from the transformed cells using the E.Z.N.A.® Plasmid DNA Mini Kit (SKU: D6942-00S) from Omega Bio-Tek. Subsequently, the plasmid DNA was sent to GENEWIZ (<https://clims4.genewiz.com/RegisterAccount/Login>) for Sanger sequencing, while the primers for Sanger sequencing were designed by Eurofins Genomics, a sequencing primer design tool (<https://eurofinsgenomics.eu/en/ecom/tools/sequencing-primer-design/>). The Tm of the primers was checked through New England BioLabs (<https://www.neb.com/en-gb>). The sequences of the primers were provided in Table 2. To maximize the amount of site-directed mutated plasmid, the transformation was performed. For maxiprep, One Shot™ TOP10 Chemically Competent *E. coli* cells (Catalog number C404010) were used, and for the isolation of plasmid through maxiprep, the E.Z.N.A.® Plasmid DNA Maxi Kit (SKU: D6922-02) from Omega Bio-Tek (<https://www.omegabiotek.com/>) was utilized.

Table 2
Primer sequences for the sanger sequencing of VWF gene variants

Serial no.	Variants	Primer Sequence
1	p. Tyr 357 His	CTGGTATGGAGTATAGGCAG
2	p. Asp 1076Glu	AAGTGGAGGAAGACCCTGTG

Expression of VWF variants

Transfections were performed using human embryonic kidney cells (HEK293T) purchased from the Sigma-Aldrich (85120602-1VL) to evaluate the expression level of both wild-type and mutant VWF constructs. The cells were cultured using DMEM (Sigma-Aldrich-D6429) supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine. HEK293T cells were seeded in 6-well culture plates and maintained under standard conditions (37°C, 5% CO₂) until they reached approximately 70–80% confluence. Each wild-type VWF construct and variants generated by site-directed mutagenesis was transfected in triplicates to have 3 biological repeats for each sample. For transfection, 3 µg of purified plasmid DNA was mixed with polyethylenimine (PEI) at a 1:4 ratio (w/w), using a PEI stock concentration of 1 mg/ml. DNA and PEI were first diluted separately in serum-free Optimum, then combined and incubated at 37°C for 15–20 minutes to allow complex formation. The resulting DNA-PEI complexes were added dropwise to the culture wells containing fresh complete growth medium.

Cells were incubated for 72 hours post-transfection in a humidified 5% CO₂ incubator at 37°C. At the end of the incubation period, conditioned media were collected and centrifuged at 13000rpm for 10 minutes to remove cell debris. Supernatants were then filtered through 0.45 µm syringe filters and stored at – 80°C for subsequent analysis, as VWF is a secreted protein. To assess intracellular expression and transfection efficiency, cells were washed with PBS and lysed using RIPA buffer (Thermo Fisher Scientific) containing protease inhibitors. After harvesting, the cell lysates were centrifuged at 13000rpm and supernatant were collected and stored at – 80°C for downstream protein analysis.

Co Expression Analysis

To simulate heterozygous states, HEK293T cells were co-transfected with equal amounts (2.5 µg each) of Wild-Type (WT) and mutant VWF plasmids. The transfection followed the PEI-mediated protocol described previously, maintaining a 1:4 DNA-to-PEI ratio. Because VWF is a secreted glycoprotein, conditioned media were harvested 72 hours post-transfection, cleared of debris via centrifugation (13000 rpm, 10 min), and filtered (0.45 µm) before storage at – 80°C. Corresponding cell lysates were prepared using RIPA buffer supplemented with protease inhibitors to evaluate intracellular protein retention and overall transfection efficiency. The concentration of total protein in the conditioned medium and cell lysates was measured by Pierce™ BCA Protein Assay Kits (Catalog number: 23225).

Qualitative expression

Western blot analysis was performed to assess the expression and co expression of wild-type and mutant VWF proteins in both cell lysates and conditioned media after expressed into HEK293T cells. All three-biological repeat of each wild type and variant samples were prepared by mixing lysates and condition media with Pierce™ LDS Sample Buffer, Non-Reducing (4X) (Thermo Fisher Scientific, Cat. No. 84788) supplemented with 2 mM dithiothreitol (DTT) as a reducing agent, followed by denaturation at 90°C for 10 minutes. Forty microliters of each sample, in biological triplicates, were loaded onto a 4–12% Bis-Tris SDS-PAGE gel and electrophoresed at 150 V for 40 minutes until the dye front reached the gel bottom. Proteins were subsequently transferred to nitrocellulose membranes using Trans-Blot Turbo™ Mini Transfer Stacks (Bio-Rad) according to the manufacturer's instructions. Membranes were blocked with 5% non-fat dry milk in PBS-T (0.1% Tween-20) for 1 hour at room temperature to prevent nonspecific binding. For primary detection, the membranes were incubated overnight at 4°C with a polyclonal rabbit anti-human VWF antibody (Ref: A0082, Dako) diluted 1:5000 in blocking buffer. After extensive washing in PBS-T, the membranes were incubated at room temperature for 1 hour with an IRDye® 800CW-conjugated goat anti-rabbit IgG secondary antibody (LI-COR Biosciences) diluted 1:10000. Blots were washed three times with PBS-T and visualized using the Odyssey® CLx Imaging System (LI-COR Biosciences). Band intensity corresponding to VWF protein was quantified using Image Studio software (LI-COR). Expression levels from mutant constructs were compared with wild-type VWF in both lysates and conditioned media. All experiments were performed in biological Triplicates to ensure reproducibility and statistical validity.

Quantification of VWF Protein

To quantitatively assess VWF protein concentrations in the conditioned media of expressed HEK293T cells, an enzyme-linked immunosorbent assay (ELISA) was performed. A 96-well microtiter plate was coated with polyclonal rabbit anti-human VWF antibody (Ref: A0082, Dako) at a final concentration of 5 µg/mL in phosphate buffer saline (PBS). The plate was incubated at 4°C for 24 hours to allow antigen binding. Following incubation, wells were washed with PBS containing 1% Tween-20 (PBS-T) and then blocked using 3% dried milk powder (DMP) in PBS for 1 hour at room temperature to prevent non-specific binding. After blocking, 100 µL of diluted samples and VWF standards were added to the wells in duplicates from each biological repeat, leading to incubation at 37°C for an hour with gentle shaking.

The plate was washed three times with PBS-T, polyclonal rabbit anti-human VWF antibody conjugated to horseradish peroxidase (HRP) (LSBio, LS-C318380) was added at a 1:1000 dilution in 0.3% DMP, and the plate was further incubated at 37°C for 1 hour in the dark with rotation. Detection was carried out using o-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, P9187), freshly prepared prior to use. After adding the substrate, color development was monitored, then 50 µL of 2.5 M sulfuric acid was added into each well to stop the reaction after 6 minutes. Absorbance was measured at 490 nm using a microplate reader. Quantitative comparison was performed by interpolating sample values from the

standard curve generated using known concentrations of human VWF protein. All the variants and Wild type VWF protein biological replicates were analysed in duplicates to validate the expression.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism version 10 (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) was applied to compare VWF concentrations among the wild-type and mutant constructs. Post hoc comparisons were conducted using Tukey's multiple comparison test to determine statistically significant differences between individual groups. The analysis was carried out to compare the expression levels of each mutant variant against the wild-type VWF. A p-value ≤ 0.05 was considered statistically significant, and the null hypothesis was rejected when this threshold was met, indicating a significant difference in protein concentration. Additionally, degrees of freedom (df) were calculated to reflect the variability and sample size used in the comparison. All experiments were conducted in biological Triplicates to ensure robustness of the results.

Results

Induction of Variants and Cloning

Site-directed mutagenesis was successfully performed on the pcDNA3.1-VWF plasmid to introduce two specific mutations: p.Tyr357His, and p.Asp1076Glu. Mutation-specific primers were used in PCR amplification, and the resulting products were analyzed via gel electrophoresis using a 10 kb DNA ladder to verify successful amplification. The presence of the intended mutations were further confirmed by Sanger sequencing. Chromatogram analysis, as shown in Fig. 1, revealed the corresponding nucleotide substitutions: T > C for p.Tyr357His, and C > G for p.Asp1076Glu confirming the successful incorporation of both variants into the VWF gene sequence.

Transient Expression Analysis

Analysis of Cell lysates expressing full-length wild-type VWF and the variants p.Tyr357His, and p.Asp1076Glu, in three biological repeats showed strong and comparable VWF band intensities, indicating successful intracellular expression of all constructs (Fig. 2A). In contrast, analysis of the conditioned medium revealed variation in secreted VWF levels. The wild-type VWF and p.Asp1076Glu variant displayed similar band intensities, suggesting efficient secretion. However, the p.Tyr357His variant exhibited noticeably decreased band intensities, indicating significant reduced secretion into the medium in Fig. 2B. The band intensities have been measured through Odyssey software and represented in the form of relative intensities and the difference between condition medium to lysate in the form of ratio has also been represented in Fig. 2C.

Quantitative Analysis

The concentration of secreted VWF protein in the conditioned medium was quantitatively assessed for the wild-type full-length VWF and the variants p.Tyr357His, and p.Asp1076Glu, using ELISA, based on a standard curve generated with purified VWF at a concentration of 5 µg/ml. The wild-type VWF exhibited a concentration of 3.3 µg/ml, serving as the reference. The p.Asp1076Glu variant showed a comparable concentration of 3.0 µg/ml, indicating efficient secretion similar to the wild type. In contrast, the p.Tyr357His variant exhibited significantly reduced VWF levels, measuring 0.6 µg/ml. These findings, visualized in a corresponding bar graph, (Fig. 3) confirm that the p.Tyr357His impair the secretion of VWF, while p.Asp1076Glu does not appear to affect protein secretion efficiency.

Co transfection expression analysis

VWF expression was measured in the presence of co-expressed wild-type (WT) and variant proteins. Cells were co-transfected simultaneously with equal amounts of WT and variant plasmids, after which cell lysates and conditioned media were collected. Cell lysates expressing full-length VWF together with the p.Tyr357His variant, as well as WT VWF together with the p.Asp1076Glu variant, showed approximately equal levels of VWF expression in triplicate experiments (Fig. 4A). In the conditioned medium, co-expression of WT VWF with p.Tyr357His showed restored VWF expression compared with cells expressing the variant alone, suggesting that WT VWF rescues the secretion defect of the p.Tyr357His variant. In contrast, co-expression of WT VWF with p.Asp1076Glu showed comparable expression to WT alone, indicating that the p.Asp1076Glu variant does not affect VWF protein expression or secretion showed in Fig. 4B. the band intensities has been measured in the presence of cell lysates and conditioned medium and relative intensity can be compare and showed in the Fig. 4C.

Co expression quantitative Analysis

To analysed the concentration of the protein in co transfections to see how much protein expression and secretion has been restored ELISA has been performed. Both variants with wild type have almost equal concentration of secreted protein. Whether p.Tyr357His had significantly reduced expression alone but with wild type expression has been resumed showed in Fig. 5.

Statistical analysis

Quantitative comparison of VWF protein concentrations in the conditioned medium revealed statistically significant differences between the wild-type and certain mutant constructs. One-way ANOVA followed by post hoc Tukey's test was performed to assess the significance of variation in protein secretion levels among the wild type and the p.Tyr357His, and p.Asp1076Glu variants. The VWF concentrations for the p.Tyr357His (0.6 µg/ml) variant was significantly lower than that of the wild-type (3.3 µg/ml), with $p < 0.05$ indicating statistical significance. In contrast, the p.Asp1076Glu variant (3.0 µg/ml) showed no statistically significant difference from the wild type ($p > 0.05$), suggesting that this mutation possibly does not impair protein secretion.

Discussion

This study elucidates the effects of two novel missense variations, (p.Tyr357His, and p.Asp1076Glu) on the expression, biosynthesis, and secretion of the von Willebrand factor (VWF), a critical glycoprotein in haemostasis. Our findings showed that p.Tyr357His significantly reduced VWF compared to the wild type VWF construct, while p.Asp1076Glu demonstrate comparable expression levels with the wild type. These findings suggested that with substitution of specific amino acid on the specific position might interfere the VWF biosynthesis, expression and secretion. In contrast both variants showed comparable levels of VWF intracellularly as compared to wild type VWF constructs. As VWF is going into different post translational modifications within the endothelial cells that helps in the regulation of VWF multimerizations and stored into Weible-palade bodies. After activation of endothelial cells VWF is secreted²². This suggested that the expression of protein in the presence of variants within the cells does not abolish while they affect the secretion markedly. Several mechanisms explains that it might be possible because of misfolding of protein, atypical trafficking of protein from ER to Golgi apparatus, and impaired assembly of multimers²³. In addition to co expression of these variants with wild type helps in the rescue the expression and secretion that suggest under heterozygous condition, protein plays part in partial reduction of secretion for p.Tyr357His, while p.Asp1076Glu showed non-significant affect.

The p.Asp1076Glu variant entails a conservative substitution within the D3 domain of VWF. Structural modelling indicates that this alteration does not significantly disrupt the local conformation or stability of the protein. Consistent with this, experimental data demonstrate that the secretion levels and functional activity of VWF harbouring the p.Asp1076Glu mutation are comparable to those of the wild-type protein, suggesting a minimal impact on VWF's haemostatic function. The p.Tyr357His mutation results in the substitution of a tyrosine residue with histidine, potentially affecting hydrogen bonding networks within the D1 domain of VWF. This alteration may lead to subtle conformational changes that compromise the stability and secretion efficiency of the protein. Empirical observations reveal a marked decrease in the secretion of VWF carrying the p.Tyr357His mutation, underscoring its detrimental effect on protein processing and release²⁴.

This study highlights the critical role of specific amino acid residues in maintaining the structural fidelity and functional competence of VWF. Mutations that disrupt key disulphide bonds or alter hydrogen bonding networks can lead to misfolding, impaired secretion, and diminished plasma levels of VWF, culminating in the clinical manifestations of VWD. Understanding these molecular mechanisms is pivotal for the development of targeted therapeutic interventions aimed at correcting the underlying defects in VWF biosynthesis and function. These findings underscore the necessity of functional analyses to complement genetic studies in VWD. Identifying mutations that impair VWF secretion can inform diagnostic strategies and therapeutic interventions, particularly in populations with high rates of consanguinity, where such mutations may be more prevalent. Furthermore, understanding the molecular mechanisms by which specific mutations affect VWF processing can aid in the development of targeted therapies aimed at correcting these defects. Although they are novel variants but there is some limitations of the study. In this study transient transfections have been used to explore the secretions of protein which may not fully reproduce endogenous VWF expression. In this system overexpression of

protein produces non-physiological protein levels and this might also effects protein stability and translation. Western blot measures the protein abundance but functional analysis needs to be established for fully characterizations.

In conclusion, the p.Tyr357His variant adversely affect VWF secretion, likely through mechanisms involving impaired folding or multimerization, while the p.Asp1076Glu mutation does not exhibit such effects. These insights contribute to our understanding of the molecular pathology of VWD and emphasize the importance of integrating molecular and functional analyses in the study of bleeding disorders.

Declarations

Competing interests

The authors declare no competing interests.

Ethics declarations

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by Institute of Microbiology and Molecular Genetics Research Ethics and Biosafety Committee, University of the Punjab, Pakistan (Approval No. D/27/3–20/MMG). Human Embryonic Kidney cells (HEK293T) were purchased from the Sigma-Aldrich (85120602-1VL) used for the expression analysis of VWF protein.

Consent to participate

Not applicable. No human participants were involved in this study; only established human embryonic cell lines were used for in vitro experiments.

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Author Contribution

Arshia Latif contributed to the project design, while NA Shabana reviewed the study concept. Arshia Latif performed data collection, conducted the experiments, and carried out the analysis and interpretation of the results. The first draft of the manuscript was written by Arshia Latif, and NA Shabana critically

reviewed the manuscript and provided logistic support. All authors reviewed the results and approved the final version of the manuscript

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Data Availability

All data supporting the findings of this study are available within the paper.

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Figures

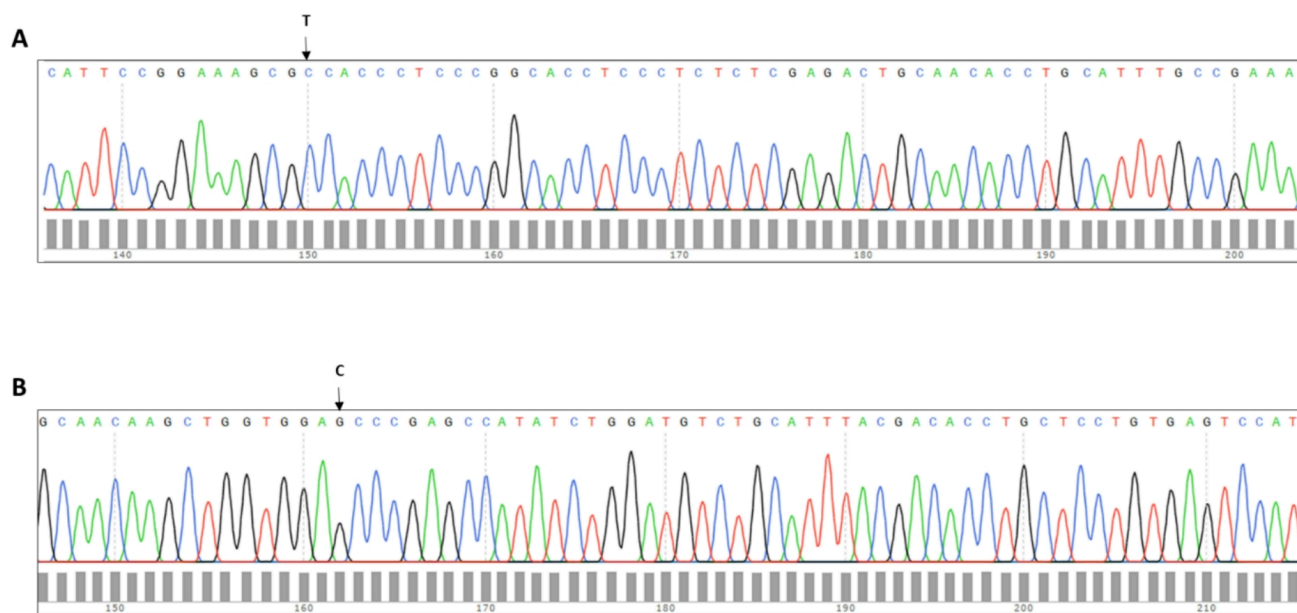


Figure 1

(A) Electropherogram showing a nucleotide change c.1069T>C encodes missense mutation p.Tyr357His. Image B: Electropherogram showing a nucleotide change c.3228C>G encodes missense mutation p.Asp1076Glu.

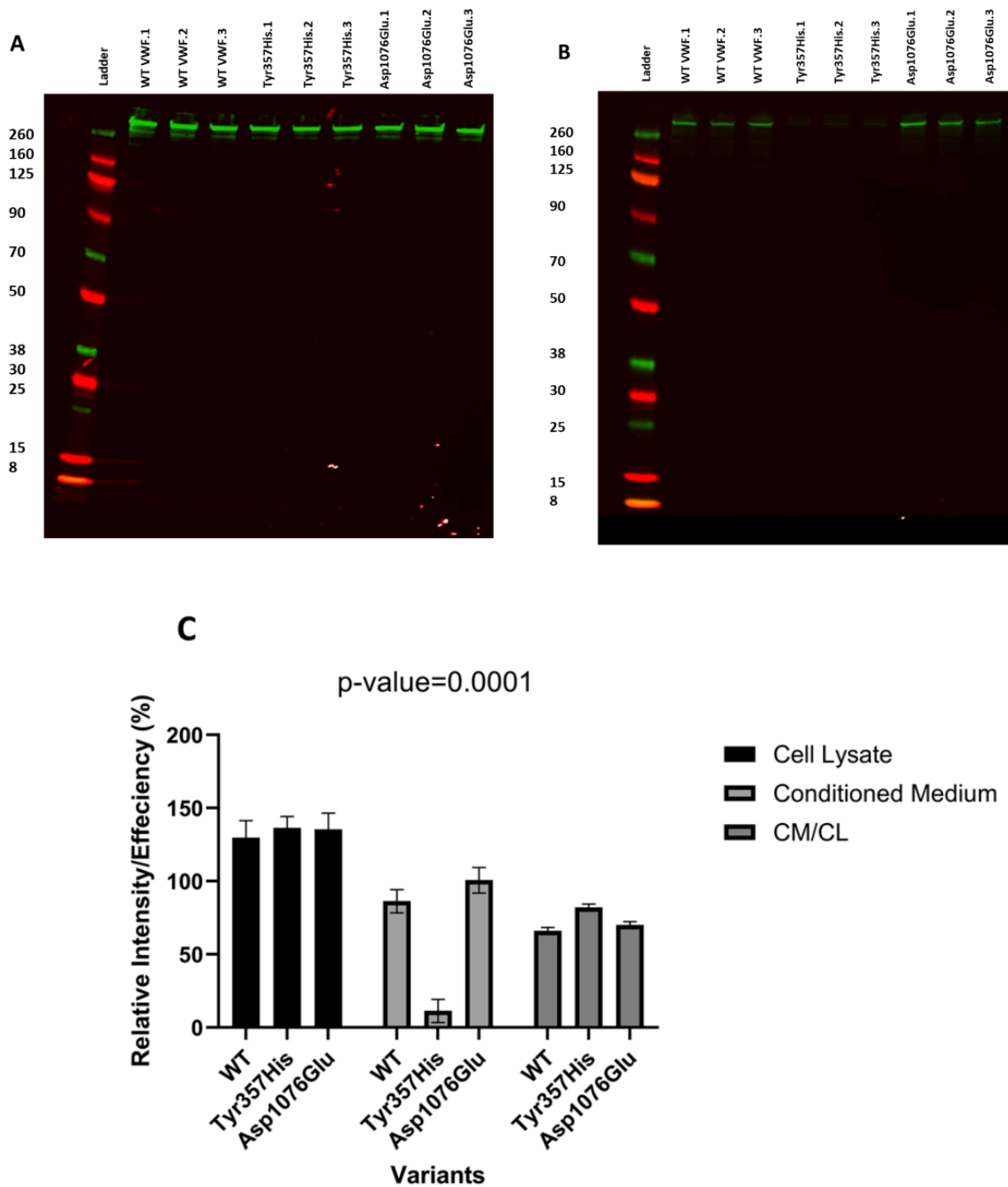


Figure 2

(A) Results of western blotting of wild type and, mutants p.Tyr357His, and p.Asp1076Glu cell lysates. Transfections of Wild type and mutants have been done in triplicates to confirm the results and run in parallel to make a good comparison. (B): Results of western blotting of three individual transfected wells of mutants (p.Tyr357His, and p.Asp1076Glu) indicates the intensity of Conditioned Medium with respect to wild type full length VWF. (C) Figure indicates the average of relative integrated band intensities of

triplicate transfected cells, in condition medium (CM) and cell lysates (Lys). While in the respective condition medium to lysate ratio of relative integrated band intensities of variants has been measured and compared them with wild type (WT) VWF.

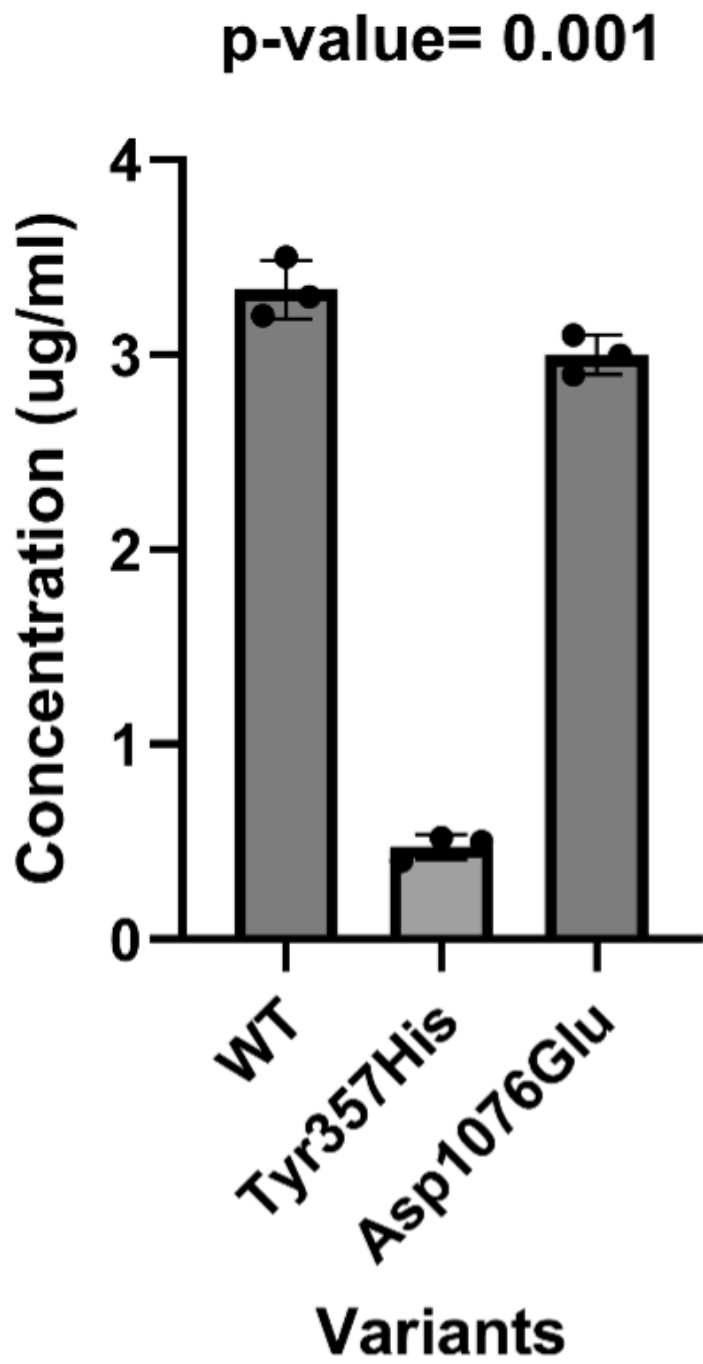


Figure 3

The bar chart showed the concentration in $\mu\text{g/ml}$ of VWF in the presence of p.Tyr357His, and p.Asp1076Glu variants and wild-type full length VWF in the conditioned medium as established with ELISA. p. Tyr357His variant showed significant reduction in the concentration in condition medium as compared to wild type VWF. While p.Asp1076Glu indicated similar secretion as wild type.

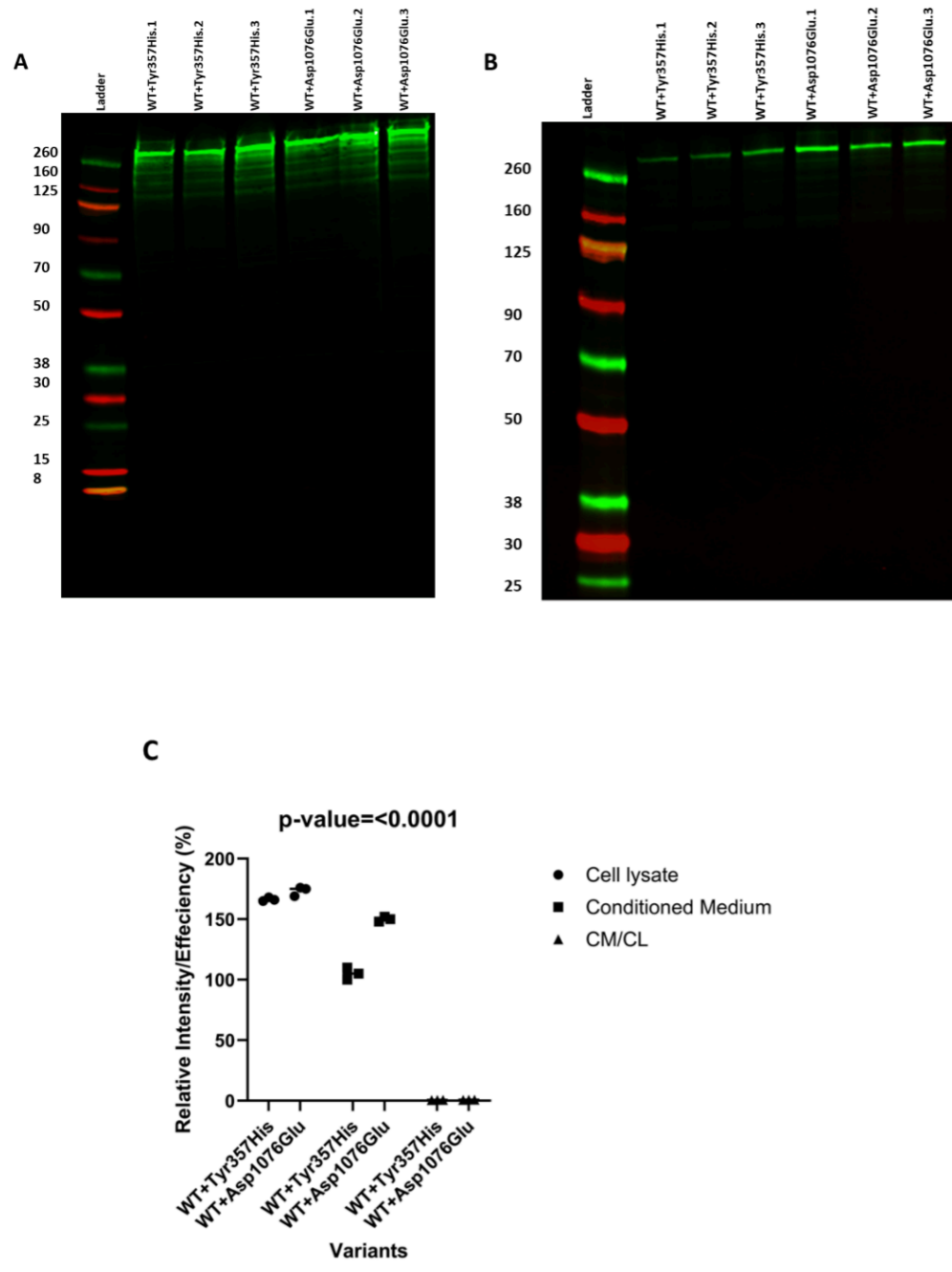


Figure 4

A Results of western blotting of co transfecting wild type VWF and, mutants WT+p.Tyr357His, and WT+p.Asp1076Glu cell lysates. Co-transfections of Wild type VWF and mutants have been done in triplicates to confirm the results and run in parallel to make a good comparison. (B): Results of western blotting of three individual co transfected wells of mutants with wild type VWF (WT+p.Tyr357His, and WT+p.Asp1076Glu) indicates the intensity of Conditioned Medium in the presence of full length VWF. (C) Figure indicates the average of relative integrated band intensities of triplicate transfected cells, in condition medium (CM) and cell lysates (Lys). While in the respective condition medium to lysate ratio of relative integrated band intensities of variants has been measured.

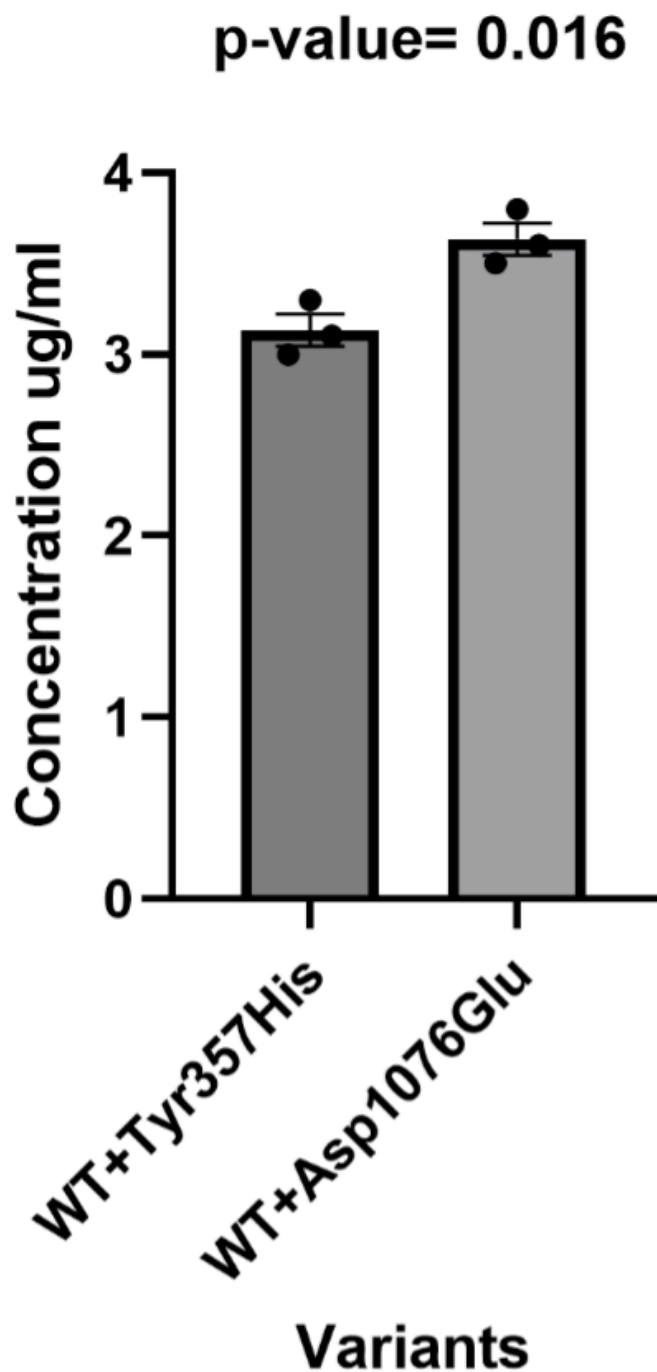


Figure 5

The bar chart showed the concentration in $\mu\text{g/ml}$ of VWF in the presence of WT+p.Tyr357His, and WT+p.Asp1076Glu variants after co transfection in the conditioned medium as established with ELISA. Both variants restore their secretion in the heterozygous condition as compared to homozygous form. Both variants were co transfected in triplicates to verify the reproducibility