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Determination of ADAMTS13 Susceptibility in Type IIA von Willebrand Disease

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Determination of ADAMTS13 Susceptibility in Type IIA von Willebrand Disease

By
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Abstract

von Willebrand Disease (vWD) is a bleeding disorder caused by a disruption in the coagulation cascade. This disruption is due to a deficiency with von Willebrand Factor (vWF), a large globular glycoprotein found in circulating blood. There are three main types of vWD— Type I, II, and III. Type I and III are expression deficiencies, and Type II is a functional deficiency. Type II is further sub-typed based on vWF's change in activity. Sub-type Type IIA results from an abnormality in vWF's inability to produce high molecular weight multimers. One mechanism believed to cause this loss of high molecular weight multimers is increased ADAMTS13 cleavage. ADAMTS13 is a metalloprotease in the blood stream that interacts only with vWF and regulates vWF's size. We characterized nine mutations suspected of causing vWD Type IIA using an *in vitro* ADAMTS13 digestion assay. We determined that six mutations— R1583Q, R1527Q, S1543F, N1635I, Q1571H and G1643S— were susceptible to ADAMTS13; while M1495L, I1509V, and A1464P were not susceptible to ADAMTS13. We then used all mutations with characterized ADAMTS13 susceptibility to determine that there is an equal prevalence percentage (i.e. chance of having a susceptible or non-susceptible mutation to ADAMTS13) in the population. Further *in vitro* studies should be done to support characterization and differentiation between vWD Type IIAII and vWD Type IIAI mutations to aid in the development of targeted therapeutics.

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Abbreviations

acronym	Word/phrase	definition
vWD	von Willebrand Disease	Bleeding disorder
vWF	von Willebrand Factor	High molecular weight protein involved in coagulation
NHP	normal human plasma	Human plasma collected from patients
FVIII	Factor VIII	Clotting protein
aa	amino acids	Building block of proteins
WPB	Weibel Palade Bodies	Storage vesicles of the cells
GPIbα	platelet binding site	Connects platelets to vWF and collagen and FVIII
Pd-vWF	plasma derived-von Willebrand Factor	Von Willebrand Factor collected from the plasma of normal, healthy patients
DDAVP	1- deamino-8 D-arginine vasopressin	Synthetic hormone that triggers the release of stored vWF as a treatment for vWD
r-vWF	recombinant- von Willebrand Factor	Synthetically-made von Willebrand Factor
CHO	Chinese hamster ovary cells	Mammalian cell line used to express recombinant vWF genes and signaling sequences
YM	Tyr1605-Met1606	ADAMTS13 Cleavage site for vWF
WT	wild-type A1-A3 vWF domains	Natural von Willebrand Factor A1-A3

Specific Aims

Specific aim 1: Based on literature review, formulate a list of all known vWD Type IIA mutations and include information such as: location in the amino acid sequence, phenotype, publications, ADAMTS13 susceptibility, and if patient plasma was used in the studies. In addition, the list will contain vWF mutations with the potential to be ADAMTS13-susceptible based on their domain location.

Specific aim 2: Develop *in vitro* digestion assays to identify vWF mutations with increased susceptibility to ADAMTS13.

Specific aim 3: Select mutations with characterized ADAMTS13 susceptibility and look in the population genomic databases, gnomAD (a public database) and Variants Viewer (a private database), to determine a prevalence rate of ADAMTS13 susceptible or non-susceptible mutations in the estimated population of vWD Type IIA individuals. Then compare of the rates to establish the odds of an individual with Type IIA having an ADAMTS13 susceptible mutation.

Background

History. von Willebrand Disease (vWD) is the most common inherited bleeding disorder and was originally known as “hereditary pseudo hemophilia” [1, 2]. vWD was discovered by Dr. Erik von Willebrand in 1926 (Figure 1). Dr. von Willebrand believed the cause of his patients’ prolonged bleed time was due to a problem in with proteins involved in coagulation process [3]. In the 1950s, vWD was recognized as a separate disease from Hemophilia, as Hemophilia involves problems with the coagulation protein Factor VIII (FVIII), and vWD is due to deficiencies in a Factor VIII co-factor now known as von Willebrand Factor [4]. In the 1960s the search for the function of this new Factor VIII co-factor began and the development of *in vitro* assays flourished. As scientific knowledge and technologies progressed providing the DNA and amino acid sequences [5] for vWF, *in vitro* assays became more specific. These more fined-tuned *in vitro* assays furthered the knowledge of vWF mechanisms of action (Figure 1) [6].

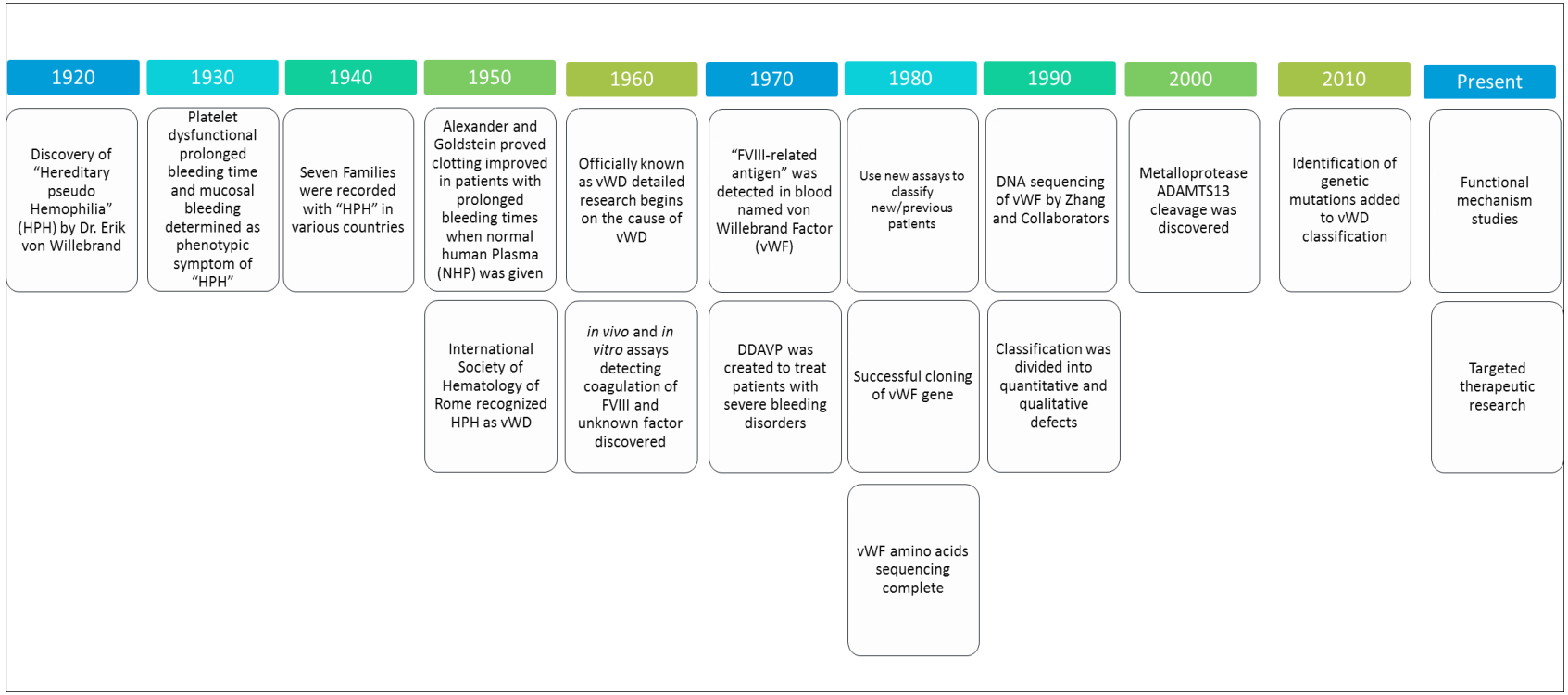


Figure 1. *Time line for vWD.* A linear representation of the history of vWD starting with Dr. Eric von Willebrand's recognition of a new bleeding disorder in 1920 through current research.

von Willebrand Factor (vWF) protein. The blood protein vWF is a large glycoprotein that initiates the coagulation cascade by binding platelets, collagen and FVIII together. When all four components— platelets, vWF, collagen and FVIII— are bound together, a blood clot (also known as a platelet plug) is formed (Figure 2) [7, 8]. The platelet plug prevents blood from leaving the damaged blood vessel and stimulates endothelial cell regrowth to repair the wound.

One important characteristic of vWF that allows the protein to form clots is its ability to assemble large multimers of 20+ monomers long [1]. vWF monomers are secreted from weibel palade bodies (WPB), storage vesicles found in endothelial cells [6, 9]. vWF monomers first dimerize prior to creating large multimers. After dimerization and multimer assembly, vWF multimers become fully functional and are released into the blood stream [6]. When vWF is not in multimer form, the protein is less effective in its ability to form clots due to reduced binding affinity for collagen and platelets [7, 10, 11]. vWF multimers that function properly are known as high molecular weight vWF multimers (>10 monomers long).

vWF contains 14 functional domains (Figure 3A). Domains A1, A3 and D3 contain binding sites for platelets (GPIb-alpha), collagen and FVIII [1], while domain A2 contains the sequence for the protease ADAMTS13 cleavage site (Figure 3A). Mutations within these domains are reportedly most responsible for non-functional vWF; and the resulting dysfunctions include structural and binding abnormalities in vWF [7, 12-15].

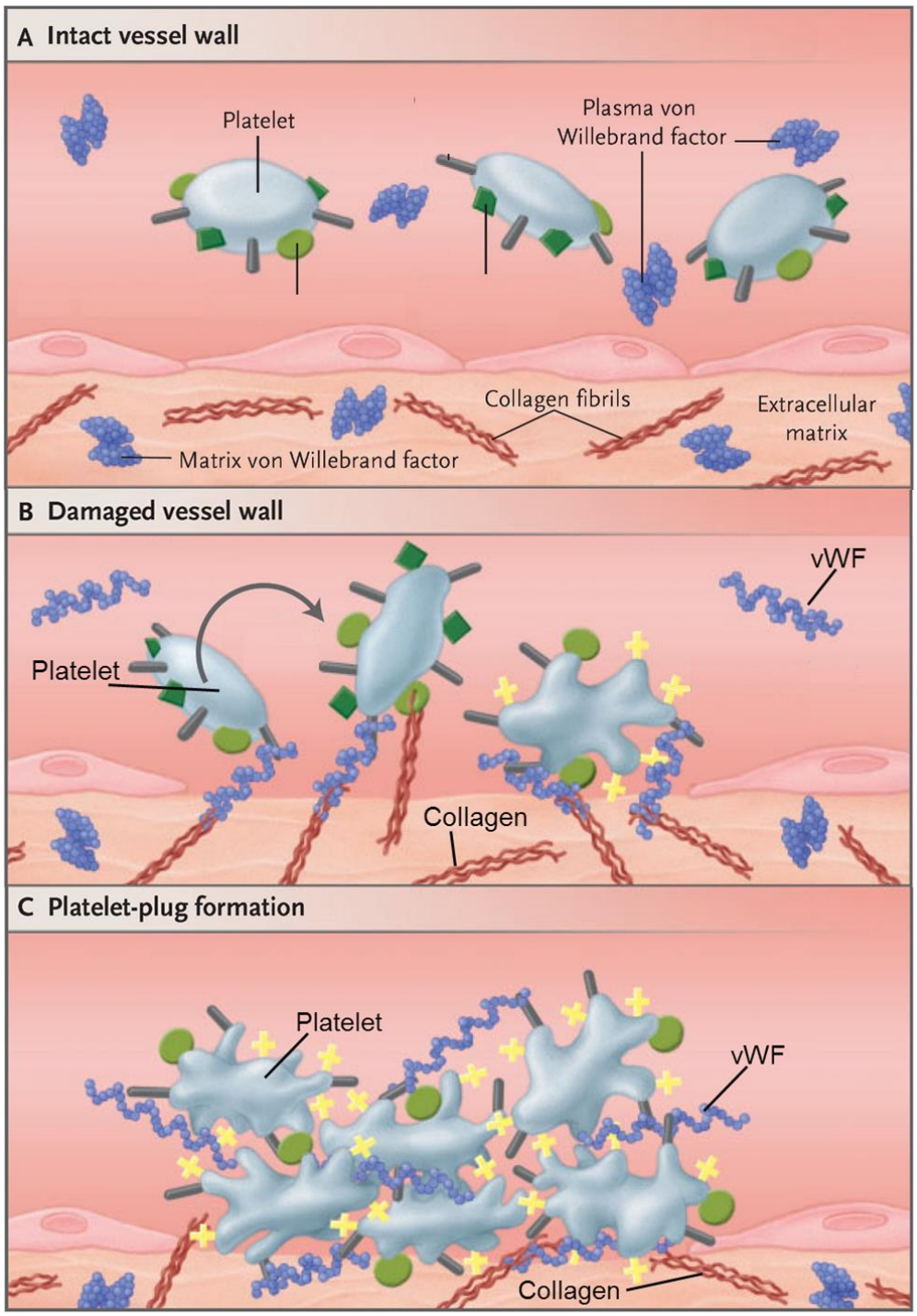


Figure 2. *Coagulation cascade and platelet plug formation*. Key components under normal blood flow conditions (A). Initial response of coagulation proteins to vessel injury. vWF is elongated by increase shear force in the vessel and binds to platelets, collagen and FVIII (B). Platelet plug formation is achieved once a critical mass of platelets, vWF and associated coagulation proteins bind together to seal the vessel wall (C). Figure reproduced with permission from N Engl J Med 2004;351:683-94, Copyright Massachusetts Medical Society.

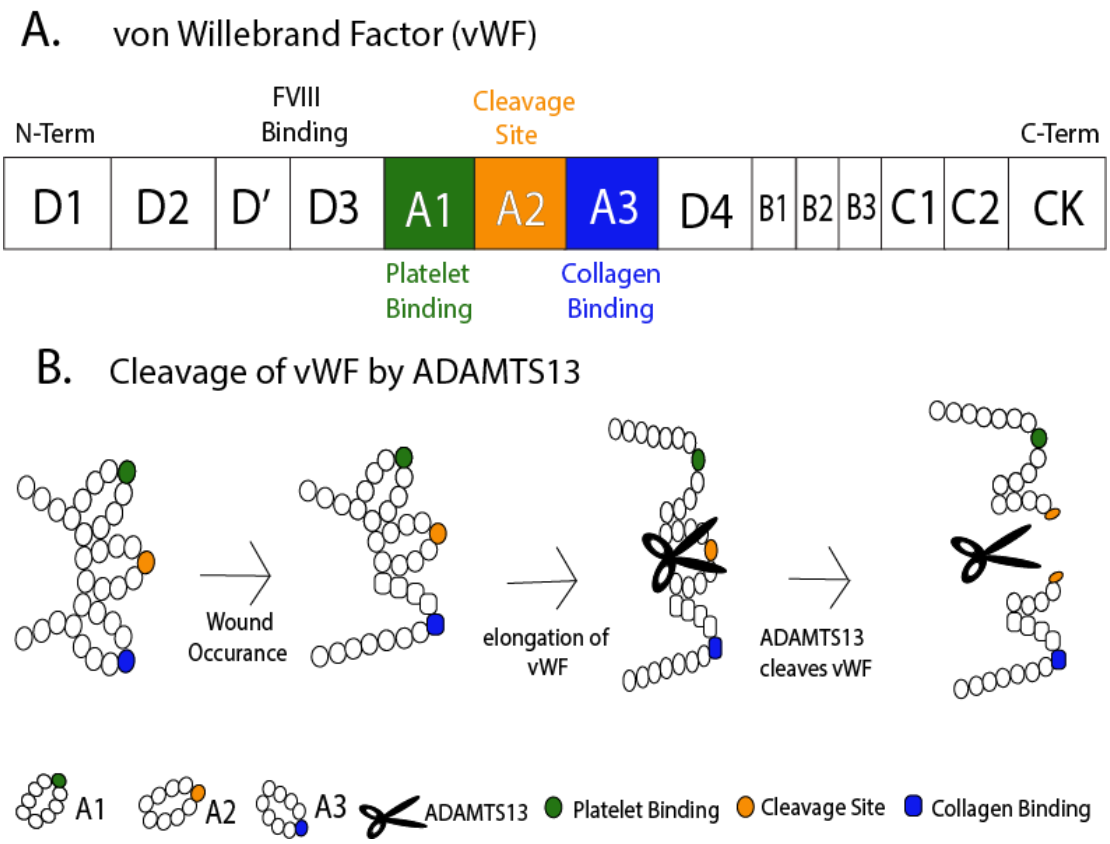


Figure 3. *Cartoon representation of vWF.* The 14 domains of the vWF gene indicating the location of functional binding of FVIII(black), platelet(green), cleavage site(blue) and collagen(purple) (A). The lengthening of vWF once a wound occurs is caused by the increasing shear force. This increase of shear force opens the A2 domain exposing the cleavage site (blue circle), leaving it susceptible to the metalloprotease ADAMTS13 (mini-scissors) (B).

Regulation of von Willebrand Factor (vWF) by ADAMTS13. The metalloprotease ADAMTS13 was discovered in 2001, and subsequent studies established its importance in vWF cleavage and blood homeostasis by regulating the size of vWF multimers [16]. Though ADAMTS13 comes from a large family of metalloproteases, it only recognizes vWF as a substrate [17]. This is considered unusual given that the ADAMTS family of enzymes usually interacts with multiple substrates [17].

The elongation of vWF via shear force not only increases the probability of binding to platelets and collagen but also exposes the vWF's cleavage site, the Tyr1605 - Met1606 (YM) bond, leaving it open to ADAMTS13 (Figure 3B) [13, 18-23]. An abnormally high increase in vWF

cleavage can cause von Willebrand Disease Type IIA [16], as large multimers are necessary to maintain a balance between proper binding and cleavage. When mutations in vWF result in improper folding or gaps in folding, the protein becomes less stable, which increases its susceptibility to ADAMTS13 cleavage.

von Willebrand Disease classification. There are three types of von Willebrand disease (vWD) — Type I, II, and III [24]. Patients with Type I and III vWD have deficiencies in vWF expression, while Type II is due to a functional deficiency in vWF. Mutations that result in expression deficiencies exhibit normal binding of vWF to all clotting components, but the cells produce lower quantities, or in rare occasions, none. Mutations that result in functional deficiencies express the normal levels of vWF monomers, but some aspect of its function— binding, multimerization, or cleavage susceptibility— is altered. These functional deficiencies (Type II) are further classified into five specific subtypes: IIA(I), IIA(II), IIB, IIN, and IIM (Figure 4).

The most common form of vWD is Type I [21], which is characterized by a low expression vWF protein. Type I is generally a mild form and has a range of symptoms from no phenotypic effects to prolonged bleeding times. The low protein expression does not affect the function of vWF, but it does lengthen the coagulation time [8, 14, 24].

Type II is the second most common form of vWD [12] and is diagnosed based on vWF over-activity or inactivity. vWF malfunction leads to platelet plug problems resulting in severe blood loss and hematomas (bruising caused by bleeding under the skin) [4, 25]. As Figure 4 demonstrates, vWD Type II subtypes include IIA (group I and II), IIB, IIN and IIM [21]. Type IIA mutations result in a loss of high molecular weight multimers of vWF which subsequently leads to a reduction in platelet binding. Multiple mechanisms have been implicated as a cause of the decreased length of multimers, which has led to the further subgrouping of Type IIA [22].

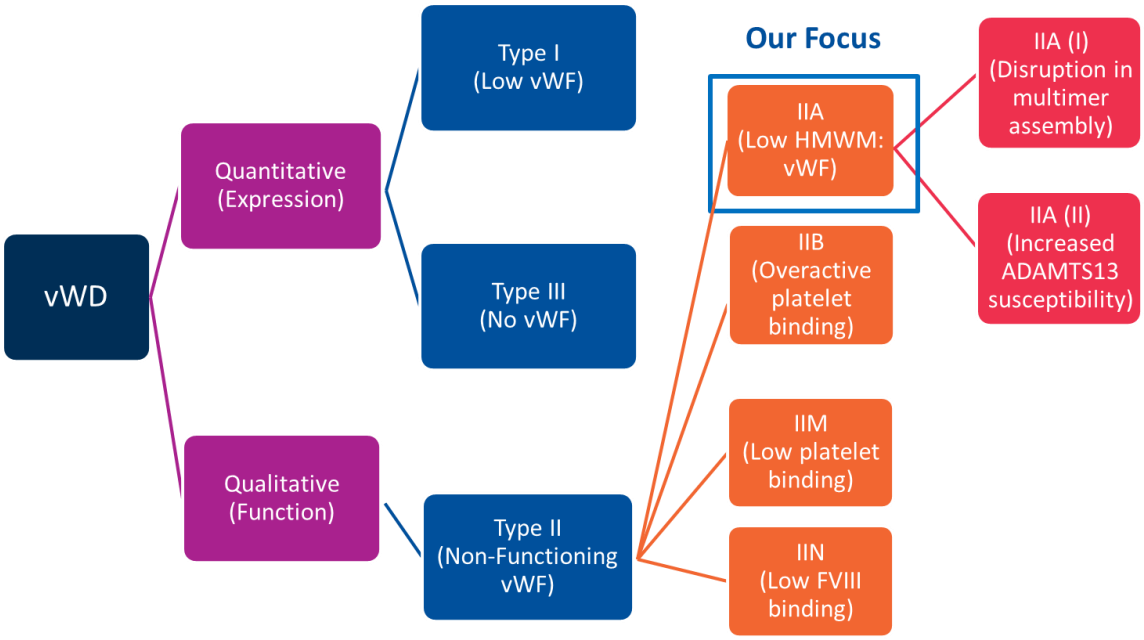


Figure 4. *Classification of von Willebrand Disease (vWD).* A diagram of vWD separated based on quantitative (expression) or qualitative (functional) deficiencies. The focus of this study is outlined in blue.

Type IIA group I (IIAI) mutations result in decreased multimer size due to an interruption in the assembly of multimers [1], whereas Type IIA group II (IIAII) mutations increase ADAMTS13 susceptibility and thus result in lower levels of high molecular weight vWF multimers [23, 24]. Type IIB mutations increase vWF's affinity to platelets, leading to faster clearance and thus causing an ineffective platelet plug and continuous bleeding [1]. Type IIN mutations prevent Factor VIII binding, which causes the platelet plug to be released early and prevents endothelial cell stimulation and regrowth [24]. Finally, Type IIM mutations result in decreased platelet binding due to a disruption in the platelet binding site, GPIb-alpha. GPIb-alpha binding leads to conformational changes in the platelets which is necessary for effective platelet plugs to form [26]. If vWF does not bind to platelets, then platelet aggregation does not occur and bleeding results [6, 24, 27].

Type III is the rarest and most severe form of vWD. Type III is due to a defect that results in the absence of vWF which in turn prevents initiation of the coagulation cascade. Those

individuals with Type III vWD are often misdiagnosed as Hemophilia A patients due to the amount of bleeding that occurs from a wound [7, 24]. Research is ongoing to delineate the mechanism and characterize the genetic mutations associated with Type III vWD [1, 14, 24].

Current *in vitro* tests to determine classification. The identification of vWF as a blood protein critical in coagulation [6] brought about the development of *in vitro* assays involving coagulation and collagen binding. The most common procedures are SDS-agarose gel electrophoresis to determine multimer size distribution of vWF and enzyme-linked immunosorbent assays (ELISAs) to quantify binding to collagen, FVIII and platelets as well as levels of vWF. Both procedures, however, provide inconsistent results which can lead to misclassification of the disease.

von Willebrand Disease (vWD) Treatment. There are currently two main types of treatment for vWD: plasma- or chemical-based. For plasma-based treatments, either normal human plasma (NHP) or plasma-derived vWF (Pd-vWF) are administered to the patient. NHP, which temporarily supplies the patient with healthy blood protein factors that include von Willebrand Factor, kick-starts the formation of platelet plugs [4, 5]. Pd-vWF is purified vWF from plasma with no other blood factors. The alternative chemical-based therapeutic is 1- deamino-8 D-arginine vasopressin (DDAVP), a synthetic hormone that triggers the release of stored vWF [3, 5, 28]. Both treatments have specific uses and limitations. Plasma-derived therapeutics are normally administered after a bleeding event has occurred [3, 6] and works for all types of vWD. The limitations for plasma-derived therapeutics are the short half-life and possible allergic reactions of recipients. DDAVP, on the other hand, has no effect on individuals with functional deficiency of vWD, such as those with Type II or Type III vWD. DDAVP is effective only for Type I patients who store fully functional vWF [1, 3].

A new therapeutic using full-length recombinant-vWF (r-vWF) to replace Pd-vWF is currently in clinical trials [29]. Some of the benefits of r-vWF are fewer allergic reactions and a longer half-life in comparison to Pd-vWF [29]. However, there are currently no alternative treatment options for functional deficiencies of vWD other than alleviating the bleeding symptoms by injecting fully functional coagulation blood proteins.

Contributions of this study. In recent years, researchers have tried to simplify vWD classification while trying to elucidate the roles of vWF, ADAMTS13, platelets and other blood factors in the coagulation pathway. Identifying the blood proteins involved and their mechanism of action will allow for the development of more targeted therapeutics that can enhance the quality of life for patients with vWD [4, 30]. Deficiencies in vWF function can have mild to severe effects, including dizziness and internal bleeding. While great strides have been made in identifying and defining vWD, there are still no comprehensive, universally-accepted diagnostic methods [1, 19, 24]. As a result, the variance in laboratory testing often leads to misdiagnoses and improper medical counseling of patients [14]. However, the increased usage and advancement of gene sequencing has aided in the discovery of new mutations on the vWF gene, some of which may be involved in the Type IIA phenotype. By classifying these mutations, we can link phenotype to molecular mechanism, which may allow for more targeted therapy.

Research Design and Methods

Critical review of literature to identify and sort vWD Type IIA mutations. Characterized, uncharacterized mutations, and potential Type IIA mutations were identified in the literature [1, 2, 8, 10, 12, 19-22, 24, 28, 30-41]. The mutations were organized into a table (Supplemental Table 1) listing its location within the amino acid sequence and the name of the amino acid that resulted

from each mutation. Supplemental Table 1 also lists the effect of each mutation on ADAMTS13 susceptibility, the domain in which the mutation is located, the current vWD classification, whether patient blood was tested, assays used to determine ADAMTS13 susceptibility, the vWD subtype classification, and the associated publications.

Plasmid construct design to test vWF mutations. UniProt (http://www.uniprot.org/uniprot/P04275) and the databases managed by the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/nuccore/NC_000012.12?strand=2&report=genbank&from=5948874&to=6124770) were used to determine the human vWF amino acid (A.A.) and nucleotide sequences for domains A1-A3, which started at nucleotide14263 and ended at nucleotide15943. Using the mutations identified from the literature described above, 15 plasmids were designed, each with a single point mutation in the A1-A3 domain of the vWF gene. Another plasmid was designed to contain the wild-type vWF domains A1-A3. In addition, the plasmids were designed with a 6xHis-tag was placed on the end of the vWF gene fragment and a FLAG-tag was placed at the beginning of the gene fragment. A signal sequence was also included to promote protein secretion. The complete plasmid sequences were sent to ATUM (DNA2.0, Newark, CA) for production.

Transfection and vWF Protein Expression. von Willebrand Factor domain A1-A3 plasmids containing different mutations (ATUM (DNA 2.0)) were transfected into Chinese Hamster Ovary (CHO) cells via electroporation using MaxCyte STX (MaxCyte, Gaithersburg, MD). Cells were grown for a period of five days. On the fifth day, cells and media were transferred into a 30mL conical tube and harvested by centrifugation at 15,000g. The supernatant and cell pellet were separated and analyzed using western blot for vWF expression. Briefly, samples were run on a 4-

12% Bis-Tris gel (Invitrogen, Carlsbad, CA) in 1x MOPS buffer for 1.5 hours at 140V. Proteins were then transferred using the iBlot system (Invitrogen) onto a nitrocellulose membrane (Invitrogen) and blocked with Starting Block (ThermoScientific, Waltham, MA) for 1 hour. Membranes were then incubated overnight with a His-Detector Nickel-Alkaline Phosphatase (SeraCare, Milford, MA) at a 1:400 dilution. Membranes were then washed in 1xTris-buffered saline with 0.05% NP-40 detergent (TBSN) and 1x Tris-buffered saline (TBS). Western Blue Stabilized Substrate for Alkaline-Phosphatase (Invitrogen) was used to visualize expression of the protein.

Fast Protein Liquid Chromatography (FPLC) affinity purification of vWF protein domains.

The vWF protein domains were purified from the supernatant (see above) using a His-Trap (GE, Boston, MA) 5mL column with nickel resin. The protein was eluted with increasing concentrations of imidazole, ranging from 500mM to 1M. A 150mM of sodium chloride (NaCl) solution was added to the supernatants prior to loading onto the column. An ÄKTA system (GE Health, Boston, MA) was used to automate the loading, binding, wash, and elution of the column as well as fraction collection of the samples. Once the purification method was complete, 65µL aliquots of the wash, flow-through, column cleaning and fractions were collected and combined with 25µL of LDS sample buffer 4x (ThermoFisher, Waltham, MA) and 10µL of sample reducing Agent 10x (ThermoFisher). These samples were then loaded onto a 4-12% Bis-Tris gel (Invitrogen), run at 180V for 65 minutes in MOPS buffer, and transferred using the iBlot system (Invitrogen) onto nitrocellulose membrane. The membrane was blocked in Starting Block (ThermoScientific) for 1 hour and incubated overnight with Nickel- AP (SeraCare). Western Blue Stabilized Substrate Alkaline-Phosphatase (Invitrogen) was then used to visualize the bound Nickel-AP and His-tagged protein. The fractions containing the protein of interest were combined, and buffer was changed

from 1M imidazole to 1x phosphate-buffered saline (PBS) using VivaSpin500 concentrators (GE Health) that had a10kDA molecular weight cut off. The protein concentration was determined by measuring absorbance at 280nm using a NanoDrop (ThermoScientific).

Shear vortex assay vWF digestion gel. A mix-mate (Eppendorf, Hamburg, Germany) was used to replicate shear force in the body at 2500rpms for 2 hours. Using 20µL as the total volume, each sample contained purified-von Willebrand Factor (150nM) from one of the 16 constructs, ADAMTS13 (A13) (25nM), 1x vortex shear buffer (20mM Hepes pH 7.0, 5mM $CaCl_2$, and 1mg/mL bovine serum albumin (BSA)). Each sample was treated in the presence and absence of EDTA, which prevents A13 from cleaving vWF. To analyze the resulting vWF cleavage, 8µL of LDS buffer (ThermoFisher) and 3µL of reducing agent (ThermoFisher) were added to the samples. Samples were then heated at 90°C for 5 minutes, and 10µL per sample were loaded onto a 4-12% Bis-Tris gel in 1x MOPS for 1.5 hours at 140Vs. The gel was then transferred onto nitrocellulose utilizing the iBlot system (Invitrogen). Once transfer was complete, membranes were blocked for an hour in Starting Block (ThermoScientific) and placed in anti-His-HRP antibody (R&D systems, Minneapolis, MN) at a dilution of 1:2000 overnight. The membrane was then washed once with TBSN and 3 times with TBS to remove excess antibody, and an ECL kit (GE Health) was then used to visualize binding/ banding using FluoroChem-M (Protein Simple, San Jose, CA) to capture the fluorescence.

Urea- and non-urea- treated samples on vWF digestion gel. The addition of various concentrations of urea (VWR, Radnor, PA) was used to denature the protein for cleavage (further explained in Results section). vWF constructs ranging from 30.43nM-150nM, A13 ranging from 30.43nM-800nM, urea ranging from 1M to 5M, and EDTA (0.02M) were mixed together. Samples were incubated at 37°C from 3 hours to overnight. LDS sample buffer (25% of total volume) and

reducing agent (10% of total volume) were added and then heated at 90°C for 5 minutes. Samples were loaded onto a 4-12% Bis-Tris gel (Invitrogen) in 1x MOPS buffer and run at 180V for 65 minutes. The gel was then transferred using the iBlot system (Invitrogen) onto nitrocellulose. The membrane was blocked in Starting Block (ThermoScientific) for one hour. After blocking, anti-His-tag antibody (1:2000; R&D systems), or anti-FLAG-tag antibody (1:2000; R&D systems) was added for overnight incubation. Membranes were washed of excess antibody, and ECL kit (GE Health) was then utilized to visualize the bound substrate and antibody with a FluoroChem-M (Protein Simple).

Database exploration for estimated number of vWD Type IIA individuals. A search of two genomic databases was carried out to determine allele frequency for each mutation. The databases searched were gnomAD (public, http://gnomad.broadinstitute.org/) and Variant (BioMarin, San Rafael, CA). vWD is heterozygous dominant disorder meaning a mutation needs to be present on only allele. Thus, the allele frequency for each vWF mutation was tallied and multiplied by two. Each frequency was then compared to the estimated population of individuals with vWF Type IIA, which was calculated based on current population on Earth (7.6 billion people) and cited incident rate of 1 in 50,000. This frequency ratio then helped to determine the percent of incidence of increased ADAMTS13 susceptibility. Finally, those percentages were used to determine the expected number of individuals with increased susceptibility vs individuals with non-susceptible mutations.

Results

Von Willebrand Disease (vWD) Type II, which is known as a functional deficiency of von Willebrand Factor (vWF), is not easily sub-classified. This study looks at the literature and genetic

databases to identify vWF mutations suspected of being Type IIA and analyzes the mutations *in vitro* assays to determine which mutations lead to vWF susceptibility to ADAMTS13 cleavage, which will help classify the vWD Type IIA group II (Type IIAII) phenotype.

Critical Review. After compiling and reading through the literature on vWD, a table was formulated to include all possible or known vWD Type IIA mutations. In total, sixty-six mutations within vWF A1-A3 domains were assembled into Supplemental Table 1. The table lists the amino acid location, exact amino acid switch, susceptibility to ADAMTS13 (if known), domain location, vWD classification (if known), whether patient blood was used, the assays that were performed to determine results, and publications associated with the mutation. From this list of sixty-six, fifteen mutations were chosen for further analysis (Table 1). According to previous studies, mutations I1380V, D1472H, and Y1584C are not susceptible to ADAMTS13 and were therefore chosen as negative controls [28, 31, 32]. For positive controls, mutations S1506L, V1565L and R1597W were chosen based on their increased susceptibility to cleavage by ADAMTS13 [8, 19, 23, 28, 31, 33, 42, 43]. The wild-type gene was chosen to be a baseline due to its natural susceptibility to ADAMTS13 cleavage. The other nine mutations— A1464P, M1495L, I1509V, R1527Q, S1543F, Q1571H, R1583Q, N1635I, and G1643S— are of unknown susceptibility and were chosen based on their A1-A3 domain location [1, 2, 8, 10, 12, 19-22, 24, 28, 30-41]. Based on location R1583Q and N1635I are the two mutations closest to the cleavage site so we expect these two mutations to be more susceptible. A1464P and M1495L are the farthest from the cleavage site, so we expect them to be less susceptible to ADAMTS13.

Table 1. Table of fifteen mutations chosen for further analysis. [1]

Loc.	A. A. switch	Mut.	A13 Susc.	Dom.	Type	Pat.	Assays	Pub.
1380	Ile-> Val	I1380V	NO	A1	?	NO	Plasmids, Transfection, Elisa Agarose Gel Immunofluorescent Adamts13 Assay	Jacobi, Bowen, Pruss
1464	Ala-> Pro	A1464P	?	A1	IIA/B	?	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castman, Gill, White-Adams, Bodo, Sanders, De Groot
1472	Asp-> His	D1472H	NO	A1	IIA	NO	Plasmids Recombinant Protein Proteolysis By Adamts13	Jacobi, Bowen, Pruss, Davies
1495	Met-> Leu	M1495L	?	A1	IIA/B	?	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castman, Gill, White-Adams, Bodo, Sanders, De Groot
1506	Ser-> Leu	S1506L	YES	A1	IIA (I) & (II)	YES	Transfection, SDS Gels, PCR, Elisa, Tagging, Western Blotting And Agarose Gel, Plasmids, Immunofluorescent ADAMTS13 Assay Heteroduplex Generations, Phenotype Analysis	Ahmad, Springer, Schneppenheim, Bowen, Jacobi, Hassenpflug, Castaman, Davies, Rodeghiero
1509	Ile-> Val	I1509V	?	A2	IIA	?	Phenotype, Mutation Screening, Expression *In Silico* Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castman, Gill, White-Adams, Bodo, Sanders, De Groot
1527	Arg-> Gln	R1527Q	?	A2	IIA	?	Phenotype, Mutation Screening, Expression *In Silico* Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castman, Gill, White-Adams, Bodo, Sanders, De Groot
1543	Ser-> Phe	S1543F	?	A2	IIA	?	Transfection, SDS Gels, PCR, Elisa, Tagging, Western Blotting And Agarose Gel	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castman, Gill, White-Adams, Bodo, Sanders, De Groot
1565	Val-> Leu	V1565L	YES	A2	?	YES	PCR, Heteroduplex Generations, Phenotype Analysis Proteolysis Studies	Ahmad, Springer, Schneppenheim, Bowen, Jacobi, Hassenpflug, Castaman, Davies, Rodeghiero
1571	Gln-> His	Q1571H	?	A2	IIA	?	PCR, Heteroduplex Generations, Phenotype Analysis Proteolysis Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castman, Gill, White-Adams, Bodo, Sanders, De Groot, Davies
1583	Arg-> Gln	R1583Q	?	A2	IIA	?	Transfection, SDS Gels, PCR, Elisa, Tagging, Western Blotting And Agarose Gel	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castman, Gill, White-Adams, Bodo, Sanders, De Groot
1584	Tyr-> Cys	Y1584C	NO	A2	?	NO	Plasmids Recombinant Protein Proteolysis By Adamts13	Jacobi, Bowen, Pruss
1597	Arg-> Trp	R1597W	YES	A2	IIA(II)	YES	Phenotype, Mutation Screening, Expression In Silico Studies, Transfection, SDS Gels, PCR, Elisa, Tagging, Western Blotting And Agarose Gel	Ahmad, Springer, Schneppenheim, Bowen, Jacobi, Hassenpflug, Castaman, Davies, Rodeghiero, Zanardelli
1635	Asn-> Ile	N1635I	?	A2	IIA	?	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castman, Gill, White-Adams, Bodo, Sanders, De Groot
1643	Gly-> Ser	G1643S	?	A2	IIA	?	Plasmids Recombinant Protein Proteolysis By Adamts13	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot

Location of the mutation (Loc.), amino acid sequence in which amino acids have been switched (A.A. Switch), the susceptibility of cleavage (A13 Susc.), which domain the mutation is located (Dom.), the subtype of von Willebrand Disease (Type), whether patient blood was used (Pat.), the assays used to determine the susceptibility and vWD classification (Assay), and the 1st author of the articles in which the mutation were found (Pub.).

vWF Plasmid construct design and transfection. Expression plasmids were designed, produced, and transfected into Chinese hamster ovary (CHO) cells for protein production. The plasmids contained the first three amino acids of the vWF pro-peptide, which served as a signal sequence for vWF secretion (Pruss et.al 2012), a FLAG-tag at the start of the sequence, the vWF domains A1-A3 (along with the associated mutations), and a 6x His-tag at the end of the genetic sequence (Figure 5A). Electroporation (Figure 5B) was used to introduce the vWF plasmids into CHO cells for expressing recombinant vWF (r-vWF) proteins.

After transfection, the supernatants were collected and tested for levels of expression using western blot analysis. Transfections with the wild-type (WT) construct and most of the other "mutant" constructs resulted in relatively high expression of each monomer at a molecular weight of 66.4kDa (Figure 5C). However, constructs containing the mutations S1506L, R1597W and S1543F exhibited low protein expression, with only a faint band at the expected weight of 66.4kDa (Figure 5C). According to Ahmad, Pruss and Berber as well as other researchers [1, 2, 8, 10, 18, 23, 28, 30, 31, 35, 36, 38, 44], both S1506L and R1597W are more susceptible to ADAMTS13 cleavage while S1543F is of unknown ADAMTS13 susceptibility, this could be due to the plasmids design.

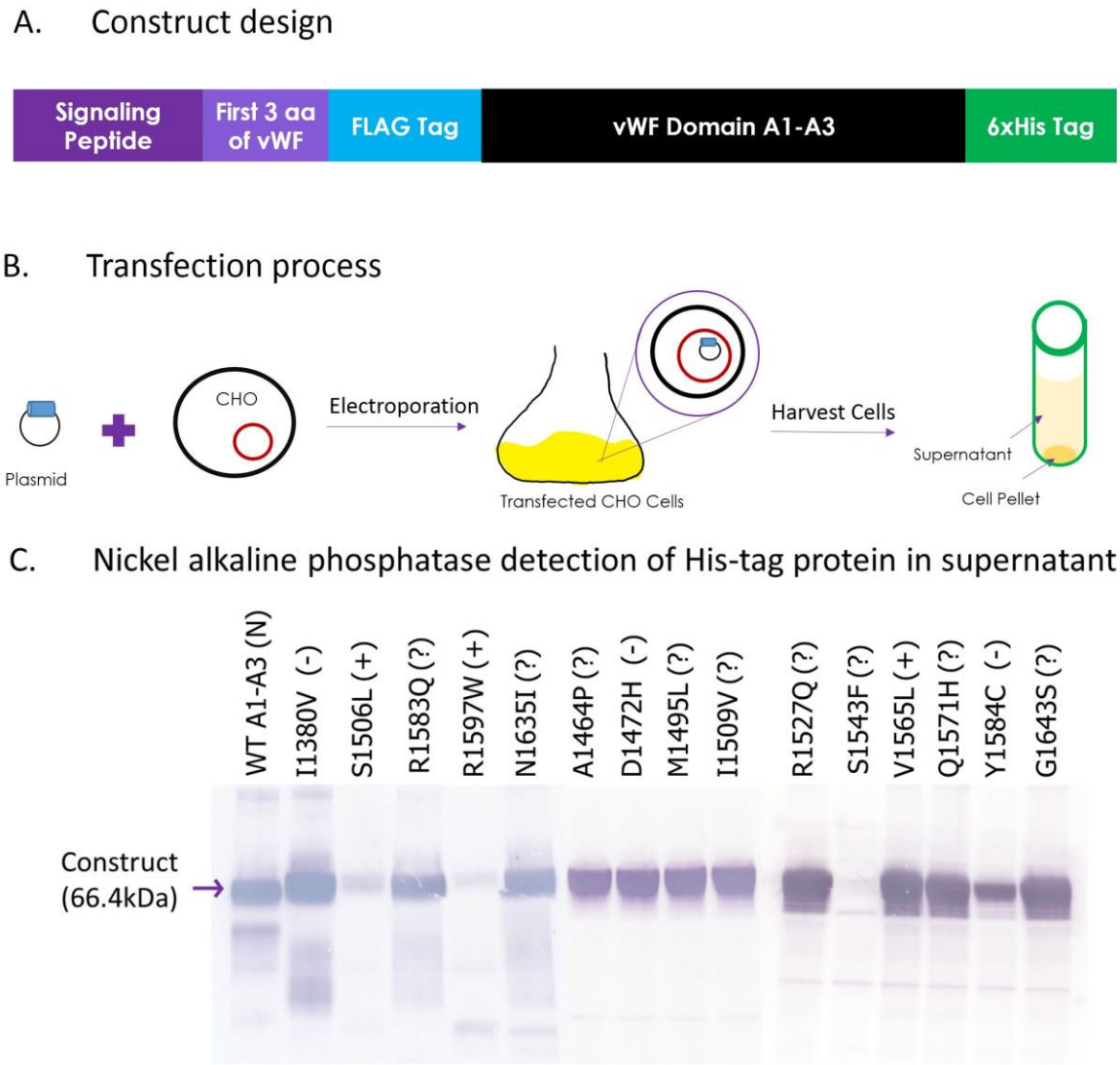


Figure 5. *Process of transfection and expression.* The plasmid design for r-vWF constructs (A). Process used for transfection is mapped out from plasmid to harvest (B). Supernatants were collected and tested for levels of protein expression by western blot using nickel-alkaline phosphatase-labeled antibody to detect His tag (C). Labels above lanes list mutation names and whether those mutations are ADAMTS13 Susceptible (+), non-Susceptible (-) or of unknown susceptibility (?) according to literature.

FPLC Purification of vWF constructs. Each protein construct was purified by fast protein liquid chromatography (FPLC) using a 5mL HI-trap nickel resin column. The manufacturer's buffer recommendations were used for the first round of purification, which included solutions containing sodium phosphate at a pH of 7.4 with 0.5M NaCl and an elution buffer containing 500mM imidazole. The fractions were still impure therefore round two of purification included the addition of

5mM of imidazole in the wash and the imidazole in the elution buffer was increased to 1M. Round 3 of purification the buffers were optimized by increasing the wash buffer's molarity of imidazole to 10mM and adding 10mM imidazole to the binding buffer (Supplemental Table 2). The host protein was still present in the fractions and the protein was considered not purified. To prevent the host protein from binding to the column, 150mM NaCl was added to the supernatant prior to loading onto the column for the final round of purification. The combination of the additional 150mM NaCl in the sample along with 10mM of imidazole in both the wash and binding buffers along with 1M imidazole at a 50% gradient produced pure protein fractions (Figure 6). The UV-280nm trace of the wash and elution graph shows a high peak in the wash where the host protein was removed from the column prior to elution (Figure 6A). The first peak on the UV-280 graph over 1A3-1A5 depicts the host protein along with some of our tagged protein as noted by the total protein gel (Figure 6B). The second smaller peak which covers 1B2-1B5 is the purified r-vWF (Figure 6A). The purity of the protein was confirmed by a total protein stained gel which showed minimal host protein in fractions 1B1-1B5 (Figure 6B). This was further verified by a western blot using nickel-AP for detection of the His tag, which revealed only the construct at monomer weight of 66.4kD in fractions 1B4-1B5 (Figure 6C). The fractions containing the pure protein (1B4 and 1B5) were pooled, concentrated and stored for *in vitro* assays.

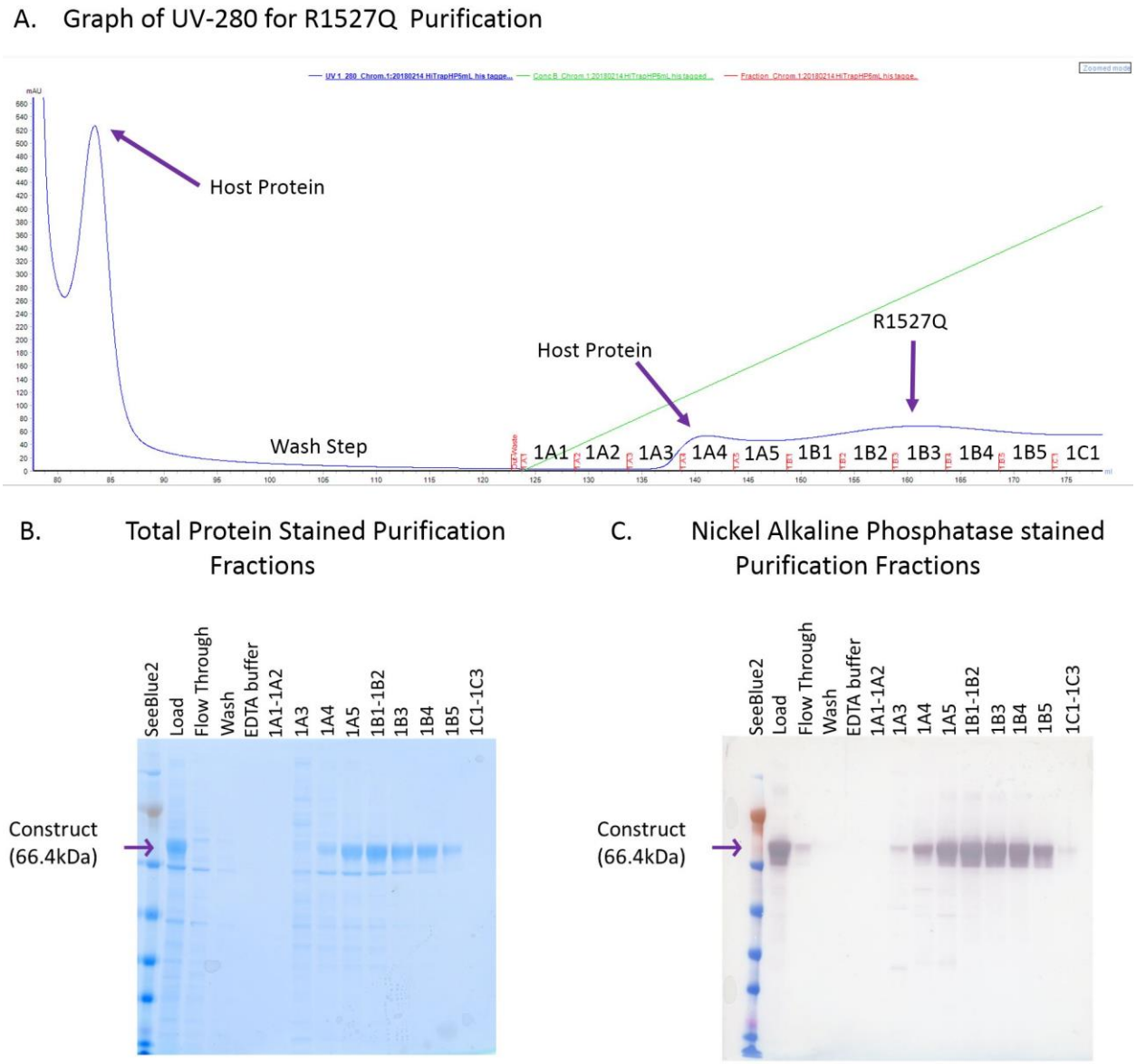


Figure 6. *Representative purification results for r-vWF constructs.* Fast Protein Liquid Chromatography Purification (FPLC) with a nickel resin column with r-vWF supernatants. UV280nm trace graph for elution of column showing peaks containing protein, the fractions are mechanically the fraction collection nomenclature is read as cassette number one, the row shown by a letter (A, B, &C) and which tube in that row (1-5), ex. 1A1 (A), a total protein gel stained for purity check (B), and western blot to check and identify fractions containing our purified protein (C).

Shear Vortex Assay. Once we successfully expressed and purified 16 variants of recombinant von Willebrand Factor (r-vWF), the next step was to test the proteins in a shear force assay. A shear vortex assay mimics the condition of shear force, the amount of force that is created when blood is pumped throughout the body, in the blood using a Mix Mate [34, 36]. The stability of vWF

can be judged based on the number of bands that appear on a gel, with ADAMTS13-cleaved samples showing up as two distinct bands. The upper band shows vWF monomers and the lower band shows the cleaved parts. Increased or severe cleavage will show a darker or solitary lower band of cleavage parts. While no cleavage or blocked cleavage will show the higher monomer band only (Supplemental Figure 1). Assay reactions contained purified vWF truncated protein (150nM), vortex buffer, the presence or absence of ADAMTS13 (25nM), and the presence or absence of EDTA. The samples were either subjected to shear force (mixing at high speeds) or left standing still at room temperature as a control. The samples for r-vWF wild-type were expected to mimic that of full length vWF (Supplemental Figure 1), by showing both a monomer band and cleaved parts band, however the results for the assay proved to be insufficient at distinguishing cleaved fragments (Supplemental Figure 2). No cleavage banding was seen in any of the constructs when tested. We expected to see a more intense cleavage and for the samples with increased susceptibility to ADAMTS13 and a less intense to non-existent band for mutations less susceptible to ADAMTS13 cleavage.

Urea vWF digestion optimization. Due to the lack of an observed cleavage product in the shear force vortex assay, urea was used to denature the vWF protein, thus exposing the ADAMTS13 cleavage site. After testing various concentrations of urea, we determined that 1.5M urea gave the optimal results (Supplemental Figure 3) as that concentration allowed a cleavage band at 31.3kDa to be visible.

The vWF digestion reaction in the presence or absence of urea at various time points ranging from zero minutes to one hour resulted in the full-length construct at molecular weight of 66.4kDa and a cleavage product at molecular weight 31.3kDa (Figure 7A). Contrary to the experiments with varying urea concentrations (Supplemental Figure 3), the samples containing urea and ADAMTS13

did not show a clear cleaved product compared to the non-urea samples (Figure 7A). The longer the fifteen samples were incubated in the presence of ADAMTS13 the more cleavage product accumulated (Figure 7B). Samples incubated for three hours and overnight had stronger, though not significant larger, intensity but still required long exposure times to see the cleavage product. These experiments were repeated four times with the same results (data not shown). The absence of a 31.27kDa band (His-Tagged c-terminus) in the presence of urea warranted a second look at the activation buffer of Hepes and Calcium Chloride pH 7.2 to activate the enzymes being used.

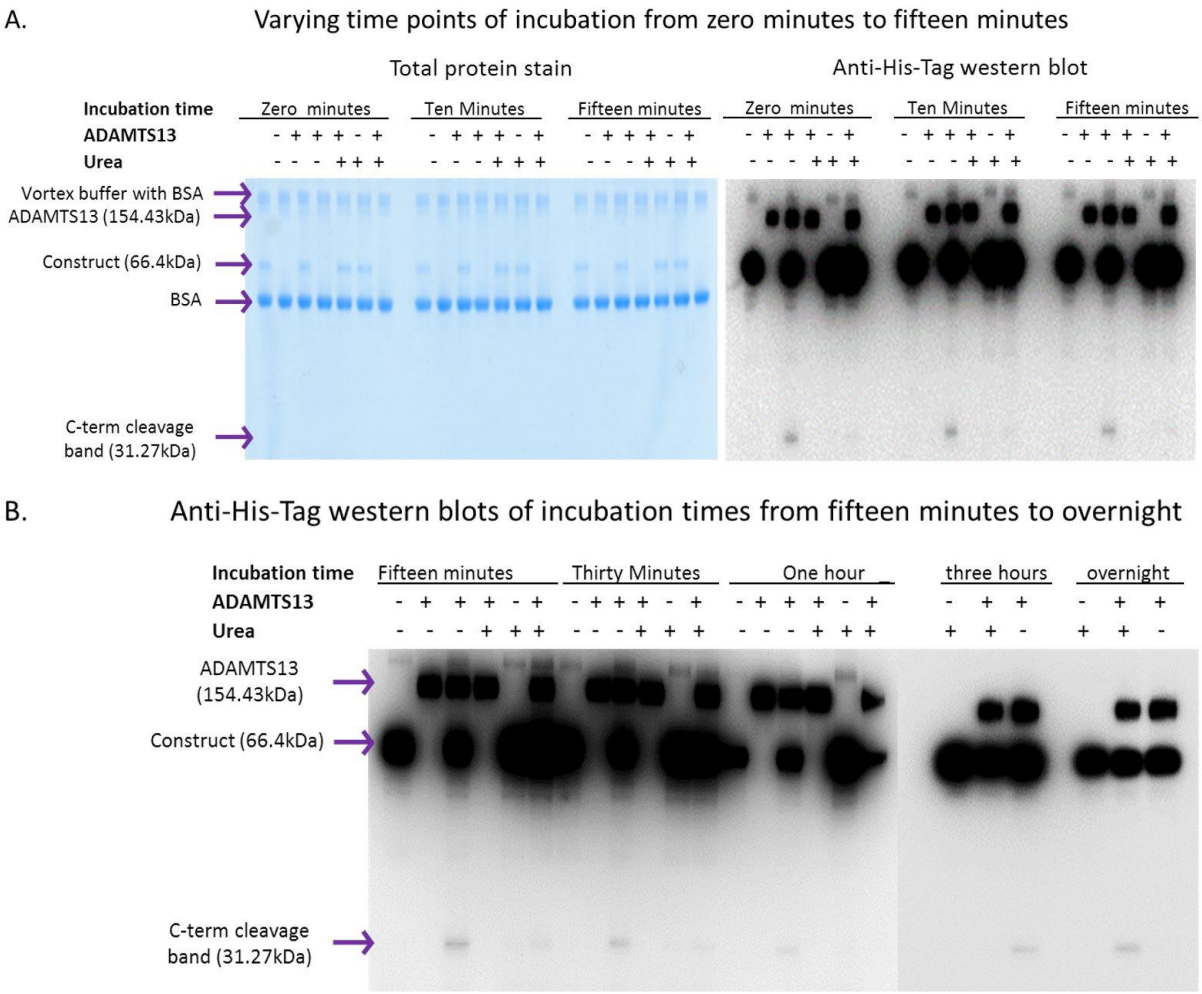


Figure 7. *Optimization of 1.5M Urea Samples by Testing Time Points and Gel Load.* The key above the membranes shows blot/gel conditions. The plus sign (+) indicates the inclusion of the component listed to the left while a minus sign (-) shows the lack of sample components. The Wild Type construct for vWF was used for the time point determination. Western blots showing sample incubation times starting at zero minutes to fifteen minutes (A) and fifteen minutes through overnight incubation times (B) N= 4.

Activation buffer optimization for vWF digestion. While the vortex buffer (20mM Hepes pH7.4, 5mM $CaCl_2$, and 1mg/mL BSA) was sufficient for cleavage to occur, we reviewed the literature and identified six activation buffers to optimize the digestion assay (Figure 8). The activation buffers tested were as follows: (1) vortex buffer without BSA; (2) Anderson buffer (10mM Tris pH7.1, 0.25mM $ZnCl_2$, 5mM $CaCl_2$,150mM NaCl) [6]; (3) Zanardelli buffer (20mM Tris pH7.8, 5mM $CaCl_2$, 150mM NaCl) [19]; (4) Interlandi buffer (10mM Hepes pH7.2, 2mM $CaCl_2$) [20]; (5) Pruss buffer (5mM Tris pH8.1, 10mM $BaCl_2$) [33]; and (6) Julia buffer (50mM Hepes pH7.1 150mM NaCl, 5mM $CaCl_2$) [10]. Results indicate that Anderson, Zanardelli, Pruss, and Julia buffers allow faint cleavage bands at 31.3kDa, but not to the same extent as the vortex buffer without BSA (Figure 8A). However, the Interlandi buffer produced the strongest cleavage band though not of significant increase (Figure 8B). Further comparison was done amongst the buffers (Figure 8C). Interlandi buffer (boxed in purple) resulted in the strongest cleavage product at 31.3kDa (Figure 8C). The optimization of activation buffers and urea in the samples allowed for a standard exposure time of 2 minutes and 30 seconds for all proceeding blots, with clear cleavage bands in the r- von Willebrand Factor. It is important to note that the cleavage band with and without urea showed no significant difference in clarity or intensity. From this point forward, urea was not added to any of the samples due to lack of supporting evidence that the urea aids in the cleavage of vWF.

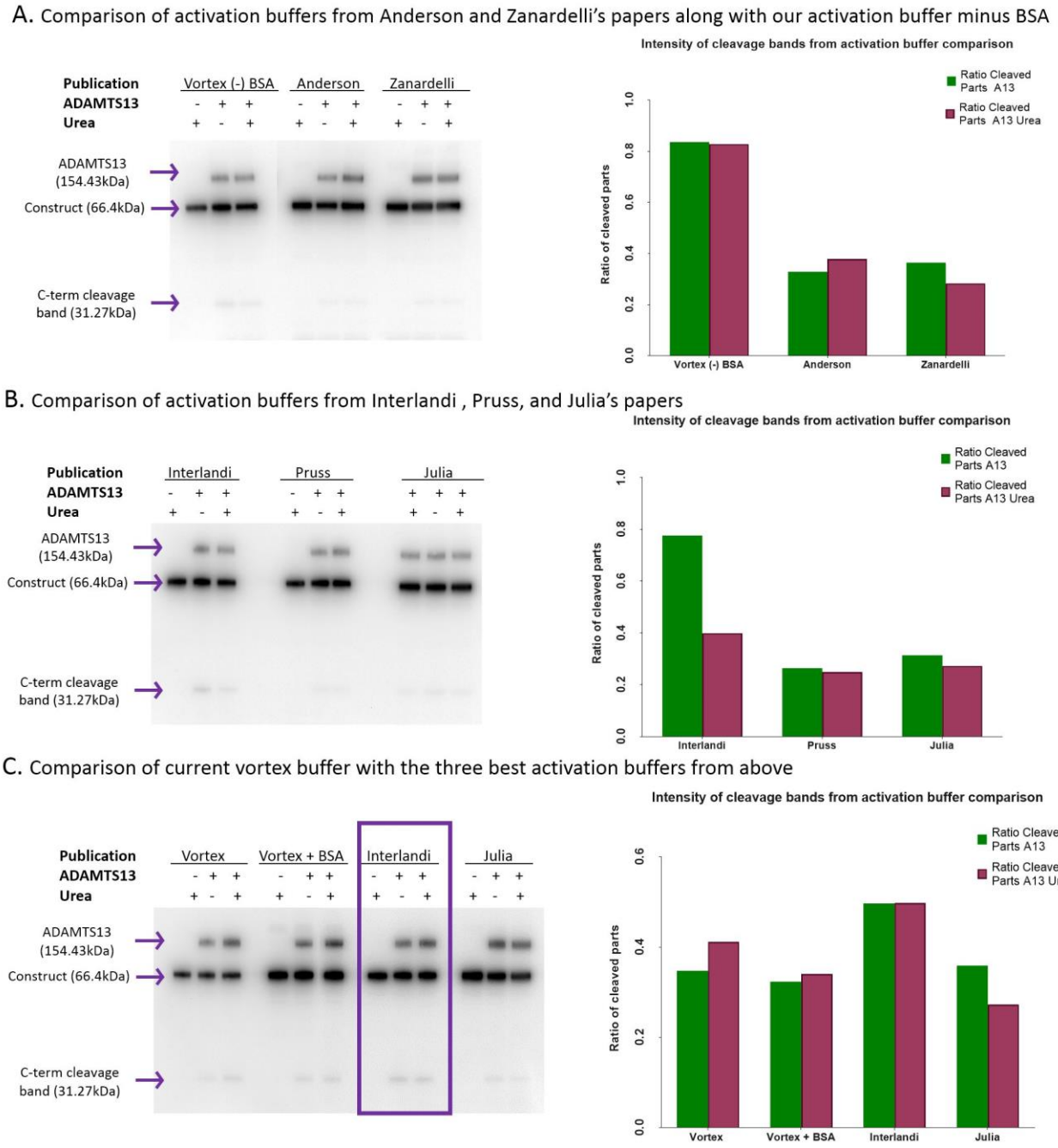


Figure 8. *Selection of Optimal Activation Buffer.* Key above blot indicates presence (+) or absence (-) of mixture components in solution. Each sample contains 30.43nM of wild-type vWF (WT), ADAMTS13 at 30.42nM, 1.5M urea and 50µL or half of the sample volume of appropriate 2x activation buffer. N=3.

Non-urea vWF digestion optimization. After selection of 2x Interlandi buffer as our activation buffer, different concentrations of recombinant vWF (r-vWF) and ADAMTS13 were compared. Specifically, the 1:1 molar ratio of r-vWF to ADAMTS13 was compared to a 1:2 molar ratio by increasing ADAMTS13 from 30.22nM to 60.86nM. The change in ratio was used to increase the interaction between ADAMTS13 and r-vWF protein (Figure 9A). The new ratio of 1:2 produced a more intense cleavage band at 31.3kDa. Both the r-vWF wild type (WT) construct and the r-vWF I1380V construct, a non-susceptible mutant, were tested. Western blot analysis of the His-tag C-terminus showed a faint but definitive cleavage product at 31.3kDa in the reactions with WT protein, while a significantly fainter cleavage product was apparent in the I1380V mutant construct (Figure 9B). Western blot analysis of FLAG-tagged r-vWF revealed a faint band for the WT construct at 54.1kDa and a definitive band for the I1380V mutant at the same molecular weight (Figure 9B). The anti-FLAG tag labels the N-terminal half of vWF protein while the anti-His tag labels the C-terminus half. Therefore, it is important to note that while a faint band at 31.3kDa was observed in the I1380V sample using the anti-His-tag antibody, at the defined band at 54.13kDa in the anti-FLAG blot indicates that the protein in the sample was cleaved since full-length proteins are 66.4 kDa (Figure 9B).

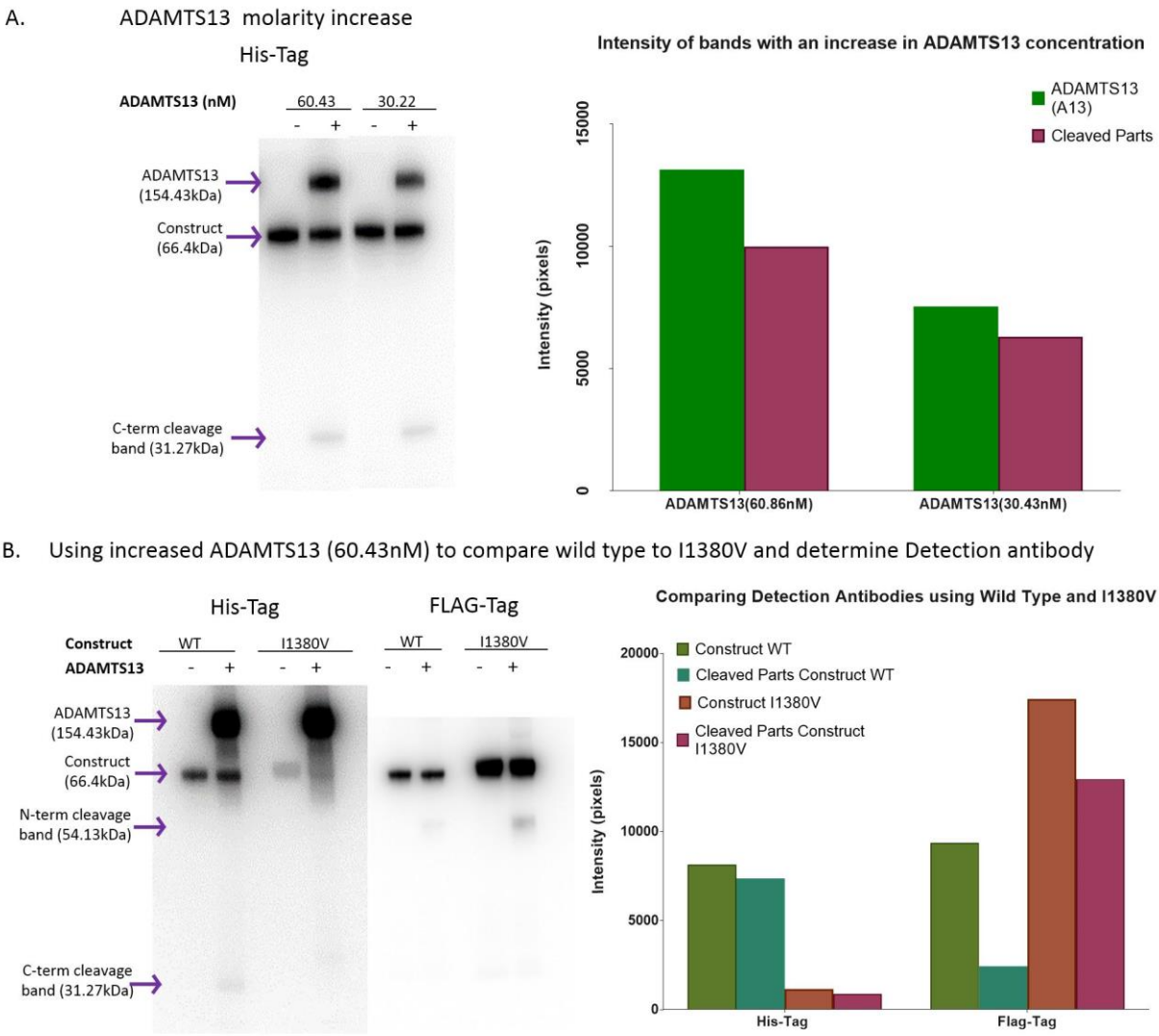


Figure 9. *ADAMTS13 molarity increase and construct comparison ratio optimization.* Key above blots indicate presence (+) or absence (-) of ADAMTS13 (60.43nM or 30.22nM) and constructs Wild Type (WT) at 30.43nM. Comparing cleavage bands with 60.43nM and 30.22nM of ADAMTS13 (A). Selecting 60.43nM of ADAMTS13, WT and I1380V were compared detecting both the N-terminus (FLAG) and C-terminus (His) cleavage products (B). N= 4.

Further optimization was done on the vWF digestion reactions by varying total volume and total incubation time. The concentration of vWF (30.43nM) and ADAMTS13 (60.43nM) remained the same while total volume of the sample was minimized to increase the interaction of ADAMTS13 with r-vWF protein. Wild type (WT) construct at 50µL total volume shows a slightly stronger cleavage band at 31.3kDa suggesting this new volume produced a more optimal cleavage

condition. The western blots also indicated that the wild type (WT) incubated at 37°C overnight (O.N.) provided the stronger cleavage bands at both 31.3kDa for His-tag and 54.1kDa for FLAG-tag (Figure 10). Our final optimized assay reactions consisted of r-vWF (30.42nM) in the presence or absence of ADAMTS13 (60.43nM) at a total volume of 50uL and were incubated overnight at 37°C.

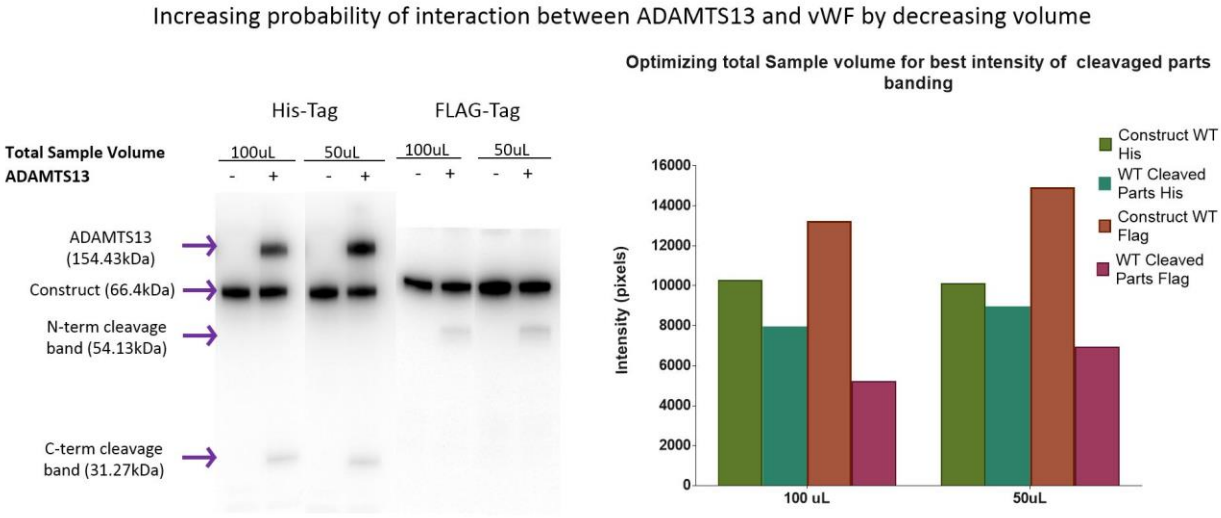
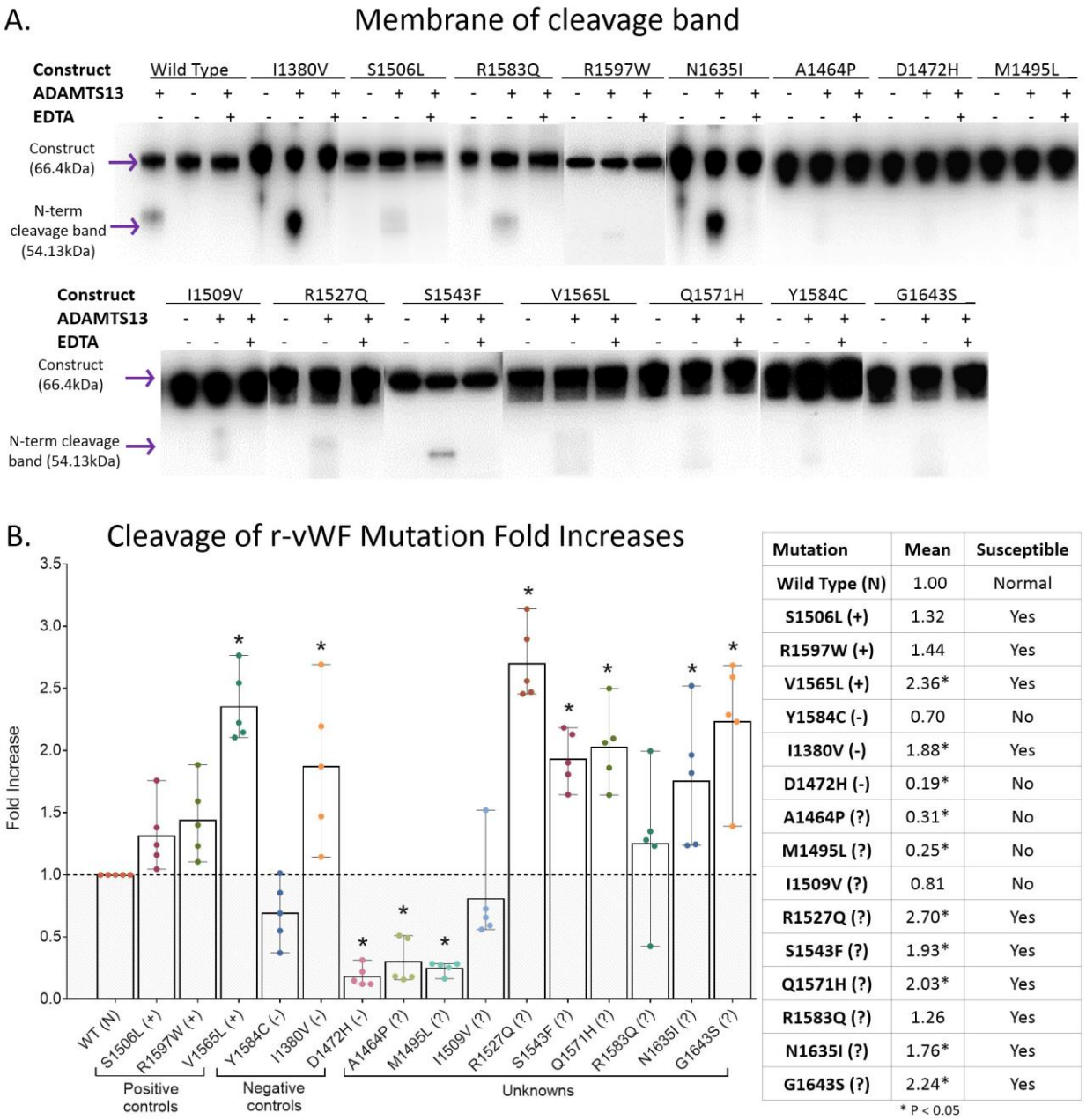


Figure 10. *Concentration optimization of Samples using Interlandi activation buffer.* Key above indicates presence (+) or absence (-) of 30.43nM Wild Type construct (WT) 60.86nM ADAMTS13, sample volume 50µL or 100µL. N= 4.

Analysis of digestion gel. Digestion reactions were made for all sixteen r-vWF proteins and were incubated overnight at 37°C. After incubation, EDTA was added to stop the reaction. Each sample was assayed five times to confirm reproducibility; and results were acquired by western blot using FLAG-tag detection (Figure 11A), which detects the 54.3kDa cleavage product. Since the N-terminus detection gave a stronger signal, this was used for quantification of monomer and cleaved parts instead of the His-tagged C-terminal end. The percentage of cleavage products was determined by measuring the intensity of the sample monomers (66.4kDa) and cleaved fragments (54.13kDa) using both the FluoroChem-M and ImageJ software.

The ratio of full-length monomer to cleaved product for r-vWF wild-type protein (WT) was normalized to a value of one, while the mutated r-VWF were calculated as fold increase or decrease relative to the wild-type (Figure 11B). A representative gel of each protein's digestion pattern is shown in Figure 11A. A graphical representation of the fold increase and the means for each sample are shown in Figure 11B. A two-way ANOVA indicated that our positive control construct V1565L and negative control construct D1472H had a significant increase and decrease in cleavage, respectively. The two-way ANOVA also indicated that the mutant constructs M1495L and A1464P were significantly less susceptible to ADAMTS13, while the five mutations R1527Q, S1543F, N1635I, Q1571H, and G1643S produced constructs significantly more susceptible to ADAMTS13 (p-values < 0.05). Surprisingly, one of our negative controls, I1380V, was significantly more susceptible to ADAMTS13. Results from Figure 11B indicate that constructs containing the mutations I1380V, S1506L, R1583Q, R1597W, N1635I, R1527Q, S1543F, V1565L, Q1571H, and G1643S were more susceptible to ADAMTS13 than the wild-type (WT) r-vWF construct, while A1464P, D1472H, M1495L, I1509V, and Y1584C yielded constructs that were less susceptible to ADAMTS13.



Mutation	Mean	Susceptible
Wild Type (N)	1.00	Normal
S1506L (+)	1.32	Yes
R1597W (+)	1.44	Yes
V1565L (+)	2.36*	Yes
Y1584C (-)	0.70	No
I1380V (-)	1.88*	Yes
D1472H (-)	0.19*	No
A1464P (?)	0.31*	No
M1495L (?)	0.25*	No
I1509V (?)	0.81	No
R1527Q (?)	2.70*	Yes
S1543F (?)	1.93*	Yes
Q1571H (?)	2.03*	Yes
R1583Q (?)	1.26	Yes
N1635I (?)	1.76*	Yes
G1643S (?)	2.24*	Yes

Figure 11. *Fold percentage of cleavage banding.* Key above membranes indicates type of r-vWF mutation plus or minus ADAMTS13 and absence or presence of EDTA. One example of the five digestion gels for each r-vWF protein is shown using anti-FLAG detection. Each membrane has a full-length monomer band at 66.4kDa and an N-terminus cleavage product at 54.13kDa (A). The fold increase in a bar/scatter plot graph with a dotted line representing the normalized WT cleavage product. The means for each r-vWF protein is found to the left of the graph; N=5. An ANOVA was run indicating significant increase or decrease with a P value < 0.05 (B).

Database Ratio. After the mutations were characterized as either susceptible or non-susceptible to ADAMTS13 cleavage, the mutations were searched in the gnomAD (public) and Variants Viewer

(private) databases. These databases compile thousands of individuals' genetic sequences that can be used for bioinformatics analysis. For this study, the allele frequencies (allele count/allele number) from the European non-Finnish (ENF) population of sequences were obtained. The allele frequencies were doubled due to vWD being a heterozygous dominant mutation; and once doubled the allele frequencies were multiplied by the current world wide population (WWP) of 7.6 billion people to create an estimated number of individuals (Table 2A). A percentage representing the prevalence for each mutation was created by dividing the estimated number of individuals with that mutation by the sum of all the individuals estimated to have each mutation and then multiplying by 100. One mutation (M1495L) was not found in the databases, and five (R1597W, I1509V, N1635I, Q1571H and G1643S) did not have a reported allele mutation for the ENF population. The remaining nine mutations, S1506L, V1565L, Y1584C, I1380V, D1472H, A1464P, R1527Q, S1543L and R1583Q were found in the ENF population, and the percentages were determined (Table 2A). The percentages for the nine mutations found in the EFN population on both databases were summarized in terms of their ADAMTS13 susceptibility (Table 2B). The six ADAMTS13 susceptible mutations —S1506L, V1565L, I1380V, R1583Q, R1527Q, and S1543L— had a total estimated allele frequency of 0.17. The allele frequency of 0.17 was used to estimate approximately 1,297,818,431 or 48.01% of individuals that have an ADAMTS13 susceptible mutation (Table 2B). The three non-susceptible mutations—Y1584C, D1427H, and A1464P— produced an allele frequency sum of 0.19 which resulted in an estimated 1,405,161,240 people or 51.99% of individuals (Table 2B).

Table 2. *Database ratios to determine major or minor mechanism of action.*[2]

A. Estimated patients with vWD Type IIA using allele frequency

Mutation	Allele Frequency European Non Finish (Allele count/ Allele number*2)	vWD Type IIA patients in world population (Allele Frequency ENF * 7.6 billion people)	Percentage of individuals in our subset (estimated vWD Type IIA individuals/summed vWD Type IIA*100)
S1506L (+)	7.15e-05	543,342.27	0.020
R1597W (+)	0	0	0
V1565L (+)	0.17	12,89,360,791	47.70
Y1584C (-)	0.008	64,134,448.98	2.37
I1380V (+)	0.001	7,369,895.49	0.27
D1472H (-)	0.18	1,339,272,000	49.55
A1464P (-)	0.0002	1,754,791.041	0.067
M1495L (-)	Not	In	Database
I1509V (-)	0	0	0
R1527Q (+)	3.60e-05	273,189.67	0.010
S1543L (+)	1.79e-05	135,605.32	0.005
Q1571H (+)	0	0	0
R1583Q (+)	1.78e-05	135,607.74	0.005
N1635I (+)	0	0	0
G1643S (+)	0	0	0

B. Identification whether ADAMTS13 susceptibility is a major or minor mechanism

9 ADAMTS13 Characterized Mutations found in databases	Summed Percentages of estimated allele frequency	Summed number of individuals in world population	Percentage of individuals
6 Susceptible to ADAMTS13 (+)	0.17	1,297,818,431	48.01%
3 Non-Susceptible to ADAMTS13 (-)	0.19	1,405,161,240	51.99%

[2] Allele frequency of individuals sequenced in the two databases multiplied by two, the number of individuals estimated to have each mutation in population and the percentage of patients with each mutation (A). The summed allele frequency, number of individuals and percentages of the 9 characterized mutation separated by susceptibility to ADAMTS13 (B).

Discussion

von Willebrand Disease (vWD) is a bleeding disorder in which coagulation is hindered by a defect of the blood protein von Willebrand Factor (vWF). These defects are either abnormalities in expression or function and are often classified into broad groups, thus leading to generalized, suboptimal treatment. Sub-group classification of vWD Type II is not only difficult to do but also uncommon, due to the lack of specialized treatment. Once a patient is diagnosed with vWD Type II, there is only one therapeutic option— plasma derived von Willebrand Factor (Pd-vWF). The root cause of the Type II high molecular weight multimer loss known as Type IIA is not well studied and no therapeutics are currently in development for this particular subtype of vWD. Identifying mutations within the A1-A3 domains of vWF and sub-classifying them into potential mechanisms of action for Type IIA—specifically by determining susceptibility to ADAMTS13 cleavage— can aid research involved with developing targeted therapeutics.

A lack of commonality in determining vWD classification has led to multiple forms of nomenclature currently being used to identify vWD Type II subtypes. The goal of this project was to identify previously discovered yet uncharacterized mutations for vWD Type IIA and use *in vitro* assays to determine ADAMTS13 susceptibility. The three functional domains of von Willebrand Factor (vWF) with the highest reports of mutations leading to high molecular weight multimer loss are domains A1-A3. Of the sixty-six mutations found, fifteen were chosen based on the location of the mutation in these domains. In this study, we used recombinant-vWF(r-vWF) containing domains A1-A3 to classify nine, previously uncharacterized mutations.

Once the mutations were identified, protein constructs were designed to express r-vWF proteins. The proteins were purified and subsequently used in various *in vitro* assays to evaluate which vWF mutations resulted in increased ADAMTS13 susceptibility. Initially, two of the three known susceptible mutations, S1506L and R1597W, as well as one uncharacterized mutation,

S1543F, secreted poorly resulting in low expression in the supernatant. Since vWD Type IIAI is known to have issues with multimerization, and vWF forms multimers as it secretes, it was originally believed these three mutations were in fact vWD type IIAI mutations leading to secretion problems. However, this was not the case, protein was seen in the supernatant after the purification. Through purification and buffer exchange, S1506L, R1597W, and S1543F were all successfully concentrated to sufficient levels for further analysis using a digestion gel assay where susceptibility to ADAMTS13 was determined. While no published study has addressed the issue of low expression, one possible explanation is that the truncated protein does not fold properly, and this in turn limits its secretion. Further *in vitro* tests such as real-time cell secretion assays should be used to fully explore the low secretion of S1506L, R1597W and S1543F. The other thirteen mutant constructs— I1380V, R1583Q, N1635I, A1464P, D1472H, M1495L, I1509V, R1527Q, V1565L, Q1571H, Y1584C, and G1643S along with wild-type— were expressed and secreted in large quantities.

Optimization of purification buffers was necessary due to excessive contamination by host proteins that had strong binding affinity to the nickel column. Adding imidazole to the binding (EQ), wash, and elution buffers improved purification efficiency. This approach was a departure from previous studies that described the use of 500mM of imidazole only in the elution buffer [15, 20, 23, 32, 41, 45, 46]. However, further optimization to remove the host proteins involved the addition of 150mM of NaCl directly to the protein supernatant prior to loading onto the column. The addition of salt and imidazole resulted in a pure enough protein product that could be used in our subsequent *in vitro* assays.

After purification, the challenge of achieving proper unfolding and enzyme cleavage began. Several strategies to induce unfolding were pursued including physical force and chemical denaturant (urea). Physical force was achieved by running the shear force assay which replicates

the physiological shear force found in the blood stream when a wound occurs [34, 36]. The shear force created by vortex mixing pulls open full-length vWF's globular form, exposing the cleavage site. Truncated A1-A3 constructs of recombinant-von Willebrand Factor (r-vWF) did not respond to shear force in the same manner as the full-length vWF protein as shown by the lack of cleavage products by the shear force assay (Supplemental Figure1). One plausible explanation may be associated with the truncated form of r-vWF missing tail ends which under normal circumstances would act as an anchor for vWF, thus allowing the force to open the globular domains revealing the hidden cleavage site. Since physical force was unable to unfold the r-vWF, a chemical denaturant, urea, was used. Although 1.5M urea seemed to be the optimal concentration for cleavage (which was consistent with previous studies investigating full-length vWF [15, 20, 23, 32, 41, 45, 46]), we found that urea was not necessary for cleavage to occur. This finding contradicted Interlandi et al., who demonstrated that the presence of urea was necessary for cleavage to occur [20]. This contradiction could be due to construct length or design of the recombinant protein. In our study, truncated forms were expressed, and smaller size protein fragments likely require less effort in unfolding than the full-length proteins, which was most often used in previous studies [1, 12, 15, 20, 23, 32, 34, 41, 45, 46].

The protein activation buffer composition was also compared to various buffers from literature. The activation buffer is the solvent that provides the necessary metals and salts for proteins and enzymes to interact and function. Most of the activation buffers we looked at were Hepes based with a pH around 7.4 with a few being Tris based with a pH of around 8. In addition, six of the seven buffers had $CaCl_2$ as the metal of activation component either in combination with another such as $ZnCl_2$ or by itself. In the end, the simplest buffer with a low concentration of Hepes and $CaCl_2$ worked the best with little need of a denaturant to open the globular r-vWF for cleavage.

The concentration of substrate and sample volume for the digestion gel assay were also

evaluated and optimized. The concentration of ADAMTS13 was increased two-fold (30.43nM to 60.43nM) to increase the interaction between vWF and the enzyme. Along with increasing the ratio of ADAMTS13 to r-vWF, the total volume of the reaction sample was decreased, and this volume change along with 1:2 ratio of r-vWF to ADAMTS13 resulted in the optimal condition for vWF cleavage by ADAMST13.

When comparing the detection of cleaved fragments using the FLAG-tag N-terminal end versus the His-tag C-terminal end, we found that greater detection of the monomer protein constructs was achieved using the FLAG-tag antibody. The lack of detection using His-tag could indicate a structural change in the A1-A3 domains, which may cause the C-terminus end of the protein to fold inward thus hiding the His-Tag. However, though a native gel would need to be run to support this explanation. Detected levels of each construct via the FLAG-tag was used to calculate the fold increase or decrease of each cleavage product compared to control.

Once the protein expression, purification, and digestion assay were optimized, five independent cleavage experiments were carried out on the constructs. Cleavage fold increase was determined for each sample and normalized to the wild-type. Our results (Figure 11B) indicate that the three positive controls—S1506L, R1597W and V1656L— were indeed more susceptible to ADAMTS13 cleavage than the wild-type vWF, Consistent with previous studies [8, 19, 23, 28, 31-33, 42, 43], the two negative controls —D1472H and Y1584C— showed non-susceptibility to ADAMTS13. The third negative control, I1380V, showed an increased susceptibility rather than a decrease. However, it should be noted that the construct for I1380V in our study only contained domains A1-A3 whereas other studies used full length r-vWF proteins. Six of the nine uncharacterized mutations —–R1583Q, N1635I, R1527Q, S1543F, Q1571H, and G1643S— were determined to be susceptible to ADAMTS13 whereas the three remaining mutations— M1495L, I1509V, and A1464P —showed minimal cleavage indicating non-susceptibility to ADAMTS13.

Based on the fold increases calculated, a pattern of increased susceptibility was observed in mutations that centered on the cleavage site of Y1605-M1606. Since the cleavage site is located in the middle of three beta sheets, these mutations may create space between the sheets leaving the site open and susceptible to cleavage. However, further analysis is necessary to definitively determine whether the increased susceptibility to ADAMTS13 cleavage of these mutant vWF proteins is a result of altered protein structure.

Of the 15 mutations studied, only nine mutations were found in the databases for the European non-Finnish population, which was chosen because it contains the largest number of sequences from individuals in the population. The allele frequencies were obtained from the two databases and used to calculate the number of individuals in the world that carry the mutation for increased ADAMTS13 susceptibility. Based on the number of individuals calculated from the allele frequencies, we concluded that the frequency of Type IIA(II) individuals— those who have an increased susceptibility to ADAMTS13— is essentially equal to the frequency of Type IIA(I) individuals—those with a discrepancy in assembly of high molecular vWF (49% vs. 51%, respectively). This knowledge shows that two therapeutics should be considered for Type IIA patients: one that targets the increased susceptibility and one that aids in multimer assembly. Further research should be done to look into the mechanism of action for Type IIA (I) or non-susceptible mutations since it is unknown how the assembly of multimers is affected. However, these calculations were done using very specific parameters and may not represent the population as a whole, for reasons described in the limitation section below.

Limitations of research. It is important to note that this study was not without its limitations. Our *in vitro* analyses used shortened recombinant vWF instead of full length-vWF protein multimers. Previous studies involving vWD Type II mutations in truncated from showed accurate folding and function [13, 34, 39, 42, 47], which led us to believe that the truncated protein for the A1-A3

domains would fold in the same manner as normal full length. However *in vivo* and *in vitro* proteins don't always act the same therefore these mutated proteins could function and fold differently in normal physiological conditions and under high stress conditions. Another limitation of this work is that we only selected mutations within the A1-A3 domains. Theoretically, there could be mutations outside these domains that also influence ADAMTS13 susceptibility. In addition, an individual may have a mutation within these domains or on the vWF gene and show no phenotypic symptoms. This study also assumes that the only two mechanisms cause multimer loss in vWD Type IIA individuals— ADAMTS13 susceptibility (IIA group II) and problems with dimerizing upon secretion (IIA group I). Furthermore, assumptions were also made when setting up the experiments, such as mutations in the A1-A3 Domains result in vWD Type IIA.

It is not common practice for individuals with vWD Type II to be genetically sequenced for diagnosis. As a result, the lack of sequenced individuals with vWD Type II mutations and inconsistent nomenclature limits the ability of the databases to differentiate between the multiple types of vWD. Another hindrance in the database is that not all individuals with vWD have phenotypic symptoms, and for vWD Type II individuals, those symptoms have a drastic range from mild dizziness and bruising to uncontrolled bleeding and blood clots. The milder symptoms of dizziness and bruising are relatively insignificant, and an individual experiencing these minor symptoms is unlikely to go to the doctor and be diagnosed. As a result, a large number of individuals may not be identified as having vWD. Lastly, these databases are more focused on recessive diseases and are more often used for estimating individuals with vWD Type III. Taken together, these factors suggest that the number of individuals with vWD Type II are greatly overestimated. Understanding these limitations can improve future studies and contribute to identifying the cause of multimer loss in vWD Type IIA and properly characterizing these mutations.

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Supplemental Figures:

Supplemental Table 1. *Chart of vWD Mutation from literature.* Continued on next page. [3]

Loc.	A. A. switch	Mut.	A13 Susc.	Dom.	Type	Pat.	Assays	Pub.
528		N528S	NO	?	IIA(I)	?	Review Paper	Berber
1099	Cys-> Phe	C1099P	YES	D3	IIA(I)	YES	Plasmids, Transfection, ELISA Agarose Gel Immunofluorescent Adamts13 Assay	Jacobi
1190	Cys-> Ser	C1190S	NO	D3	?	YES	Plasmids, Transfection, ELISA Agarose Gel Immunofluorescent Adamts13 Assay	Jacobi
1195	Asp-> Tyr	D1195Y	no	?	II	YES	VWF Full-length With Mutations Platelet Binding Assays And Multimer Analysis	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot
1229	Val-> Gly	V1229G	?	?	IIA	YES	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad
1231	Asn-> Thr	N1231T	?	?	IIA	YES	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad
1272	Cys-> Ser	C1272S	NO	A1	IIA(I)	YES	Plasmids, Transfection, ELISA, Agarose Gel Immunofluorescent ADAMTS1313 Assay, SDS Gels, PCR, Tagging	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot
1272	Cys-> Arg	C1272R	YES	A1	IIA(I)	YES	Plasmids, Transfection, ELISA Agarose Gel Immunofluorescent ADAMTS13 Assay, Phenotype, Expression In Silico Studies	Jacobi, Ahmad
1272	Cys-> Tyr	C1272Y	YES	A1	IIA(I)	YES	Plasmids, Transfection, ELISA Agarose Gel Immunofluorescent ADAMTS13 Assay	Jacobi
1308	Arg-> His	R1308H						
1380	Ile-> Val	I1380V	NO	A1	?	YES	Plasmids, Transfection, ELISA Agarose Gel Immunofluorescent ADAMTS13 Assay	Jacobi, Bowen, Pruss
1464	Ala-> Pro	A1464P	?	?	IIA/B	YES	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot
1472	Asp-> His	D1472H	NO	A1	IIA	YES	Plasmids Recombinant Protein Proteolysis by ADAMTS13	Jacobi, Bowen, Pruss, Davies
1495	Met-> Leu	M1495L	?	?	IIA/B	YES	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot

[3] location (Loc) of the mutation (Mut) in the amino acid sequence which amino acids have been switched (A.A. switch), the susceptibility of cleavage (A13 Susc.), which domain the mutation is located on (Dom), the subtype of von Willebrand Disease (Type), the assays used the determine the susceptibility and type (assay), and whether or not patient blood was used in the assays (Pat) and the 1st author of the articles the mutation was found (Pub).

Supplemental Table 1. *Chart of vWD Mutation from literature.* Continued on next page.

Loc	A. A. switch	Mut	A13 Susc.	Dom.	Type	PAT	Assays	Pub
1502	Phe-> Ala	F1502A	**YES**	A2	IIA	NO	Tensile Force, Velocity Pulling, Plasmid, Expression Cleavage Assay	Interlandi
1503	Leu-> Arg	L1503R	**NO**	A1	?	YES	Plasmids, Transfection, ELISA, Agarose Gel Immunofluorescent ADAMTS13 Assay	Jacobi
1505	Gly-> Glu	G1505E	**YES**	A2	IIA(II)	?	Transfection, SDS Gels, PCR, Elisa, Tagging	Hassenpflug
1505	Gly-> Arg	G1505R	**YES**	A2	IIA (I)	?	Transfection, SDS Gels, PCR, Elisa, Tagging	Hassenpflug
1505	Leu-> Arg	L1505R	**YES**	A2	IIA(II)	?	Review Paper	Berber
1506	Ser->Leu	S1506L	**YES**	A1	IIA (I) & (II)	YES	Transfection, SDS Gels, PCR, Elisa, Tagging, Western Blotting And Agarose Gel, Plasmids, Immunofluorescent, ADAMTS13 Assay heteroduplex Generations, Phenotype Analysis	Ahmad, Springer, Schneppenheim, Bowen, Jacobi, Hassenpflug, Castaman, Davies, Rodeghiero
1509	Ile-> Val	I1509V	**?**	?	IIA	YES	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot
1527	Arg-> Gln	R1527Q	**?**	?	IIA(II)	YES	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot
1528	Met-> Val	M1528V	**YES**	A2	IIA(II)	?	Transfection, SDS Gels, PCR, Elisa, Tagging, Western Blotting And Agarose Gel	Hassenpflug
1543	Ser-> Phe	S1543F	**?**	?	IIA	YES	Transfection, SDS Gels, PCR, Elisa, Tagging, Western Blotting And Agarose Gel	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot
1565	Val-> Leu	V1565L	**YES**	A2	?	YES	PCR, HETERODUPLEX GENERATIONS, PHENOTYPE ANALYSIS Proteolysis Studies	Ahmad, Springer, Schneppenheim, Bowen, Jacobi, Hassenpflug, Castaman, Davies, Rodeghiero
1568	Ile-> Asn	I1568N	**YES**	A2	IIA(II)	YES	Plasmids, Transfection, Elisa Agarose Gel Immunofluorescent Adamts13 Assay	Jacobi
1571	Gln-> His	Q1571H	**?**	A2	IIA	YES	PCR, Heteroduplex Generations, Phenotype Analysis Proteolysis Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot, Davies

Supplemental Table 1. *Chart of vWD Mutation from literature.* Continued on next page.

Loc.	A. A . Switch	Mut.	A13 Susc.	Dom.	Type	Pat.	Assays	Pub.
1572	Gln-> His	Q1572H	**NO**	A2	IIA	?	Plasmids Recombinant Protein Proteolysis By Adamts13	Pruss
1579	Gly-> Arg	G1579R	**YES**	A2	IIA(II)	YES	Plasmids, Transfection, ELISA Agarose Gel Immunofluorescent ADAMTS13 Assay	Jacobi
1583	Arg-> Gln	R1583Q	**?**	?	IIA	YES	Transfection, SDS Gels, PCR, ELISA, Tagging, Western Blotting And Agarose Gel	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot, Baronciani
1583	Arg-> Ala	R1583A	**NO**	A2	IIA	No	PCR, Cleavage Assay, Surface Plasma Resonance	Zanardelli
1584	Tyr-> Cys	Y1584C	**NO**	NO	I			Jacobi, Bowen, Pruss
1587	Asp-> Ala	D1587A	**YES**	A2	IIA	No	PCR, Cleavage Assay, Surface Plasma Resonance	Zanardelli
1597	Arg-> Gln	R1597Q	**NO**	A2	IIA	No	PCR, Cleavage Assay, Surface Plasma Resonance	Zanardelli
1597	Arg-> Trp	R1597W	**YES**	A2	IIA(II)	YES	Phenotype, Mutation Screening, Expression In Silico Studies, Transfection, SDS Gels, PCR, ELISA, Tagging, Western Blotting and Agarose Gel	Ahmad, Springer, Schneppenheim, Bowen, Jacobi, Hassenpflug, Castaman, Davies, Rodeghiero, Zanardelli
1601	Phe-> Thr	P1601T	**NO**	A1	IIA	YES	Plasmids Recombinant Protein Proteolysis By ADAMTS13 ,PCR, Heteroduplex Generations, Phenotype Analysis Proteolysis Studies	Jacobi, Bowen, Pruss
1605	Tyr-> Phe	Y1605F	**YES**	A2	IIA	?	Plasmids Recombinant Protein Proteolysis By ADAMTS13	Pruss
1605	Tyr-> His	Y1605H	**YES**	A2	IIA	?	Plasmids Recombinant Protein Proteolysis By ADAMTS13	Pruss
1605	Tyr-> Asn	Y1605N	**YES**	A2	IIA	?	Plasmids Recombinant Protein Proteolysis By ADAMTS13	Pruss
1606	Met-> Ala	M1606A	**YES**	?	?	?	SDS Gels and ADAMTS13 Assays	De Groot
1606	Met-> Leu	M1606L	**YES**	A2	IIA	?	Plasmids Recombinant Protein Proteolysis By ADAMTS13	Pruss

Supplemental Table 1. *Chart of vWD Mutation from literature.* Continued on next page.

Loc.	A. A . switch	Mut.	A13 Susc.	Dom.	Type	Pat.	Assays	Pub.
1607	Val-> Asp	V1607D	NO	A2	IIA(I)	YES	Transfection, SDS Gels, PCR, ELISA, Tagging, Western Blotting and Agarose Gel, Plasmids, Immunofluorescent ADAMTS13 Assay	Hassenpflug, Castaman, Jacobi
1609	Gly-> Arg	G1609R	YES	A2	IIA(II)	?	Transfection, SDS Gels, PCR, ELISA, Tagging	Hassenpflug
1613	Ser-> Pro	S1613P	YES	?	IIA	YES	Live Rat Model , Cell Culture Transfection	Stoddert
1614	Asp-> Gly	D1614G	NO	A2	IIA	No	PCR, Cleavage Assay, Surface Plasma Resonance	Zanardelli
1614	Asp-> Ala	D1614A	YES	A2	IIA	No	PCR, Cleavage Assay, Surface Plasma Resonance	Zanardelli
1615	Gln-> Ala	E1615A	YES	A2	IIA	No	PCR, Cleavage Assay, Surface Plasma Resonance	Zanardelli
1617	Lys-> Ala	K1617A	YES	A2	IIA	No	PCR, Cleavage Assays, Surface Plasma Resonance	Zanardelli
1625	Val-> Arg	V1625R	?	?	IIA	YES	Transfection, SDS Gels, PCR, ELISA, Tagging, Western Blotting and Agarose Gel	Castaman
1628	Ile-> Thr	I1628T	YES	A2	IIA(II)	YES	Transfection, SDS Gels, PCR, ELISA, Tagging, Western Blotting and Agarose Gel, Phenotype, Mutation Screening, Expression In Silico Studies in tensile Force, Velocity Pulling, Plasmid, Expression Cleavage Assay, Heteroduplex Generations, Phenotype Analysis	Hassenpflug, Interlandi, Castaman, Ahmad, Davies, Zanardelli
1629	Gly-> Arg	G1629R	NO	A2	IIA	No	PCR, Cleavage Assay, Surface Plasma Resonance	Zanardelli

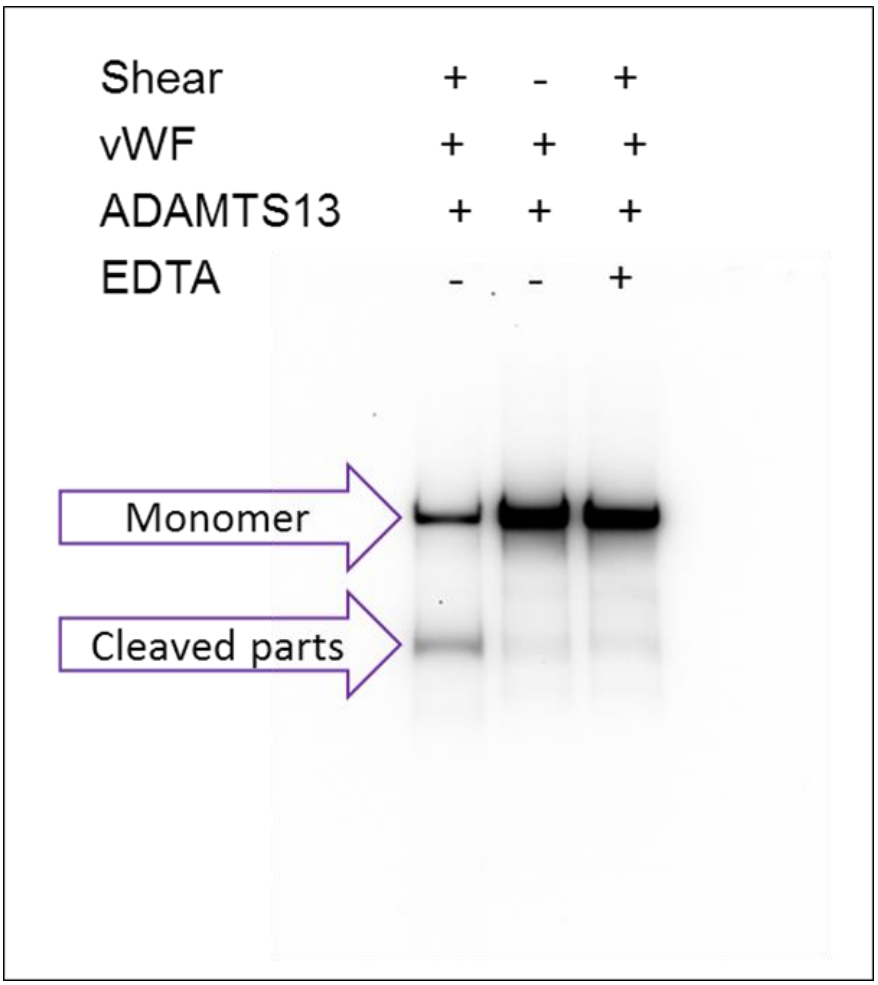
Supplemental Table 1. *Chart of vWD Mutation from literature.*

Loc.	A. A . switch	Mut.	A13 Susc.	Dom.	Type	Pat.	Assays	Pub.
1629	Gly-> Glu	G1629E	**YES**	A2	IIA(II)	YES	Transfection, SDS Gels, PCR, ELISA, Tagging, Western Blotting and Agarose Gel	Hassenpflug, Castaman
1631	Ile-> Asp	G1631D	**YES**	A2	IIA(II)	YES	Transfection, SDS Gels, PCR, ELISA, Tagging, Western Blotting and Agarose Gel, Plasmids, Immunofluorescent ADAMTS13 Assay	Hassenpflug, Castaman, Jacobi
1635	Asn-> Ile	N1635I	**?**	?	IIA	YES	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot
1638	Glu-> Lys	E1638K	**YES**	A2	IIA(II)	NO	SDS Gels, PCR, ELISA, Tagging, Phenotype, Mutation Screening, Expression In Silico Studies in tensile Force, Velocity Pulling, Plasmid, Expression Cleavage Assay, Surface Plasma Resonance	Hassenpflug, Interlandi, Zanardelli
1643	Gly-> Ser	G1643S	**?**	A2	IIA	?	Plasmids Recombinant Protein Proteolysis by ADAMTS13	Ahmad, Berber, Iannuzzi, Muia, Lillicrap, Zanardelli, Springer, Interlandi, Veyradier, Jacobi, Baronciani, Bowen, Pruss, O'Brien, Lynch, Castaman, Gill, White-Adams, Bodo, Sanders, De Groot
1647	Ala-> Asp	A1647D	**?**	?	IIA	YES	Phenotype, Mutation Screening, Expression In Silico Studies	Ahmad
1651	Ile-> Ala	I1651A	**YES**	A2	IIA	NO	Tensile Force, Velocity Pulling, Plasmid, Expression Cleavage Assay	Interlandi
1657	Leu-> Ile	L1657I	**YES**	A2	IIA	NO	Tensile Force, Velocity Pulling, Plasmid, Expression Cleavage Assay	Interlandi
1661	Ala-> Gly	A1661G	**NO**	A2	IIA	?	Tensile Force, Velocity Pulling, Plasmid, Expression Cleavage Assay	Interlandi
1665	Val-> Glu	V1665E	**?**	?	IIA	YES	Transfection, SDS Gels, PCR, ELISA, Tagging, Western Blotting And Agarose Gel	Castaman
7401	Met	M7401	**NO**	D2	?	YES	Plasmids, Transfection, ELISA Agarose Gel Immunofluorescent ADAMTS13 Assay	Jacobi

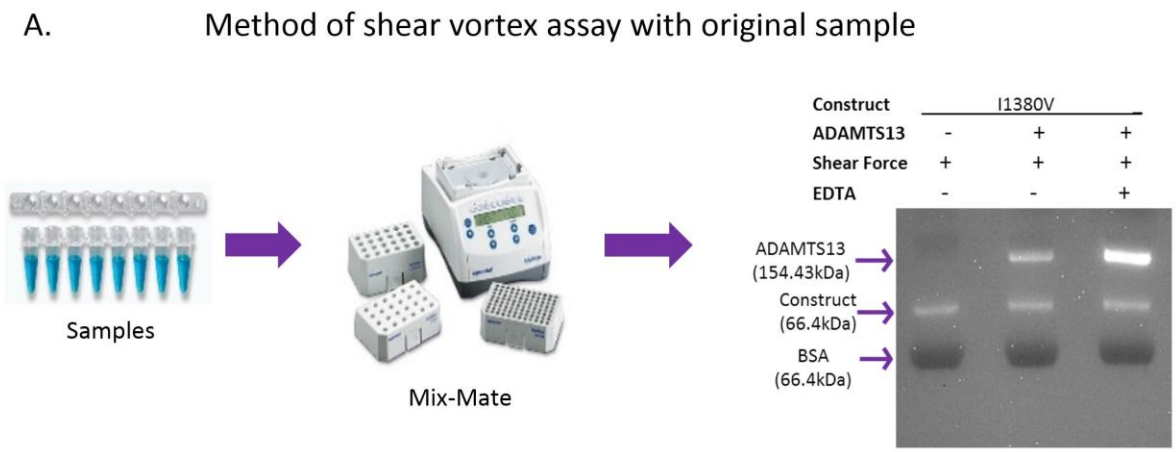
Supplemental Table 1. Optimization of buffers for supernatant purification. [4]

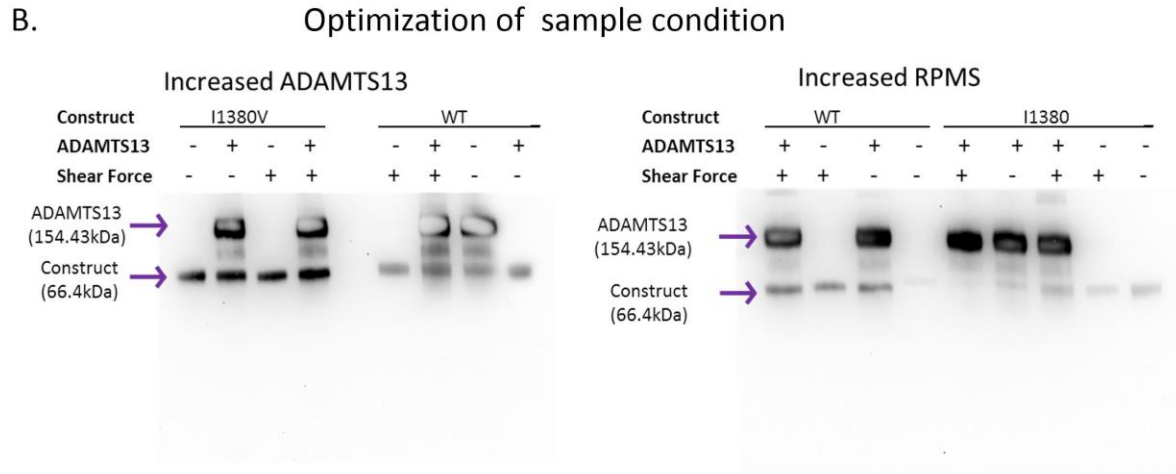
Purification Round	Purification Buffers				
	EQ/ Binding (Imidazole)	Wash (Imidazole)	Elution (Imidazole)	Supernatant (Salt)	UV-280 elution trace results
1	NONE	NONE	500mM	NONE	No protein detected
2	NONE	NONE	1M	NONE	Host and plasmid protein detected in elution
3	5mM	NONE	1M	NONE	Less Host protein detected
4	10mM	10mM	1M	NONE	Least amount of host protein detected
Final	10mM	10mM	500mM	150nM	Pure protein seen in elution

[4] *Using an FPLC or AKTA the buffers used were per the manufactures recommendation. Buffers are listed in the figure. Each constructs has a graph representing the peaks of the fractions traced with UV280nm, a Bulldog total protein stain for purity check, and nickel-alkaline phosphatase Western blot to check expression in sample loaded onto column, and fractions.*

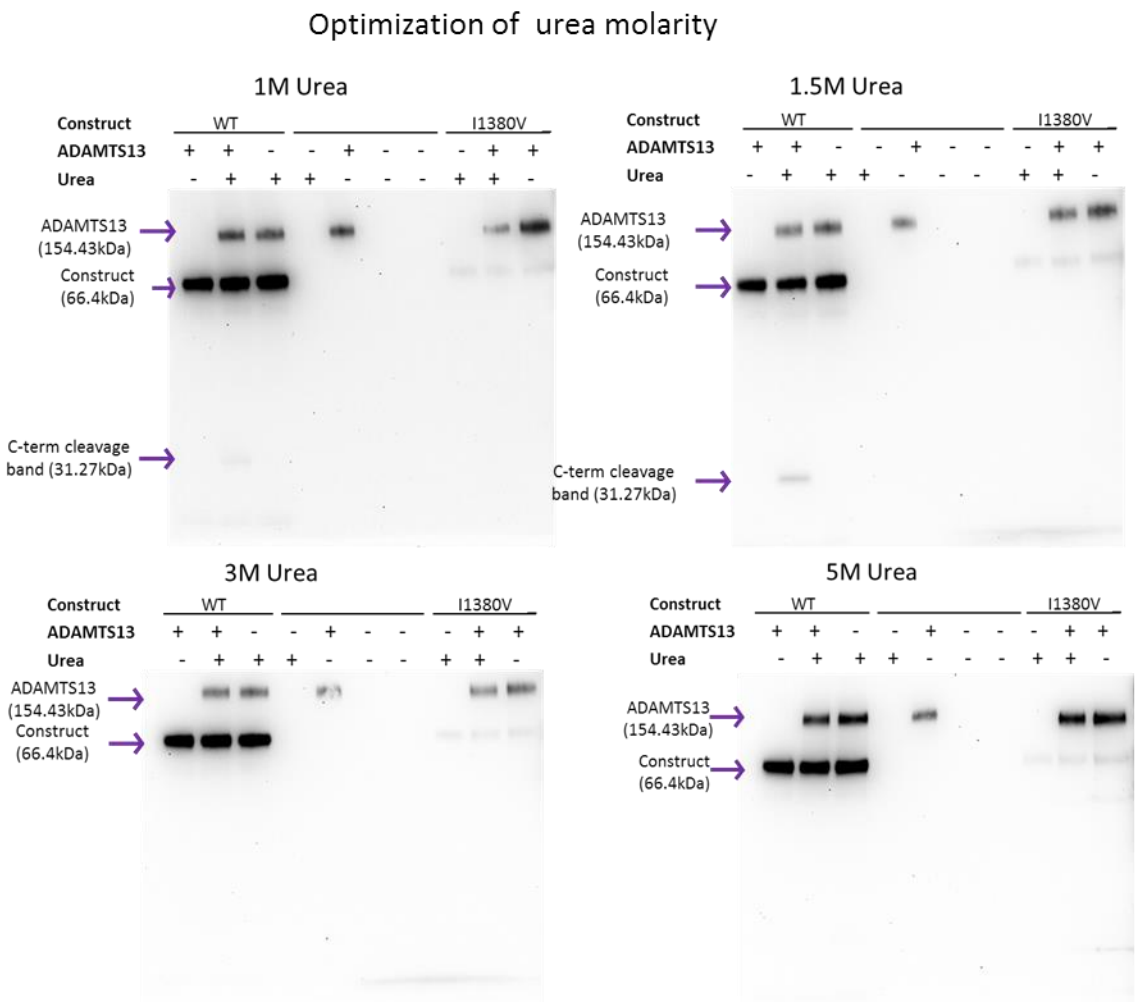


Supplemental Figure 1. *Shear Vortex Assay Full-Length vWF preliminary data.* The key above the gel shows conditions, plus sign (+) indicates inclusion while a minus sign (-) indicates the lack of protein or force applied to each sample.





Supplemental Figure 2. *Shear Vortex Assay using Purified Protein.* The key above the membranes shows conditions. The plus sign (+) indicates the inclusion of the component listed on the side in bold while a minus sign (-) shows the lack of protein or other component such as shear force. The upper blot show the original conditions while the lower blots show the optimized condition used indicated by the title above the blots.



Supplemental Figure 3. *Optimization of Urea Assay.* The key above the membranes indicates presence (+) or absence (-) of components of sample. Various concentrations of urea were tested as a means to denature vWF construct so cleavage will increase. Based on these results, 1.5M of urea was determined to have the best results.