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(54) **Title:** GAIN-OF-FUNCTION ADAMTS 13 VARIANTS RESISTANT TO AUTOANTIBODY INHIBITION AND METHODS OF USE THEREOF

(57) **Abstract:** Compositions and methods for the treatment of thrombotic thrombocytopenic purpura are disclosed.



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**GAIN-OF-FUNCTION ADAMTS13 VARIANTS RESISTANT TO AUTOANTIBODY
INHIBITION AND METHODS OF USE THEREOF**

By

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5 This application claims priority to US Provisional
Application Nos: 61/578,295, 61/731,193 and 61/34,580 filed
December 21, 2011, November 29, 2012 and December 7, 2012
respectively. Each of the foregoing applications is
incorporated herein by reference.

10 Pursuant to 35 U.S.C. §202 (c) it is acknowledged that the
U.S. Government has rights in the invention described, which
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FIELD OF THE INVENTION

15 This invention relates to the fields of physiology and
hematology. More specifically, the invention provides gain-
of-function ADAMTS13 variants which are resistant to
autoantibody inhibition and therefore useful for the treatment
of aberrant thrombus formation such as that observed in TTP,
20 myocardial infarction, and stroke.

BACKGROUND OF THE INVENTION

 Several publications and patent documents are cited
throughout the specification in order to describe the state of
the art to which this invention pertains. Each of these
25 citations is incorporated herein by reference as though set
forth in full.

 ADAMTS13 (A Disintegrin And Metalloprotease with
ThromboSpondin type1 repeats-13) cleaves ultra large (UL) von
Willebrand factor (VWF) on endothelial cells ¹ and soluble VWF
30 in the flowing blood ^{2,3} or at site of injury where VWF-rich

platelet thrombi are formed ⁴⁻⁶. This cleavage is highly specific occurring at the Tyr¹⁶⁰⁵-Met¹⁶⁰⁶ bond in the A2 domain ⁷. *In vivo*, fluid shear stress accelerates the cleavage of cell bound ULVWF ^{1;8} and soluble VWF in circulation ^{2;3}. *In vitro* addition of a denaturant such as urea ⁹ or guanidine ⁷ markedly accelerates the cleavage of soluble VWF by ADAMTS13. These findings facilitate the development of various biochemical assays for assessing ADAMTS13 activity.

The importance of VWF proteolysis is highlighted by the development of a fatal syndrome thrombotic thrombocytopenic purpura (TTP) when plasma ADAMTS13 activity is severely deficient, either due to hereditary mutations of *ADAMTS13* gene ¹⁰ or acquired formation of autoantibodies that inhibit ADAMTS13 activity ¹¹⁻¹³. Nearly all adult patients with severely deficient ADAMTS13 activity harbor polyclonal immunoglobulin Gs (IgGs) that bind the Cys-rich and spacer domains, particularly the spacer domain of ADAMTS13 ^{13,17}. Recent studies have shown that exosite 3 (i.e. Y659-Y665) and several other adjacent amino acid residues (i.e. R568 and F592) in the spacer domain comprise a major antigenic epitope for autoantibodies in TTP ^{18;19}. This region is also found to play an essential role in proteolytic cleavage of VWF under various conditions ^{6;20-24} and inhibition of arterial thrombus formation *in vivo* ⁶.

Clearly a need exists for the identification of those residues in the ADAMTS13 spacer region which modulate substrate recognition and autoantibody recognition. This information should provide guidance for the development of therapeutic compositions useful for the treatment of TTP, myocardial infarction and stroke, and other inflammatory and arterial thrombotic disorders.

SUMMARY OF THE INVENTION

In accordance with the present invention, isolated gain of function ADAMTS13 variants are provided comprising at least one amino acid change in the spacer domain of ADAMTS13,
5 provided the change is not an alanine substitution. The variants of the invention retain proteolytic activity and exhibit resistance to autoantibody inhibition. In a preferred embodiment, the variant is one of the M1 variant, the M2 variant, the M3 variant, the M4 variant or the M5 variant. In
10 a particularly preferred embodiment, the variant is the M4 variant .

Also encompassed by the present invention is a pharmaceutical composition comprising the isolated ADAMTS13 variants described above in a biologically acceptable carrier.

15 In yet another aspect, the invention provides a method for the treatment of TPP. An exemplary method comprises administration of an effective amount of the ADAMTS13 variants described above to a patient need thereof, the ADAMTS13 variants inhibiting thrombus formation in the patient. In a
20 particularly preferred embodiment, the variant exhibits increased proteolytic activity when compared to wild type ADAMTS13 and/or is resistant to autoantibody inhibition.

BRIEF DESCRIPTION OF THE DRAWINGS

25 **Figure 1 . Western blot and proteolytic activity of ADAMTS13 and mutants.** A . Western blotting with mouse anti-V5 IgG detects wild type ADAMTS13 (WT) and single point mutants at position 660 in the concentrated condition medium (~50 nM per lane) . Arrowhead and double stars indicate the intact
30 full-length ADAMTS13, -195 kDa, and degradation product, respectively. B . Relative proteolytic activity (%) of WT and single point variants assessed by the cleavage of rF-VWF73 as

described in the Materials and Methods. Means and standard deviations from three independent experiments are shown (n=3). All mutants except for R660K have activity below 20% of WT. **c.** Proteolytic degradation of multimeric VWF by ADAMTS13 and single point mutants under denaturing conditions. Plasma-derived VWF (150 nM) was incubated with 0.2 nM of recombinant ADAMTS13 and mutants in the presence of 1.5 M urea for 4 hours. The proteolytic cleavage of VWF was determined by 1% agarose gel electrophoresis and Western blotting. The sign + or - indicates the presence or absence of 10 mM EDTA in the reaction. HMW and P indicate the high molecular weight multimers and cleavage product, respectively.

Figure 2 . Characterization of single and compound

ADAMTS13 variants. **A.** Schematic domain organization of full-length ADAMTS13 (top), surface representation of exosite 3 and adjacent residues in the spacer domain of ADAMTS13 (Left), and names of various ADAMTS13 variants with amino acid substitution (Right). **B.** Western blotting with anti-V5 detects recombinant WT and variants in the conditioned medium (50 nM each lane). Arrowhead indicates full-length ADAMTS13 of ~195 kDa with little degradation. **c.** Relative specific proteolytic activity of the variants in comparison to WT is shown. The results are the mean and SD of three independent experiments (n=3). **P<0.001 is considered to be statically highly significant.

Figure 3 . Proteolytic cleavage of multimeric VWF by

ADAMTS13 and variants under denaturing conditions. **A.** Human plasma-derived VWF (150 nM) pre-denatured with 1.5 M urea was mixed with WT, M1, M2, M3, M4, and M5 (0.04 nM and 0.2 nM) in the absence or in the presence of EDTA (10 mM) (last lane) and dialyzed against 10 mM Tris-HCl, pH 8.0 containing 1.5 M urea at 37° for 4h. The cleavage of VWF was determined by agarose (1%) gel electrophoresis and Western blotting with rabbit

anti-VWF IgG (1:5,000), followed by IRDye 800CW-labeled goat anti-rabbit IgG (1:10,000). **B.** The relative activity was determined by ImageJ quantitation of the ratio of cleavage product (P, arrowhead) to high molecular weight (HMW) VWF multimers in each sample. The specific activity was normalized to that of WT (1 arbitrary unit). The results are the means $\pm$ standard error of the mean from three independent experiments.

Figure 4 . Inhibition of proteolytic activity of WT and variants by autoantibodies. Recombinant ADAMTS13 (WT) or variants (M1-M5) (final concentration of 0.2 nM) was incubated without (-) or with (+) 35 μ M of human monoclonal anti-spacer IgG (mAb II-1) (**A**) or 5-10 μ l of heat-inactivated normal human plasma (N) or TTP patient #1 plasma (P) (**B**) for 60 min. The residual activity was determined by the cleavage of pre-denatured multimeric VWF as described in the Materials and Methods. EDTA (10 mM) was included in the last lane as a negative control. The relative residual activity was determined by the ratio of product (P) to high molecular weight VWF (HMW) multimer using ImageJ and normalized to the activity in the presence of normal human plasma. The percentage of inhibition (means and standard deviation) by a panel of 12 TTP patient plasmas is shown in panel **c**. ** $p < 0.001$ indicates statistically highly significant difference between WT and three variants (M3, M4, and M5).

Figure 5 . Binding of IgG autoantibodies from TTP patients ADAMTS13 and variants. ADAMTS13 (WT) or variants (M1-M5) (50 ng) were incubated with a human monoclonal anti-spacer IgG (mAb II-1) (35 μ M) or TTP patient plasmas (5-10 μ l each, depending on plasma IgG concentrations). The immune complexes were pulled down with protein A-Sepharose 4B and detected by Western blotting with monoclonal anti-V5. The anti-V5 IgG-coupled Sepharose 4B beads were used for a positive control. Normal IgG was used for a negative control.

Figure 6. WT and variants inhibited platelet aggregation on collagen-coated surface under flow.

Whole blood (anti-coagulated with PPACK) was incubated with AlexaFluor488 conjugated anti-CD41 IgG (1 µg/ml) and WT or variants (10 nM) for 15 min. The blood was then flown through collagen-coated surface in a microfluidic device at 10 dyns/cm² for 3 min. The rate and amount of platelets accumulated on the surface were determined under an inverse fluorescent microscope with a high speed CCD camera (A). Data were analyzed by the BioFlux Mortgage software. The mean percentages of the platelet coverage from three independent experiments (n=3) at 1.5 minutes were plotted against the various ADAMTS13 variants added (B).

Figure 7. Modeling of ADAMTS13-spacer and VWF-A2

interaction. A. Surface representation of ADAMTS13-DTCS fragment; B. Close-up view of the hydrophobic cluster in the exosite of ADAMTS1-spacer domain. This pocket contains a cluster of hydrophobic residues (L591, F592, L637, P638, L668, T669, and ring of Y661 and Y665), lined by basic residues (R568, R589, R636, and R660) supported by 8 β sheets (i.e. β1, 2, 3, 6, 7, 8, 9, and 10). C. A substitution of these surface residues with those in yellow appears to increase hydrophobicity of this pocket. D. VWF-A2 (1653-1668) forms an amphipathic helix (α6). Here shown are the hydrophobic residues facing to the top and charged residues to the bottom. This amphipathic helix may govern specificity to the ADAMTS13-spacer exosite by inserting its hydrophobic side into the pocket.

Figure 8. A) ADAMTS13 domains. M=metalloprotease,

D=disintegrin, TSP=thrombospondin type-1, C=cysteine-rich, s=spacer, CUB=CUB. B) Crystal structure of ADAMTS13 DTCS with surfaces is shown with domains colored according to figure 9A. Exosites 3 and 4 are boxed. The spacer domain is enlarged

showing exosites are solvent-exposed. c) Residues of exosites 3 and 4 create a solvent-exposed interface for substrate and/or antibody binding.

Figure 9. A) Schematic of VWF-73 FRETs assay. Residues

5 corresponding to Q1599 and P1611 in the A2 domain of VWF are mutated to cysteines and labeled by fluorescein-5-maleimide . These residues are homoquenchers that fluoresce upon cleavage by ADAMTS13. B) VWF-73 FRETs assay of motif B variants.

Concentration of VWF-73 peptide=2 μ M; various concentrations
10 of variants are assayed and quantified relative to the wild type activity. $\Delta B = {}^{632}\text{LTEDRLPR}^{639} \rightarrow$ Deleted, $\Delta B1 = {}^{632}\text{LTED}^{635} \rightarrow$ Deleted, and $\Delta B2 = {}^{636}\text{RLPR}^{639} \rightarrow$ Deleted. $\Delta B1$ is not secreted, so data could not be obtained for this variant.

Figure 10. A) Multimer analysis of wild type ADAMTS13 and

15 deletion mutants. B) Multimer analysis of motif B mutations. All multimers are done with 150 nM VWF, denatured in 1.5 M urea for 4 hours at 37 °C in the presence of ADAMTS13. EDTA (20 mM) was included in B lane 1 as control.

Figure 11. A) Results of hydrogen exchange for ADAMTS13

20 MDTCs construct. Exchange was done at pH 7.3 and stopped at 0 °C. The only statistically significant difference in exchange was observed between residues 629 and 642 in the spacer domain, shown by the dagger (†). B) The region containing motif B shows the least exchange in the presence of
25 scFV 4-20. 6 unique peptides exist and are shown below the sequence. One peptide is not shown because data obtained was incomplete. c) Results of hydrogen exchange from a few representative peptides. Residues 33-45 are in the
30 metalloprotease domain and exchange readily with or without scFV 4-20 present. Residues 574-580 and 643-650 are in the spacer domain but show no interaction with scFV 4-20. Residues

632-637, 635-642, and 636-641 have decreased exchange with scFV 4-20, indicating an interaction.

Figure 12. A) Schematic representation of whole IgG and a single chain fragment of the variable region (scFVs) of human monoclonal antibody against ADAMTS13 isolated from an acquired TTP patient from B-cells through phage display library screening. B) Inhibition of plasma ADAMTS13 activity by affinity purified scFV 4-20 expressed in E. coli using a VWF-73 FRET assay. Concentration-dependent inhibition of plasma ADAMTS13 is shown with an IC₅₀ of 0.01 ug/ml.

Figure 13. Inhibition of murine ADAMTS13 activity and alteration of VWF multimers by scFv 4-20.

Figure 14. 4-20 mAb causes acquired TTP triggered by bacterial shigatoxin.

Figure 15. A graph showing survival rates in mice expressing control and inhibitory scFv after being challenged with shiga toxin.

DETAILED DESCRIPTION OF THE INVENTION

Thrombotic thrombocytopenic purpura (TTP) is primarily caused by immunoglobulin G (IgG) autoantibodies against ADAMTS13. Nearly all TTP patients harbor IgGs that bind the spacer domain of ADAMTS13, a region critical for recognition and proteolysis of von Willebrand factor (VWF). We show that a modification of an exosite in the spacer domain generates ADAMTS13 variants with reduced autoantibody binding and preserved or enhanced specific activity. Site-directed mutagenesis was used to generate a series of ADAMTS13 variants and their functional properties were assessed. Of 24 novel variants, two (i.e. M4, R660K/F592Y/R568K/Y661F and M5, R660K/F592Y/R568K/Y661F/Y665F) exhibited increased specific activity by ~4-5 and ~10-12 fold to cleave peptide VWF73 and

multimeric VWF, respectively. More interestingly, the gain-of-function ADAMTS13 variants were more resistant to inhibition by anti-ADAMTS13 autoantibodies from patients with acquired TTP, owing to reduced binding by anti-ADAMTS13 IgGs. Other
5 variants are disclosed that possess alterations in the exosite 4. These results shed more light on the critical role of the exosite in the spacer domain in substrate recognition. Our findings also help understand the pathogenesis of autoimmune TTP. The autoantibody-resistant variants provide new agents
10 for therapy of acquired TTP with inhibitors.

Another aspect of the invention relates to the treatment of stroke and other blood coagulation disorders. Data have shown that low ADAMTS13 activity is a risk factor for myocardial infarction and ischemic stroke. Indeed,
15 recombinant ADAMTS13 is being tested in a phase I clinical trial for these disorders in addition to assessing efficacy for the treatment of TTP. The variants disclosed herein would also be suitable for this purpose. Thus, ADAMTS13 variants may be administered to a patient via infusion in a
20 biologically compatible carrier. The polypeptides of the invention may optionally be encapsulated in to liposomes or other phospholipids to increase stability of the molecule. The polypeptides or complexes there of may be administered alone or in combination with other agents known to modulate
25 thrombotic events. An appropriate composition in which to deliver ADAMTS13 can be determined by a medical practitioner upon consideration of a variety of physiological variables, including, but not limited to, the patient's condition and hemodynamic state. A variety of compositions well suited for
30 different applications and routes of administration are well known in the art and described hereinbelow.

The preparation containing the purified polypeptide variant contains a physiologically acceptable matrix and is preferably formulated as a pharmaceutical preparation. The

preparation can be formulated using substantially known prior art methods, it can be mixed with a buffer containing salts, such as NaCl, CaCl_2 , and amino acids, such as glycine and/or lysine, and in a pH range from 6 to 8. Until needed, the purified preparation containing the polypeptides can be stored in the form of a finished solution or in lyophilized or deep-frozen form. Preferably the preparation is stored in lyophilized form and is dissolved into a visually clear solution using an appropriate reconstitution solution.

Alternatively, the preparation according to the present invention can also be made available as a liquid preparation or as a liquid that is deep-frozen.

The preparation according to the present invention is especially stable, i.e., it can be allowed to stand in dissolved form for a prolonged time prior to application. The preparation according to the present invention can be made available as a pharmaceutical preparation with anti thrombotic activity in the form of a one-component preparation or in combination with other factors in the form of a multi-component preparation.

Prior to processing the purified proteins into a pharmaceutical preparation, the purified proteins are subjected to the conventional quality controls and fashioned into a therapeutic form of presentation. In particular, during the recombinant manufacture, the purified preparation is tested for the absence of components from any cellular components (e.g., nucleic acids, cellular debris) possibly present during the purification process.

Another feature of this invention relates to making available a preparation which contains ADAMTS13 variants which is free from inactive intermediates and autoproteolytic degradation products.

The pharmaceutical preparation may contain dosages of between 10-1000 $\mu\text{g/kg}$, more preferably between about 10-250

µg/kg and most preferably between 10 and 75 µg/kg, with 40 µg/kg of the polypeptides being particularly preferred. Patients may be treated immediately upon presentation at the clinic with a coagulation disorder or thrombotic disorder.

5 Alternatively, patients may receive a bolus infusion every one to three hours, or if sufficient improvement is observed, a once daily infusion of the polypeptides described herein.

10

Definitions :

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specification and claims.

15 With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally
20 occurring genome of the organism from which it originates. For example, the "isolated nucleic acid" may comprise a DNA or cDNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the DNA of a prokaryote or eukaryote .

25 With respect to RNA molecules of the invention, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it
30 would be associated in its natural state (i.e., in cells or tissues) , such that it exists in a "substantially pure" form (the term "substantially pure" is defined below) .

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes used herein.

This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention.

Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "promoter region" refers to the transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns.

The term "vector" refers to a small carrier DNA molecule into which a DNA sequence can be inserted for introduction into a host cell where it will be replicated. An "expression vector" is a specialized vector that contains a gene or nucleic acid sequence with the necessary regulatory regions needed for expression in a host cell.

The term "operably linked" means that the regulatory sequences necessary for expression of a coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g. promoters, enhancers, and termination elements) in an expression vector. This definition is also sometimes applied to the arrangement of nucleic acid sequences of a first and a second nucleic acid molecule wherein a hybrid nucleic acid molecule is generated.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, of the compound of interest. Purity is measured by methods appropriate for the

compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like) .

5 The phrase "consisting essentially of" when referring to a particular nucleotide sequence or amino acid sequence means a sequence having the properties of a given SEQ ID NO:.. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel
10 characteristics of the sequence.

The term "oligonucleotide, " as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides , preferably more than three. The exact
15 size of the oligonucleotide will depend on various factors and on the particular application for which the oligonucleotide is used .

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or
20 DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length
25 of the probe will depend upon many factors, including temperature, source of probe and method of use. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain
30 fewer nucleotides.

The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically

hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary
5 nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has
10 sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such
15 hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the
20 invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or
25 double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to act functionally as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate
30 nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the

addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product .

The primer may vary in length depending on the particular conditions and requirements of the application. For example, 5 in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able to anneal with the desired template strand in a 10 manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non- 15 complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of 20 the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product .

The term "percent identical" is used herein with reference to comparisons among nucleic acid or amino acid 25 sequences. Nucleic acid and amino acid sequences are often compared using computer programs that align sequences of nucleic or amino acids thus defining the differences between the two. For purposes of this invention comparisons of nucleic acid sequences are performed using the GCG Wisconsin 30 Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin. For convenience, the default parameters (gap creation penalty = 12, gap extension penalty = 4) specified by that program are intended for use herein to compare sequence identity. Alternately, the Blastn 2.0

program provided by the National Center for Biotechnology Information (found on the world wide web at ncbi.nlm.nih.gov/blast/; Altschul et al., 1990, J Mol Biol 215:403-410) using a gapped alignment with default parameters, 5 may be used to determine the level of identity and similarity between nucleic acid sequences and amino acid sequences.

The following materials and methods are provided to facilitate the practice of the present invention.

10 **Constructs :**

QuickChange site-directed mutagenesis reagents (Stratagene) were used to replace one or a clustered of surface charged amino acid residues (R660, F592, R568, Y661, and Y665) in the $\beta 9$ - $\beta 10$ variable region of the spacer domain. 15 A pcDNA3.1 vector containing wild-type ADAMTS13-V5-His was used as a plate. The resulting variants with a desired mutation or mutations were sequenced to confirm the accuracy at the Nucleic Acid Core Facility, The Children's Hospital of Philadelphia .

20 **Preparations of recombinant ADAMTS13 and variants :**

COS7 cells were transfected with plasmid and polyethylenimine (PEI) according to the manufacture instruction (CellNTech Advanced Cell System) . Serum-free conditioned medium was collected 4 days after transfection and 25 concentrated 50-100 x using a filtration column (Millipore) in the presence of protease inhibitor cocktail (Sigma) .

ELISA;

The concentrations of ADAMTS13 and variants in the concentrated conditioned medium were determined by a Sandwich 30 enzyme-linked immunoassay (ELISA) . Briefly, a high binding microtiter plate (NUNC) was coated with 100 μ l of monoclonal

anti-Disintegrin IgG (40 µg/ml) (Custom made in Green Mountain Antibody, Vermont, NH) overnight. The remaining binding sites were blocked for 30 min with 150 µl/well of 2.5% BSA in PBS. WT and variants diluted with PBS were added and incubated for 2 hours. After being washed with PBS, monoclonal anti-V5-HRP IgG (1:1,000) was added for detection. Previously purified WT was used as a calibration. All quantifications were repeated three times for consistency.

Western blot:

The integrity of WT and variants in the concentrated conditioned medium were assessed by Western blotting after fractionation on 8% SDS-polyacrylamide gel under reduced conditions. After being transferred to a nitrocellulose membrane, recombinant WT and variants were blotted by anti-V5 IgG (1:5,000) and IRdye800CW-labeled goat anti-mouse IgG (1:20,000) (LI-COR, Lincoln) in 20 mM Tris-HCl, 150 mM NaCl containing 0.05% Tween20 and 1% casein (TBSTc). The fluorescent signal obtained with Odyssey imaging system (LI-COR, Lincoln, Nebraska) was converted to gray images.

Proteolytic cleavage of VWF73 peptide:

Maleimide-fluorescein-labeled VWF73 (rF-VWF73) (2 µM) as described previously ^{25,26} was incubated with ADAMTS13 and variants (0.2 nM) in 5 mM Bis-Tris, pH 6.0 containing 25 mM CaCl₂ and 0.005% Tween 20 in a 96-well white plate (Corning, NY). The rate of fluorescence generation was monitored at 37 °C with a fluorescent microtiter plate reader (Molecular Devices, Sunnyvale, CA) (Ex/Em 485/535nm) every minute for 30 min. Pooled normal human plasma was used as a reference.

Proteolytic cleavage of multimeric VWF under denaturing conditions :

Purified plasma VWF (150 nM) was incubated with ADAMTS13 and variants in the conditioned medium (0.2 nM and 0.04 nM) or purified WT and variants (2 nM and 10 nM) at 37 °C for 4 hours on a membrane (0.25 µm, pore size) floating over 50 ml buffer (10 mM Tris-HCl, pH 8.0 containing 1.5 M urea in a conical tube. The digested material was withdrawn and denatured with sample buffer (70 mM Tris-HCl, pH6.5, 2.4% SDS, 0.67 M urea, and 4 mM EDTA) at 60 °C for 20 minutes. The denatured VWF was fractionated with 1% (wt/vol) SeaKem HGT agarose (Cambrex, East Rutherford, NJ) gel. The protein was then transferred onto a nitrocellulose membrane and detected by Western blotting with anti-VWF IgG (1:5,000) and IRDye 800CW-labeled goat anti-rabbit IgG (1:10,000) (LI-COR Bioscience, Lincoln, Nebraska) as described previously ^{6;27}.

Inhibition of cleavage of VWF by autoantibodies from TTP patients :

Recombinant ADAMTS13 and variants (0.2 nM) were incubated with human monoclonal antibody against ADAMTS13-spacer domain isolated from a patient with idiopathic TTP (mAb II-1) (kindly provided by Dr. Jan Vorberg, Sanquin-AMC Landsteiner Laboratory, Amsterdam, the Netherlands) or with heat-inactivated (56 °C for 60 min) normal human plasma or patient plasmas (2.5-10 µl) in PBS for 30 min. The residual proteolytic activity was determined by cleavage of VWF multimers using agarose gel electrophoresis and Western blotting as described previously ^{6;27}. The percentage of inhibition was determined by comparing the residual activity in WT and variants after addition of control plasma with that after patient plasma.

Binding of patient anti-ADAMTS13 IgGs to ADAMTS13 and variants :

An immunoprecipitation plus Western blotting analysis was used to detect the antigen and antibody reaction in solution as described previously¹³. WT and variants (50 ng) were incubated 5-10 μ l of normal human plasma or patient plasma and 5 30 μ l of protein A/G Sepharose 4B (Invitrogen) in 50 mM Tris-HCl, pH 7.6 containing 0.15 M NaCl, 1% bovine serum albumin (BSA), 1% Triton X-100, and 0.1% Tween-20 (TBST) at 4 °C, overnight. After wash with TBST, the bound recombinant ADAMTS13 and variants were eluted from the beads and 10 determined by Western blotting with anti-V5 IgG (1:5,000) (Invitrogen). The amount of bound primary antibody was determined by IRDye 800CW-labeled goat anti-mouse IgG (1:20,000) (LI-COR Bioscience, Lincoln, Nebraska) in TBST containing 1% casein as previously described¹³.

15 **Model of ADAMTS13 and VWF interaction:**

The interaction between ADAMTS13-spacer domain and VWF-A2 was modeled using the HHPred server plugin in PyMol software (<http://www.pymol.org/>). The movies in the supplemental materials were made with Adobe Photoshop CS software.

20 The following examples are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

EXAMPLE I

Gain-of-Function ADAMTS13 Variants That Are Resistant to 25 Inhibition by Anti-ADAMTS13 Autoantibodies from Patients with Acquired Idiopathic Thrombotic Thrombocytopenic Purpura

Identification of the optimal residue at position of 660 for ADAMTS13 activity: We and others have previously shown that R660 in the spacer domain of ADAMTS13 plays an essential 30 role for substrate recognition (Genbank Accession No. mRNA, NM_139025.3; protein NM_6205941; Uniprot No. Q76XL8; ¹⁸⁷²⁸. A

substitution of arginine at the position of 660 with alanine (R660A) nearly abolished proteolytic activity toward various substrates^{18/28}. To determine the optimal residue at this position, we prepared a series of ADAMTS13 variants by replacing the R with 18 other amino acid residues. The resulting constructs were transiently expressed in COS-7 cells, which ran at ~195 kDa with little degradation on a SDS-polyacrylamide gel under reduced conditions (**Fig. 1A**). The specific activity was assessed by the cleavage of rF-VWF73 and VWF as described in the Methods. A replacement of R660 with any other residues except for K (M1, R660K) resulted in dramatically reduced cleavage of rF-VWF73 (**Fig. 1B**) and VWF (**Fig. 1C**). These results suggest that a positively charged residue such as arginine or lysine at position 660 in the spacer domain is required for ADAMTS13 activity.

Identification of gain-of-function ADAMTS13 variants:

Based on the preliminary results described above, we performed additional site-directed mutagenesis experiments by sequentially replacing several other charged/hydrophobic residues on the surface loop in the spacer domain (i.e. R568, F592, R660, Y661, and Y665) with K, Y, F, and K, respectively (**Fig. 2A**). The resulting ADAMTS13 variants (i.e. M2, M3, M4, and M5) were also transiently in COS7 cells. All ran at ~195 kDa on SDS-polyacrylamide gel under reduced conditions (**Fig. 2B**). The specific activity was assessed by the cleavage of both rF-VWF73 and VWF. The variants M1, M2, and M3 exhibited similar activity to WT cleaving VWF73 peptide (**Fig. 2C**) and multimeric VWF (**Fig. 3**). However, the variants M4 and M5 exhibited increased specific activity by 4-5 fold ($p < 0.001$) and 10-12 fold ($p < 0.001$) in cleaving VWF 73 peptide (**Fig. 2C**) and multimeric VWF (**Fig. 3**), respectively. These results demonstrate for the first time that gain-of-function ADAMTS13

variants can be engineered through a modification of exosite 3 in the spacer domain.

Identification of ADAMTS13 variants resistant to autoantibodies in TTP patients : Exosite 3 plus several other adjacent residues in the spacer domain contains major binding sites for anti-ADAMTS13 autoantibodies in patients with acquired TTP^{18;19}. We hypothesized that a modification in this region may alter the binding and inhibition of ADAMTS13 variants by patients' autoantibodies. To this aim, ADAMTS13 and variants (0.2 nM) were incubated for 60 min with a well-described human monoclonal antibody against spacer domain (mAb II-1) (35 μ M) isolated from a patient with acquired TTP^{6;14_16}. The mAb II-1 dramatically inhibited proteolytic activity of WT and M2, but not M1, M3, M4, and M5 (**Fig. 4A**), suggesting that R660 is critical for autoantibody inhibition. The reason why M1, but not M2 was resistant to inhibition is not clear.

When plasmas from TTP patients were used as the source of autoantibodies against ADAMTS13, proteolytic activity of WT, M1, and M2 was almost completely inhibited after 60 min of incubation (**Fig. 4B, 4C, and Table 1**), while variant M3 was only variably inhibited by the same amount of patient plasma, but variants M4 and M5 were resistant to patient plasma under the same conditions (**Fig. 4B, 4C, and Table 1**). These results indicate that the novel gain-of-function ADAMTS13 variants, especially M4 and M5, are more resistant to inhibition by both monoclonal and polyclonal anti-ADAMTS13 autoantibodies derived from patients with acquired idiopathic TTP.

Binding of patient anti-ADAMTS13 IgG to recombinant ADAMTS13 and variants: To determine whether the resistance of ADAMTS13 variants to autoantibodies was the result of impaired binding interactions between ADAMTS13 variants and autoantibodies, we performed immunoprecipitation followed by

Western blotting assay as described previously^{13;19}. As shown, mAb II-1 bound to WT and M2 consistently, but not to M1, M3, M4, and M5 (Fig. 5). This result was in a complete agreement with the antibody inhibitory activity (Fig. 4A).

Moreover, plasma polyclonal anti-ADAMTS13 IgGs from all 12 acquired idiopathic TTP patients also bound consistently to WT, M1, and M2, variably to M3, but rarely to M4 and M5 (Fig. 5). As controls, IgGs from healthy donors did not bind WT and variants detectably, while anti-V5 IgG bound to all constructs (Fig. 5). These results indicate that the inhibitory activity of either monoclonal or polyclonal anti-ADAMTS13 IgGs from TTP patients is mediated through their direct binding to exosite 3 in the spacer domain of ADAMTS13.

Model of interactions between ADAMTS13 and VWF: To gain insight into the mechanisms underlying the enhanced activity of ADAMTS13 variants, we performed molecular modeling using the existing crystal structure of ADAMTS13-DTCS fragment (Fig. 6A)²⁹ and VWF-A2 domain³⁰. As shown, the spacer domain comprises ten β -sheets (i.e. β 1-10), a pocket formed by various β sheets containing a cluster of hydrophobic residues (L591, F592, L637, F638, L668, and T669), and a ring formed by Y661 and Y665 lined by basic residues R568, R589, R660, and R636 (Fig. 6B). This pocket appears to directly interact with the α 6-helix (residues between D1653 and R1668) in the central A2 domain of VWF (Fig. 6D). The hydrophobic residues in the A2 domain presumably face exosite 3 to make strong hydrophobic contacts in conjunction with some hydrogen bonding outside of the pocket (data not shown). A substitution of R with K residue or Y with F residue or vice versa appears to alter the hydrophobicity of exosite 3 (Fig. 6C). The corresponding changes of the hydropathy index were noted as follows: R $\rightarrow$ K: -4.5 $\rightarrow$ -3.9 and Y $\rightarrow$ F: -1.3 $\rightarrow$ +2.8, thereby enhancing the interaction between VWF and ADAMTS13. Furthermore, a

substitution of F592 with Y may open up the pocket even more, thereby better engaging substrate. There was also a corresponding backbone shift which appears to take place in the β_2 , β_5 , β_6 , and β_9 sheets to compensate for the increased hydrophobicity (Fig. 6c). These changes allow greater engagement of exosite 3 with the A2 domain, particularly the amino acid residues between residues D1653 and R1668 (data not shown). Together, our findings suggest that the modification

Table 1. Clinical characteristics, plasma ADAMTS13 activity and inhibitors of TTP patients, and sensitivity of wild-type ADAMTS13 and novel variants to the inhibition by patient plasma

Pt ID#	Age (Y)	Sex	Pit count ($\times 10^9/L$)	Hct (%)	LDH (U/L)	Cr (mg/dl)	CNS Sym	A13 Act (%)	Anti-A13 Inhibitors	Anti-A13 IgG (U/ml)	Sensitivity to Pt plasma inhibition					
											WT	M1	M2	M3	M4	M5
1	44	F	15	23	695	1.1	Yes	<5	+	84.2	++	++	++	+	-	-
2	56	F	19	29	654	1.1	Yes	<5	+	35.4	++	++	++	+	+	+
3	45	F	88	28	995	0.7	Yes	<5	+	81.9	++	++	++	-	-	-
4	52	F	52	20	866	1.1	Yes	<5	+	132.0	++	++	++	-	-	-
5	79	F	7	27	1,660	1.4	Yes	<5	+	118.0	++	++	++	+	-	-
6	34	M	15	23	2,703	0.9	Yes	<5	+	127.5	++	++	++	+	-	-
7	34	M	0	33	859	1.9	No	<5	+	162.0	++	++	++	-	-	-
8	23	F	11	18	3,412	0.8	Yes	<5	+	132.0	++	++	++	-	-	-
9	42	F	23	17	3,259	0.8	Yes	<5	+	168.0	++	++	++	-	-	-
10	21	F	9	13	1,489	1.5	Yes	<5	+	72.0	++	++	++	+	+	+
11	61	F	33	28	511	0.8	Yes	<5	+	221.0	++	++	++	-	-	-
12	42	M	16	24	6,517	1.2	No	<5	+	147.2	++	++	++	+	-	-
N=12		43*	-	15.5*	23.5*	1.242*	1.1'	(83.3)	(100)	(100)	(100)	(100)	(100)	(50)	(17)	(17)

Pit, platelet; Hct, hematocrit; LDH, lactate dehydrogenase; Cr, creatinine; CNS Sym, central nervous signs and symptoms; A13 act, ADAMTS13 activity by rF-vWF73; Anti-A13, plasma anti-ADAMTS13 autoantibodies; IgG, immunoglobulin G; WT, recombinant wild-type ADAMTS13; M1-M5, refer to the legends in Fig. 1. F/M, female to male. "-", negative inhibition (<10% reduction in activity); "+", mild inhibition (10-30% reduction in activity); "++", moderate inhibition (30-50% reduction in activity); "+++", strong inhibition (>50% reduction in activity) after 50:50 mixing with patient plasma, 'indicates median values; () indicates the positive rates in percentage.

of an exosite in the spacer domain is a viable approach to improve ADAMTS13 function while reducing autoantibody binding and inhibition.

Discussion

In the present study, we have demonstrated that a positively charged residue i.e. arginine or lysine at the position 660 is critical for ADAMTS13 function (Fig. 1). This observation promotes us to test a hypothesis that replacement of the critical residues in the exosite in the spacer domain

may generate ADAMTS13 variants that are resistant to binding and inhibition by autoantibodies from patients with acquired TTP, while preserving proteolytic activity. Of 24 ADAMTS13 variants prepared, two (i.e. M4 and M5) exhibited dramatically enhanced specific activity cleaving VWF73 peptide (**Fig. 2**) and multimeric VWF (**Fig. 3**). More interestingly, these two gain-of-function variants are more resistant than WT and several other variants to inhibition by monoclonal and polyclonal autoantibodies against ADAMTS13 in patients with acquired idiopathic TTP (**Fig. 4** and **Table 1**). As shown, 10/12 TTP patient plasmas (83%) do not appear to inhibit proteolytic activity of M4 and M5, while the same amount of plasma completely inhibits proteolytic activity of WT, M1, and M2, but variably M3 under the same conditions. Plasmas from two patients weakly inhibit M4 and M5 activity (**Table 1**). These results further confirm using the gain-of-function rather than loss-of-function approaches described in the literature^{18,28} the critical role of the exosite in the spacer domain in substrate recognition and proteolytic cleavage of VWF.

Molecular modeling of interaction between VWF A2 and spacer domain shows that α 5-helix of VWF A2 domain appears to directly interact with the residues in the exosite 3 (**Fig. 6** and data not shown), primarily through hydrophobic interactions. A substitution of the residues R, F, R, Y, and Y with K, Y, K, F, and F at the positions of 568, 592, 660, 661, and 665, respectively, appears to increase hydrophobic interactions between the exosite 3 and α 5-helix of the central A2 domain (**Fig. 6** and data not shown).

Our findings also provide novel insight into the mechanism underlying pathogenesis of acquired idiopathic TTP caused by anti-ADAMTS13 autoantibodies. Despite polyclonal nature of autoantibodies against ADAMTS13 in patients with TTP^{13,31}, the inhibitory activity of anti-ADMTS13 autoantibodies

appears to be largely mediated through their binding to the exosite 3 in the spacer domain, as an alteration in this region dramatically reduced binding of anti-ADAMTS13 IgGs (Fig. 5) and inhibition by patient plasma autoantibodies (Fig. 4). Our results are consistent with those reported by Pos et al.¹⁹, in which a replacement of R568, F592, R660, Y661, and Y665 with A abolishes the binding of anti-ADAMTS13 IgGs from most TTP patients¹⁹. However, alanine substitution results in loss-of-function of ADAMTS13 variants, which have no values therapeutically.

Our findings could change the way we treat acquired TTP with inhibitors. To date, plasma exchange remains the main treatment for acquired TTP patients^{32,33}. Plasma exchange alone is found to be inadequate to restore deficiency of plasma ADAMTS13 activity and remove autoantibodies against ADAMTS13³³. Infused wild type ADAMTS13 may be rapidly neutralized by IgG autoantibodies, rendering patients persistent deficiency of plasma ADAMTS13 activity. Low plasma ADAMTS13 activity and persistence of anti-ADAMTS13 IgGs correlate with an increased rate of relapse^{11;33;34}. Other immunosuppressive therapies such as cyclosporine^{35;36}, cyclophosphamide^{37;38}, rituximab (anti-CD20 antibody)^{37;38} may reduce the antibody formation, but take weeks to months to have an effect. Therefore, autoantibody-resistant ADAMTS13 variants may have a value to instantaneously restore plasma ADAMTS13 activity when plasma exchange is not readily available or delayed. The infused ADAMTS13 variants are likely to work despite polyclonal nature of anti-ADAMTS13 IgGs. The affinity of anti-ADAMTS13 IgGs in patients with TTP toward various other domains besides spacer domain is relatively weak^{13-15;31} and the clearance of plasma ADAMTS13 as a result of binding by non-inhibitory IgGs does not appear to be the primary mechanism underlying severe

deficiency of plasma ADAMTS13 activity in patients with acquired TTP³⁹.

We conclude that subtle alterations of the exosite binding site in the spacer domain represent a viable strategy for engineering of ADAMTS13 variants with preserved or enhanced proteolytic activity but resistant to inhibition by anti-ADAMTS13 autoantibodies in patients with acquired idiopathic TTP. The gain-of-function variants provide a novel tool for understanding of the critical role of exosite interaction for proteolysis of VWF by ADAMTS13. Furthermore, the antibody-resistant variants underscore the importance of exosite 3 and adjacent residues in the spacer domain in pathogenesis of acquired TTP caused by autoantibodies against ADAMTS13. Finally, we hope that these variants with desired properties to be further developed for therapy of acquired TTP with inhibitors.

EXAMPLE II

Identification of a Novel Exosite (Glu⁶³⁴-Arg⁶³⁹) in the Spacer Domain of ADAMTS13 Required for Recognition of Von Willebrand Factor

As mentioned above, exosite binding plays a key role in cleavage of VWF by ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin type 1 repeats, 13). Two exosites that are evolutionarily conserved from zebra fish to mammals have been identified in the spacer domain by sequence alignment. Example I provides data showing that exosite 3 in the spacer domain plays a critical role for substrate recognition (*Blood* 115:2300-10, 2010), and modification of this exosite generates ADAMTS13 variants with improved specific activity but reduced autoantibody binding (*Blood* 119:3836-43, 2012).

In the present study, using a site-directed mutagenesis approach, we identified a novel exosite near exosite 3 in the

spacer domain, termed exosite 4, a region between residues Glu⁶³⁴ and Arg⁶³⁹. See Figure 8. A partial (DEx4a:deletion of Leu632-Asp635 or DEx4b:deletion of Arg636-Arg639) or complete deletion of the exosite (DEx4) significantly impaired

5 proteolytic activity towards peptidyl VWF73 and multimeric VWF. See Figures 9, and 10. Moreover, substitution of all surface exposed residues in Ex4A (LTED/AAAA) or Ex4b (RLPR/AAAA) with alanine had a similarly detrimental effect on proteolytic activity. However, a deletion or mutation of

10 ⁶³LTED⁶³⁵ to alanines caused intracellular retention of the enzyme. Further studies using deuterium-hydrogen exchange and mass spectrometry demonstrated that the residues (D632-E642) were targeted by a human monoclonal antibody (scFV4-20) isolated by phage display from an acquired TTP patient. See

15 Figures 11 and 12.

Figure 13 shows inhibition of murine ADAMTS13 activity and alteration of VWF multimers by scFv 4-20. Figure 14 shows that 4-20 mAb causes acquired TTP triggered by bacterial shigatoxin. Figure 15 is a graph showing survival rates in

20 mice expressing control and inhibitory scFV after being challenged with shiga toxin.

We conclude that the region between residues Leu632 and Glu642 is a novel exosite necessary for recognition and

25 cleavage of VWF and appears to be important for autoantibody binding in the case of TTP. Further studies demonstrated that the residues Asp635 and Arg636 in exosite 4 play a critical role for substrate recognition. We conclude that the region between residues Glu634 and Arg639 is a novel exosite

30 necessary for recognition and cleavage of VWF.

Reference List

1. Dong JF, Moake JL, Nolasco L et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood* 2002;100:4033-9.
- 5 2. Tsai HM. Shear stress and von Willebrand factor in health and disease. *Semin Thromb Hemost* 2003;29:479-88.
3. Vincentelli A, Susen s, Le Tourneau T et al. Acquired von Willebrand syndrome in aortic stenosis. *N Engl J Med* 2003;349:343-9.
- 10 4. Banno F, Chauhan AK, Kokame K et al. The distal carboxyl-terminal domains of ADAMTS13 are required for regulation of in vivo thrombus formation. *Blood* 2009;113:5323-5329.
5. Chauhan AK, Motto DG, Lamb CB et al. Systemic antithrombotic effects of ADAMTS13. *J Exp Med* 15 2006;203:767-76.
6. Xiao J, Jin SY, Xue J et al. Essential Domains of ADAMTS13 Metalloprotease Required for Modulation of Arterial Thrombosis. *Arterioscler .Thromb .Vase .Biol .* 2011 ;31 :2261-2269.
- 20 7. Tsai HM. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood* 1996;87:4235-44.
8. Dong JF, Moake JL, Bernardo A et al. ADAMTS-13 metalloprotease interacts with the endothelial cell-derived ultra-large von Willebrand factor. *J Biol Chem* 25 2003;278:29633-9.
9. Furlan M, Robles R, Lammle B. Partial purification and characterization of a protease from human plasma cleaving

von Willebrand factor to fragments produced by in vivo proteolysis. Blood 1996;87:4223-34.

10. Levy GG, Nichols WC, Lian EC et al. Mutations in a member of the ADAMTS gene family cause thrombotic
5 thrombocytopenic purpura. Nature 2001;413:488-94.

11. Tsai HM, Lian EC. Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. N Engl J Med 1998;339:1585-94.

12. Tsai HM, Raoufi M, Zhou W et al. ADAMTS13-binding IgG are
10 present in patients with thrombotic thrombocytopenic purpura. Thromb Haemost 2006;95:886-92.

13. Zheng XL, Wu HM, Shang D et al. Multiple domains of ADAMTS 13 are targeted by autoantibodies against ADAMTS 13 in patients with acquired idiopathic thrombotic
15 thrombocytopenic purpura. Haematologica 2010;95:1555-1562.

14. Luken BM, Turenhout EA, Hulstein JJ et al. The spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with thrombotic thrombocytopenic purpura. Thromb Haemost 2005;93:267-74.

20 15. Luken BM, Kaijen PH, Turenhout EA et al. Multiple B-cell clones producing antibodies directed to the spacer and disintegrin/thrombospondin type-1 repeat 1 (TSP1) of ADAMTS13 in a patient with acquired thrombotic thrombocytopenic purpura. J Thromb Haemost 2006; 4 :2355-
25 2364.

16. Luken BM, Turenhout EA, Kaijen PH et al. Amino acid regions 572-579 and 657-666 of the spacer domain of ADAMTS13 provide a common antigenic core required for binding of antibodies in patients with acquired TTP.
30 Thromb Haemost 2006;96:295-301.

17. Soejima K, Matsumoto M, Kokame K et al. ADAMTS-13 cysteine-rich/spacer domains are functionally essential for von Willebrand factor cleavage. *Blood* 2003;102:3232-7.
18. Pos W, Crawley JT, Fijnheer R et al. An autoantibody epitope comprising residues R660, Y661, and Y665 in the ADAMTS13 spacer domain identifies a binding site for the A2 domain of VWF. *Blood* 2010;115:1640-1649.
19. Pos W, Sorvillo N, Fijnheer R et al. Residues Arg568 and Phe592 contribute to an antigenic surface for anti-ADAMTS13 antibodies in the spacer domain. *Haematologica* 2011DOI : 10.3324/haematol.2010.036327 .
20. Ai J, Smith P, Wang S, Zhang P, Zheng XL. The proximal carboxyl-terminal domains of ADAMTS13 determine substrate specificity and are all required for cleavage of von Willebrand factor. *J Biol Chem* 2005;280:29428-34.
21. Gao W, Anderson PJ, Majerus EM, Tuley EA, Sadler JE . Exosite interactions contribute to tension-induced cleavage of von Willebrand factor by the antithrombotic ADAMTS13 metalloprotease . *Proc Natl Acad Sci U S A* 2006;103:19099-04.
22. Gao W, Anderson PJ, Sadler JE . Extensive contacts between ADAMTS13 exosites and von Willebrand factor domain A2 contribute to substrate specificity. *Blood* 2008; 112 :1713-1719 .
23. Zheng XL, Nishio K, Majerus EM, Sadler JE . Cleavage of von Willebrand factor requires the spacer domain of the metalloprotease ADAMTS13. *J Biol Chem* 2003;278:30136-41.
24. Zhou W, Dong L, Ginsburg D, Bouhassira EE, Tsai HM. Enzymatically active ADAMTS13 variants are not inhibited

by anti-ADAMTS13 autoantibodies: a novel therapeutic strategy? J Biol Chem 2005;280:39934-39941.

25. Raife TJ, Cao w, Atkinson BS et al. Leukocyte proteases cleave von Willebrand factor at or near the ADAMTS13 cleavage site. Blood 2009;114:1666-74.
26. Zhang L, Lawson HL, Harish VC et al. Creation of a recombinant peptide substrate for fluorescence resonance energy transfer-based protease assays. Anal.Biochem . 2006;358:298-300.
27. Cao WJ, Krishnaswamy s, Camire RM, Lenting PJ, Zheng XL. Factor VIII accelerates proteolytic cleavage of von Willebrand factor by ADAMTS13. Proc Natl Acad Sci U S A 2008;105:7416-21.
28. Jin SY, Skipwith CG, Zheng XL. Amino acid residues Arg(659), Arg(660), and Tyr(661) in the spacer domain of ADAMTS13 are critical for cleavage of von Willebrand factor. Blood 2010;115:2300-2310.
29. Akiyama M, Takeda s, Kokame K, Takagi J, Miyata T. Crystal structures of the noncatalytic domains of ADAMTS13 reveal multiple discontinuous exosites for von Willebrand factor. Proc Natl Acad Sci U S A 2009;106:19274-19279.
30. Zhang Q, Zhou YF, Zhang CZ et al. Structural specializations of A2, a force-sensing domain in the ultralarge vascular protein von Willebrand factor. Proc.Natl.Acad.Sci.U.S.A 2009;106:9226-9231.
31. Klaus C, Plaimauer B, Studt JD et al. Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura. Blood 2004;103:4514-9.

32. George JN. How I treat patients with thrombotic thrombocytopenic purpura: 2010. *Blood* 2010;116:4060-4069.
33. Zheng XL, Richard KM, Goodnough LT, Sadler JE. Effect of plasma exchange on plasma ADAMTS13 metalloprotease activity, inhibitor level, and clinical outcome in patients with idiopathic and non-idiopathic thrombotic thrombocytopenic purpura. *Blood* 2004;103:4043-9.
34. Zheng XL, Sadler JE. Pathogenesis of Thrombotic Microangiopathies. *Annu .Rev .Path .Mech .Dis .* 2008;3:249-277.
35. Cataland SR, Jin M, Zheng XL, George JN, Wu HM. An evaluation of cyclosporine alone for the treatment of early recurrences of thrombotic thrombocytopenic purpura. *J Thromb Haemost* 2006;4:1162-4.
36. Cataland SR, Jin M, Lin S et al. Cyclosporin and plasma exchange in thrombotic thrombocytopenic purpura: long-term follow-up with serial analysis of ADAMTS13 activity. *Br J Haematol* 2007;139:486-93.
37. Fakhouri F, Teixeira L, Delarue R, Grunfeld JP, Veyradier A. Responsiveness of thrombotic thrombocytopenic purpura to rituximab and cyclophosphamide. *Ann Intern Med* 2004; 140 :314-5 .
38. Zheng XL, Pallera AM, Goodnough LT, Sadler JE, Blinder MA. Remission of chronic thrombotic thrombocytopenic purpura after treatment with cyclophosphamide and rituximab. *Ann .Intern .Med .* 2003;138:105-108.
39. Shelat SG, Smith AG, Ai J, Zheng X.L. Inhibitory autoantibodies against ADAMTS-13 in patients with thrombotic thrombocytopenic purpura bind ADAMTS-13 protease and may accelerate its clearance in vivo. *J Thromb Haemost* 2006;4:1707-17.

While the invention has been described in detail and with reference to specific examples thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and

5 scope thereof.

What is claimed is :

1. An isolated gain of function ADAMTS13 variant comprising at least one amino acid change in the spacer domain
5 of ADAMTS13, said variant retaining proteolytic activity and exhibiting resistance to autoantibody inhibition, with the provision that the amino acid change is not an alanine substitution .

2. The isolated ADAMTS13 variant of claim 1, selected
10 from the group consisting of the M1 variant, the M2 variant, the M3 variant, the M4 variant and the M5 variant.

3. The isolated ADAMTS13 variant of claim 2 which is the M4 variant.

4. The isolated ADAMTS13 variant of claim 2 which is the
15 M5 variant.

5. A pharmaceutical composition comprising the isolated ADAMTS13 variant of claim 1, in a biologically acceptable carrier .

6. A method for the treatment of TPP comprising
20 administration of an effective amount of the ADAMTS13 variants of claim 1 in a patient need thereof, said ADAMTS13 variants inhibiting thrombus formation in said patient.

7. The method of claim 6, wherein said variant exhibits increased proteolytic activity when compared to wild type
25 ADAMTS13 and is selected from the group of variants consisting of M4 and M5 .

8. The method of claim 6, wherein said ADAMTS13 variant is resistant to autoantibody inhibition.

9. The method of claim 6, wherein said effective amount of said ADAMTS13 variant is infused into a patient.

10. The method of claim 6, wherein said variant is delivered to said patient in an AAV vector comprising a
5 nucleic acid sequence encoding said variant.

11. An ADAMTS13 variant comprising an alteration in exosite 4 between amino acid residues Glu⁶³⁴ and Arg⁶³⁹, said variant exhibiting reduced proteolytic activity relative to an ADAMTS13 molecule lacking said alteration.

10 12. The ADAMTS13 variant of claim 11, comprising a partial deletion of exosite 4.

13. The ADAMTS13 variant of claim 11, comprising a complete deletion of exosite 4.

15 14. The ADAMTS13 variant of claim 11, wherein all surface exposed residues in exosite 4 are replaced with alanine .

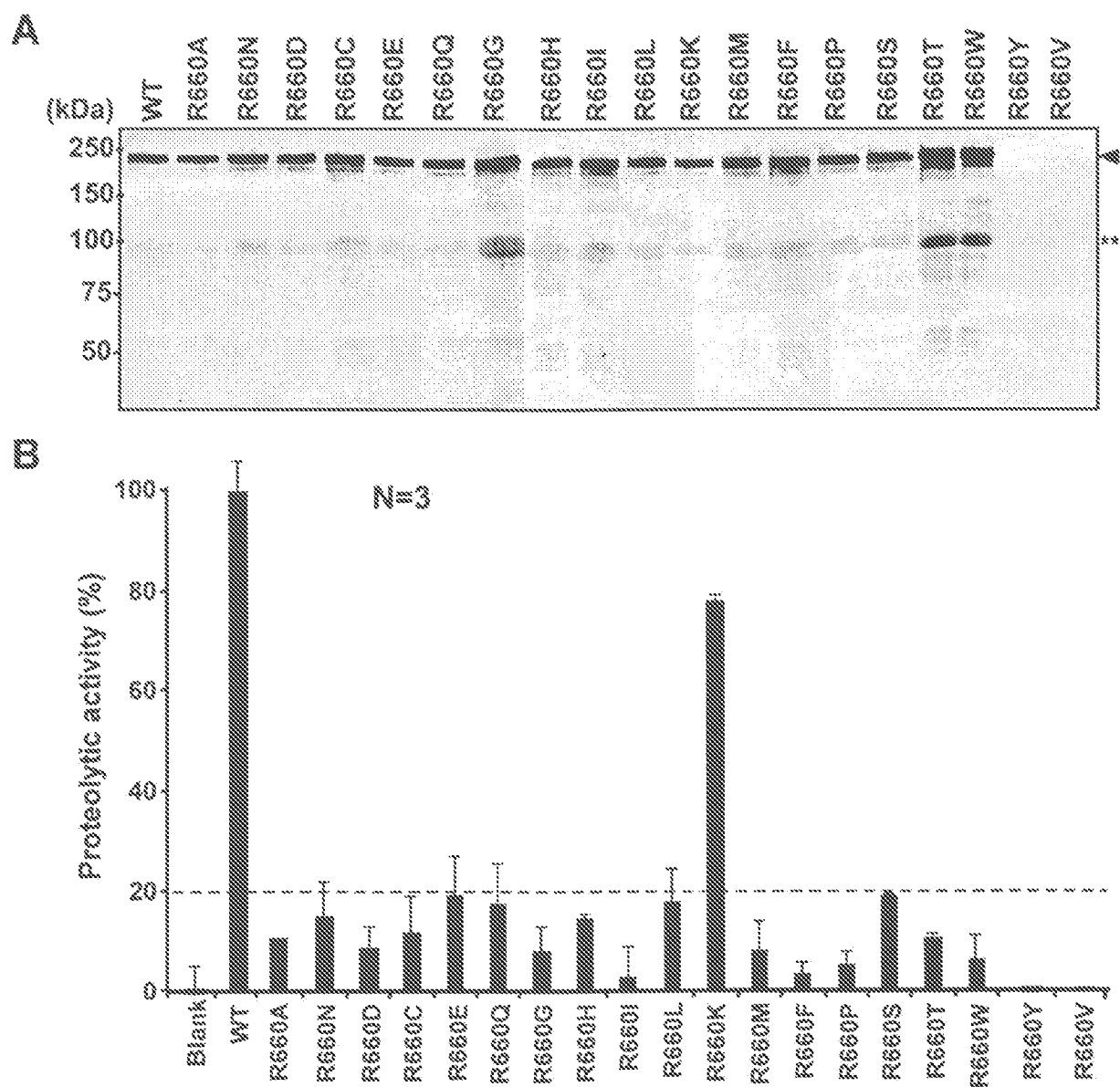


Figure 1

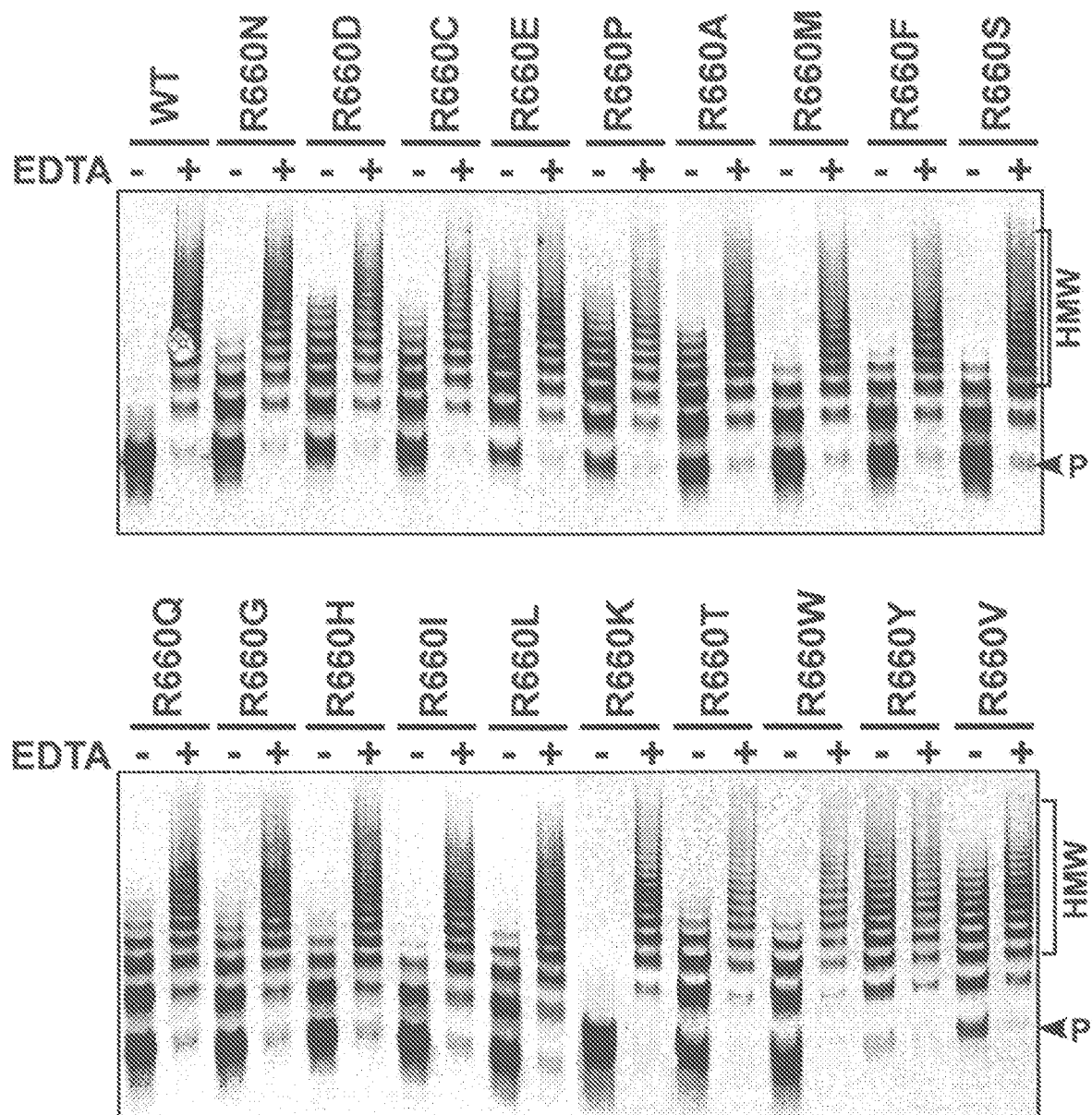


Figure 1C

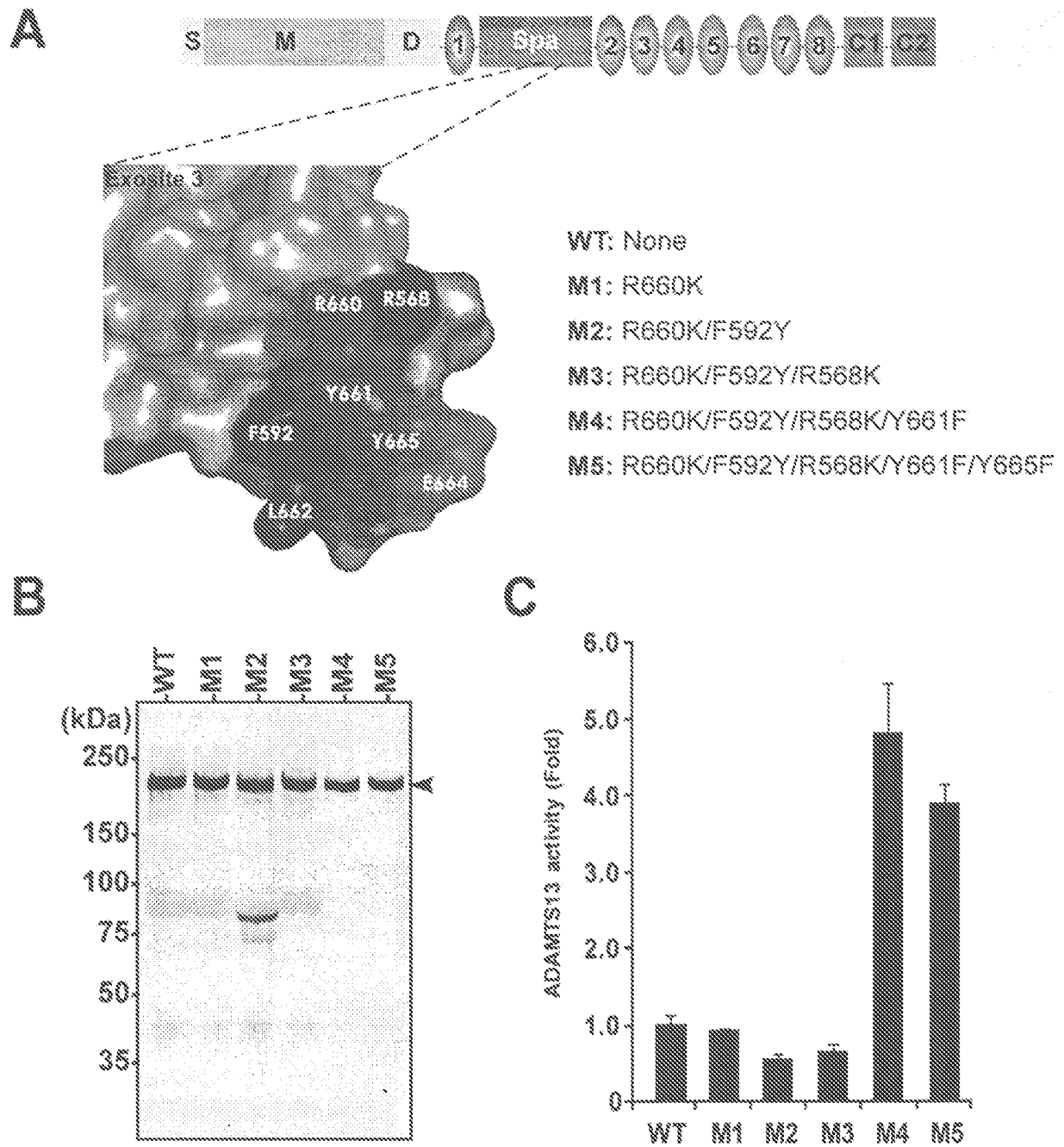


Figure 2

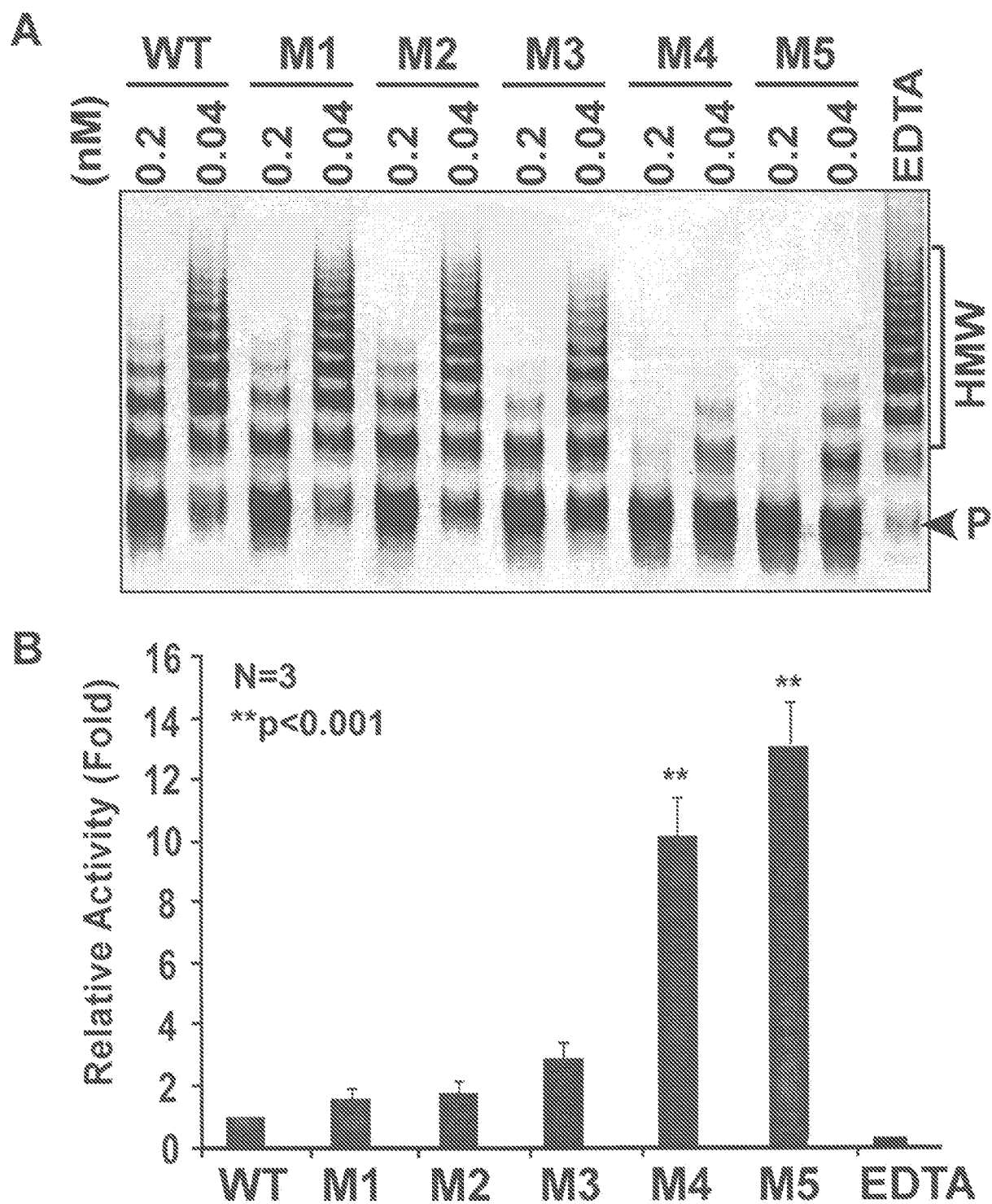


Figure 3

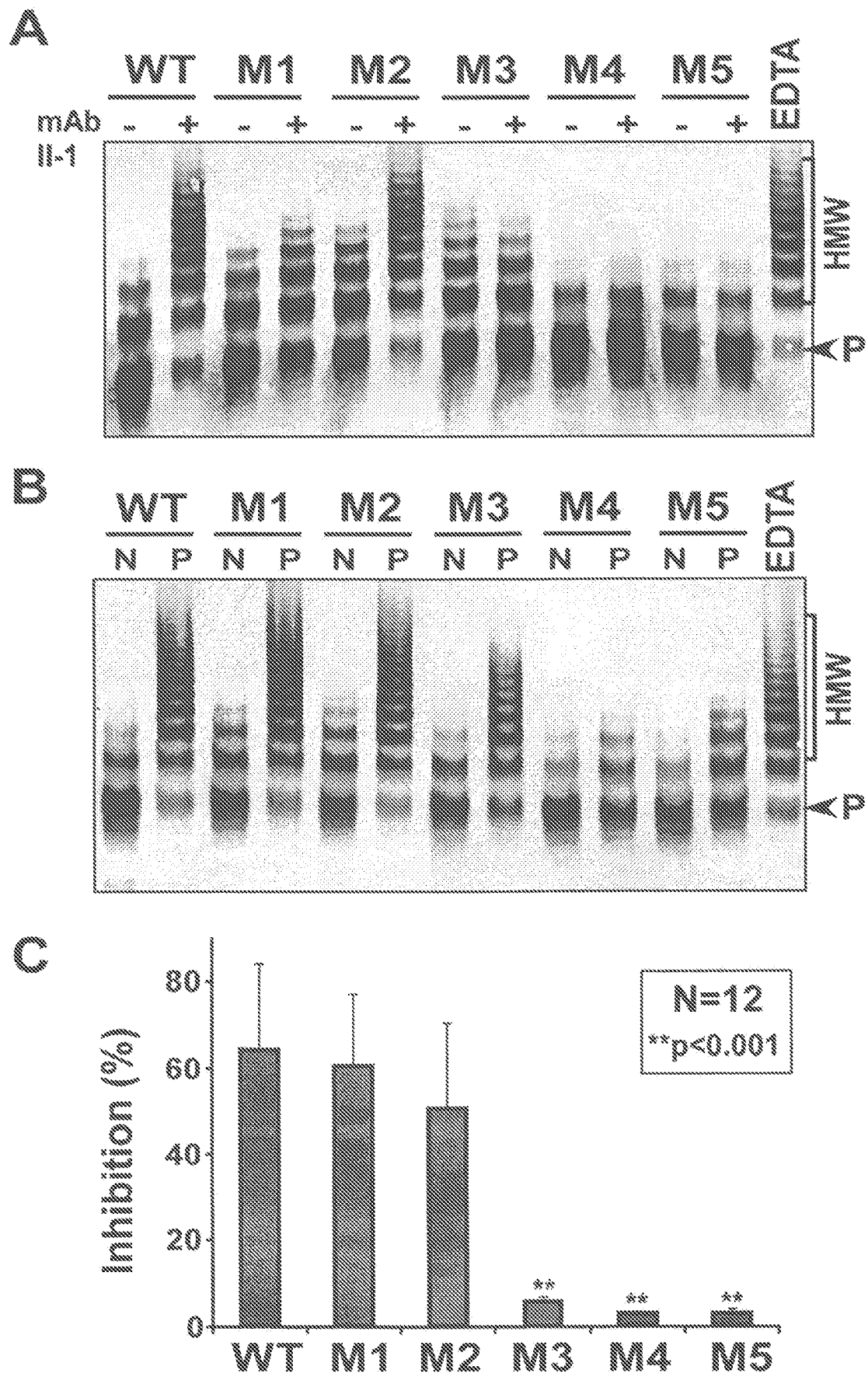


Figure 4

6/19

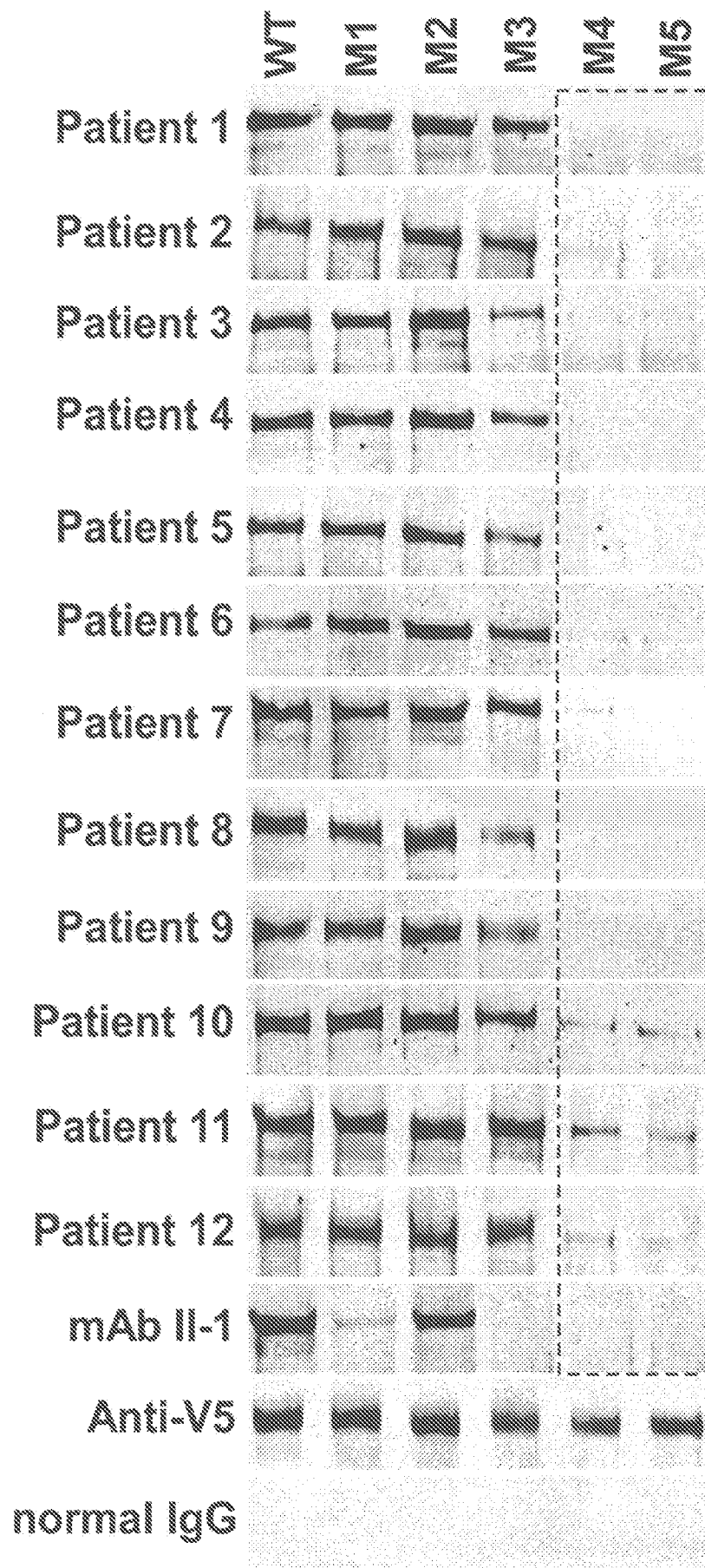


Figure 5

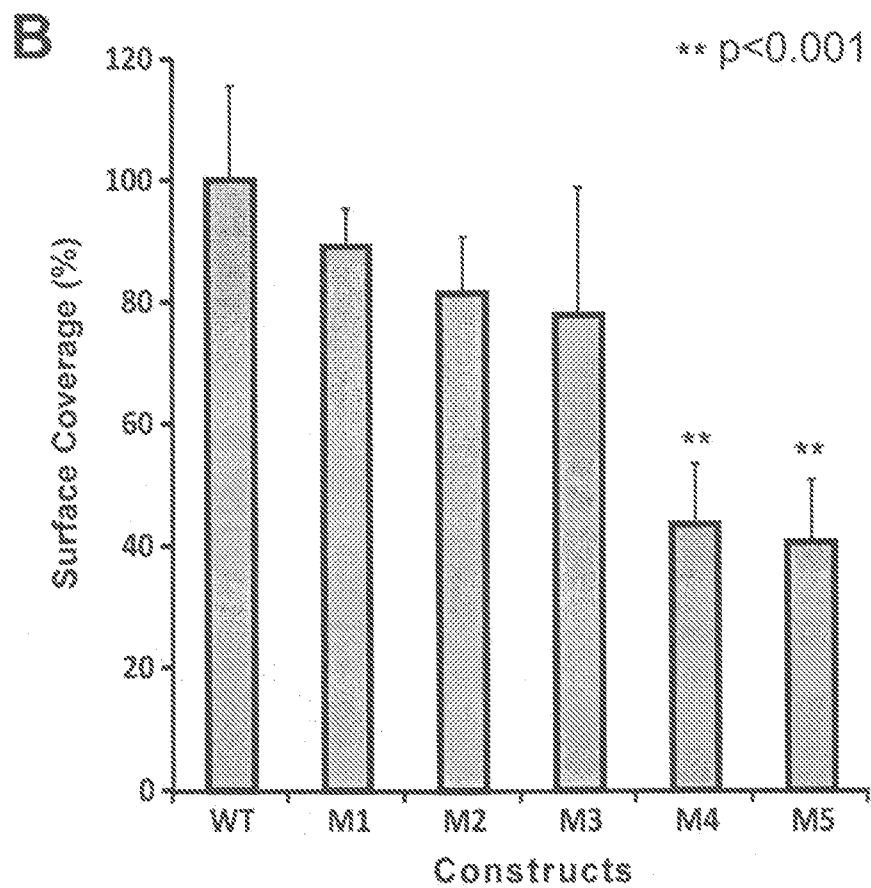
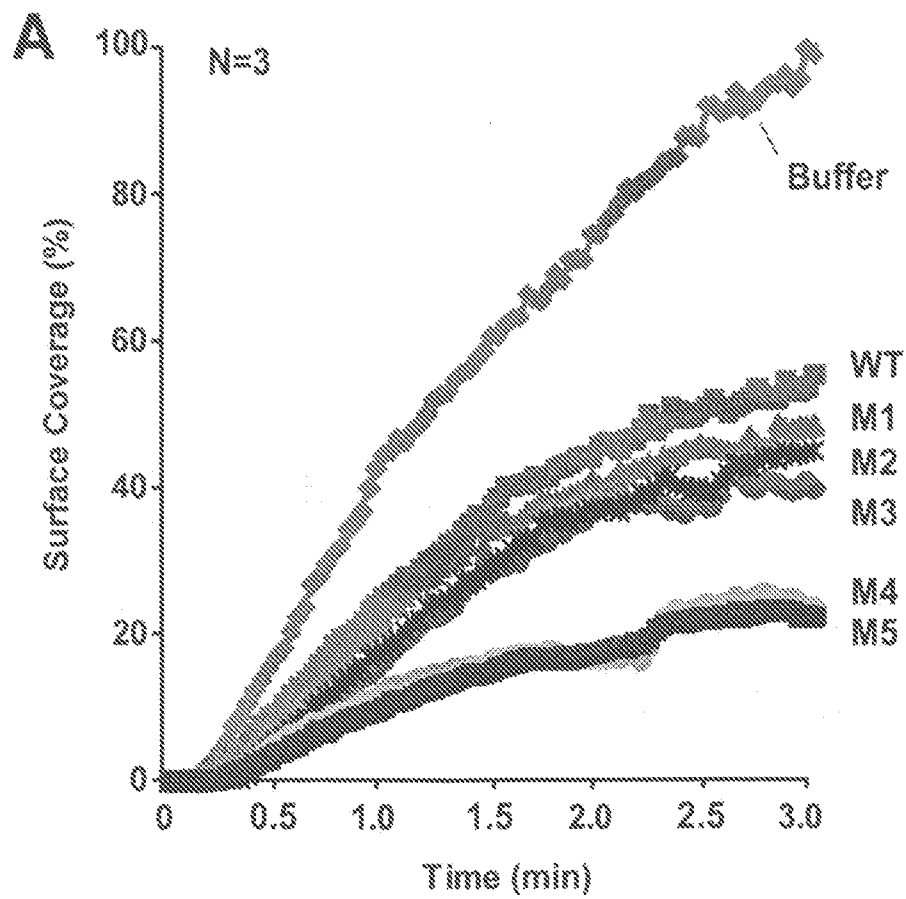


Figure 6

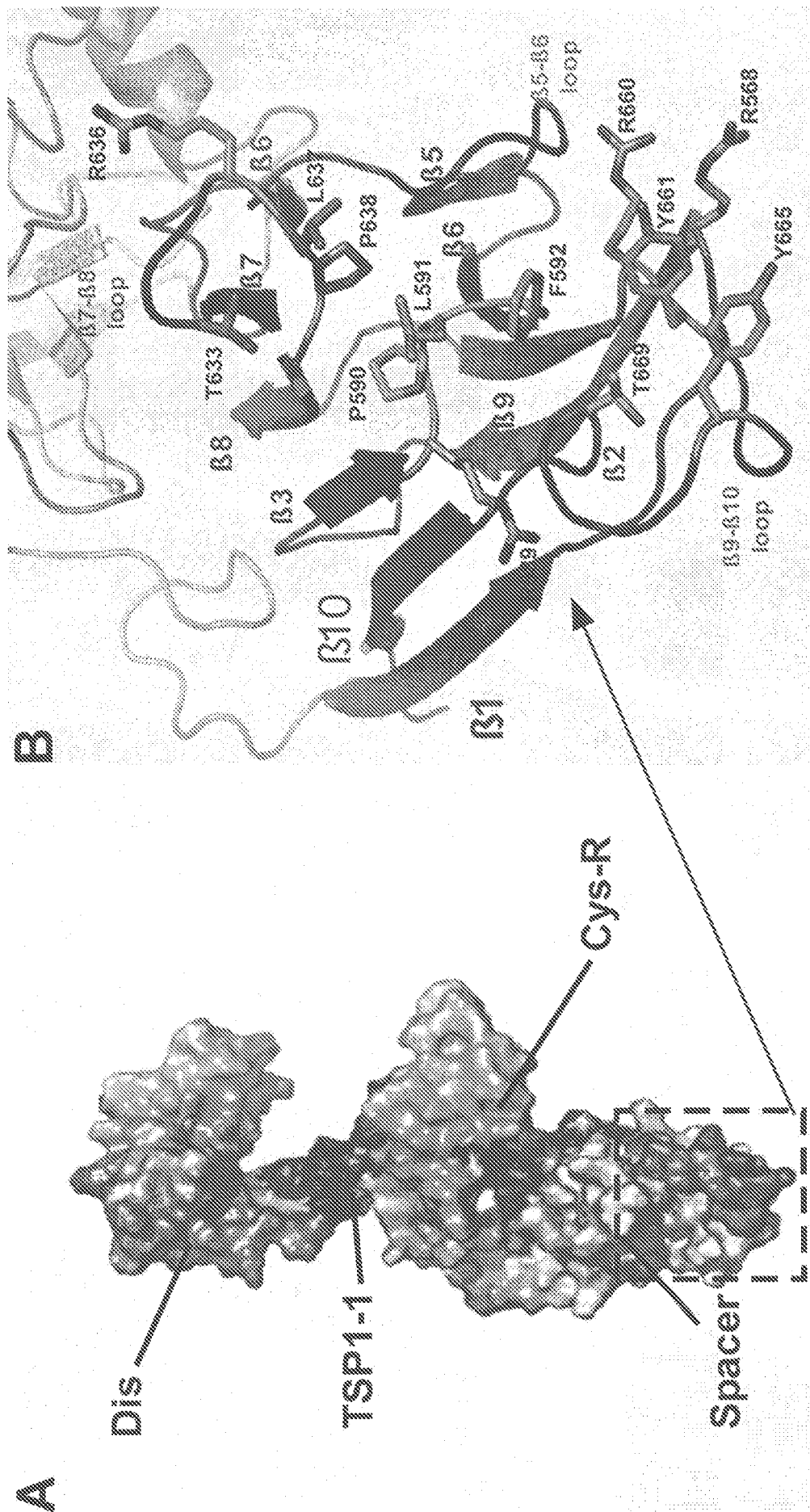


Figure 7

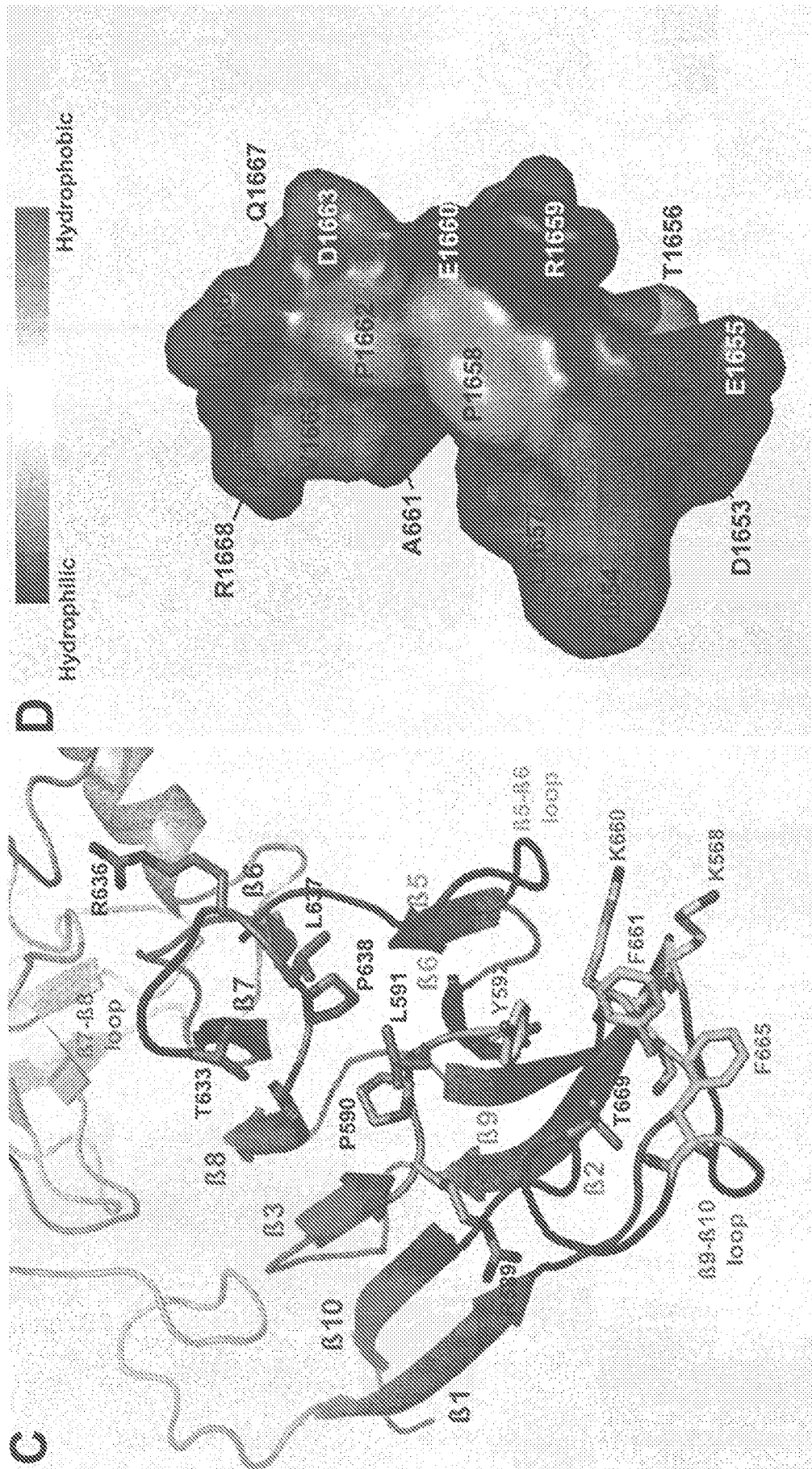


Figure 7

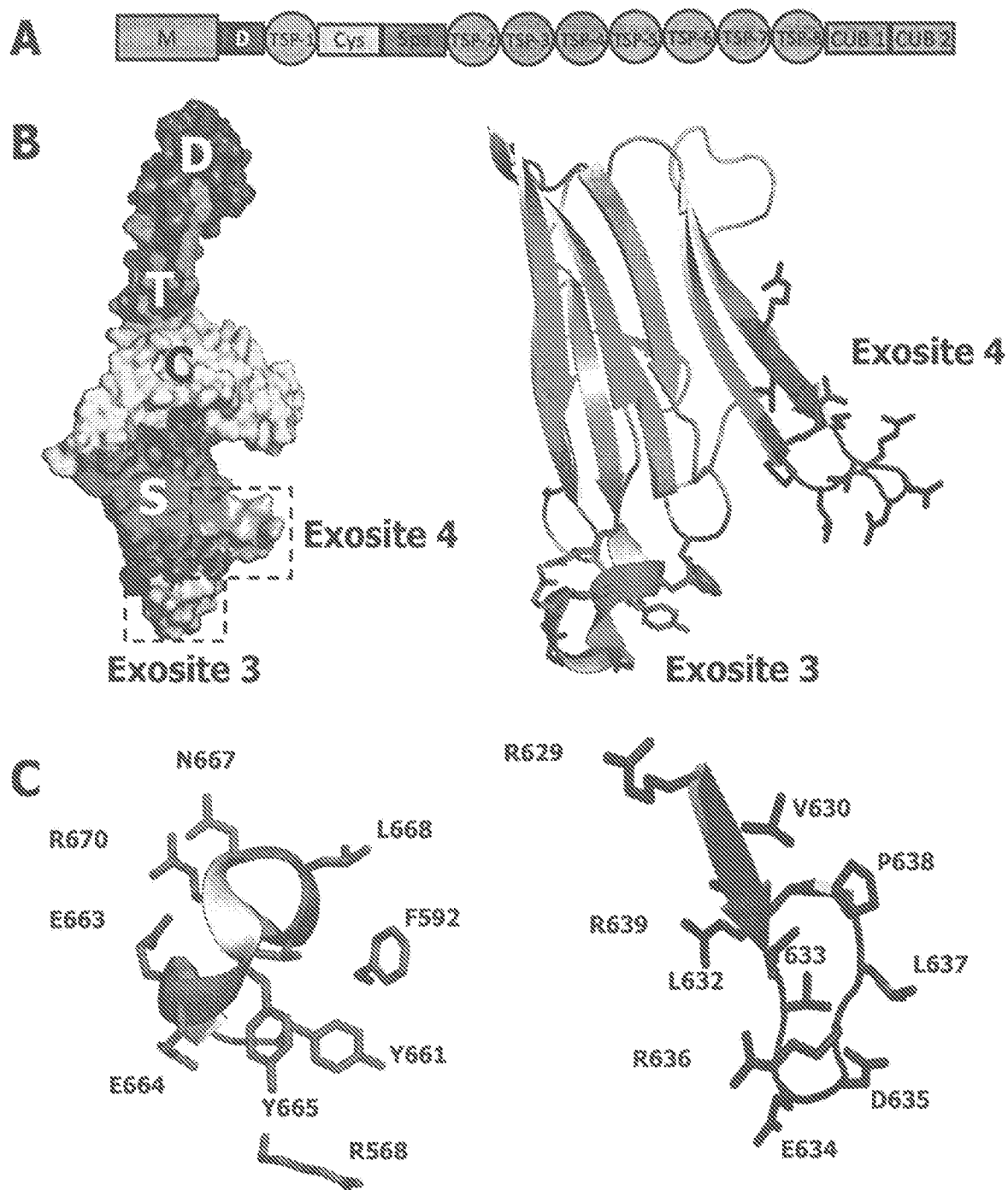


Figure 8

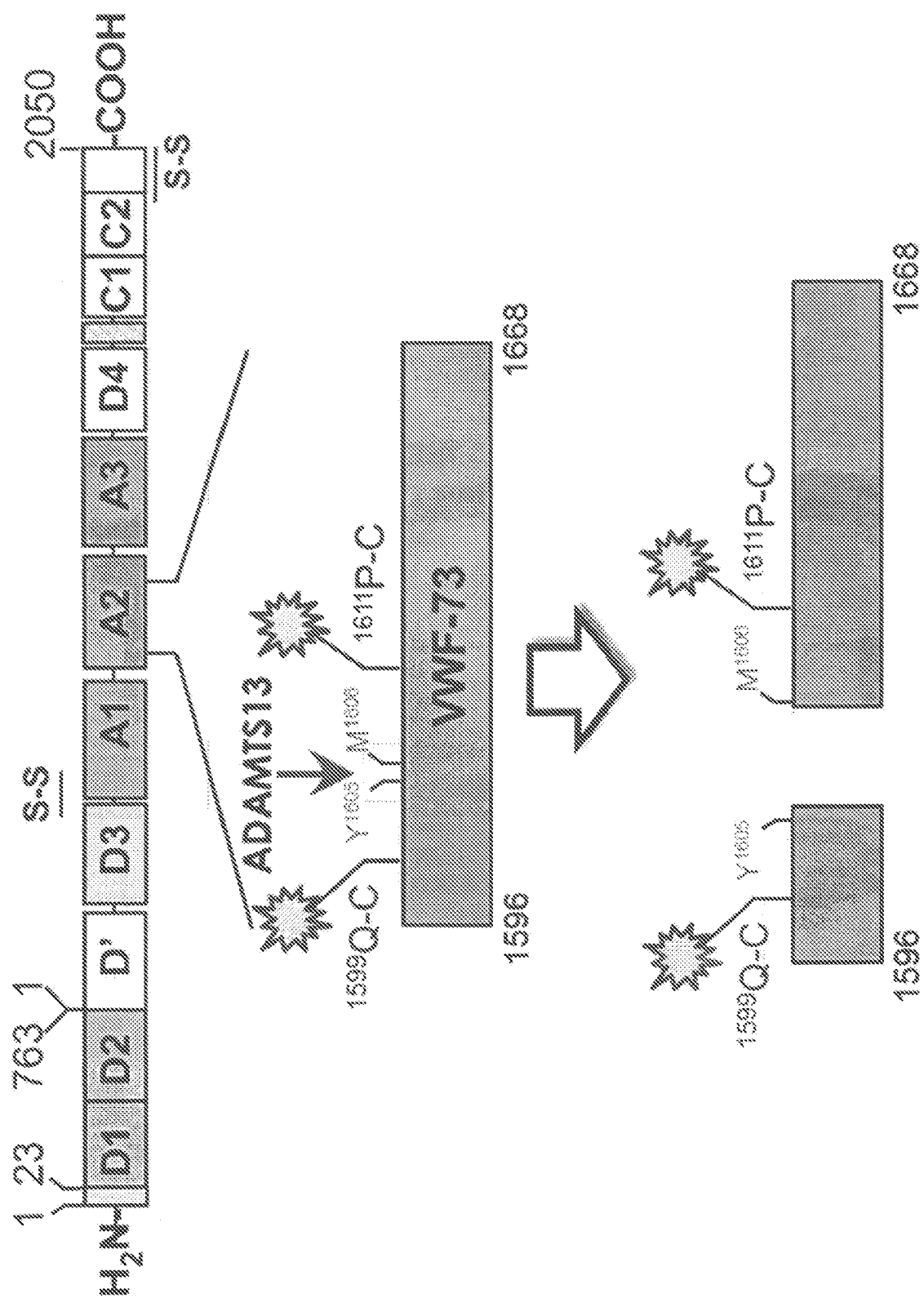


Figure 9A

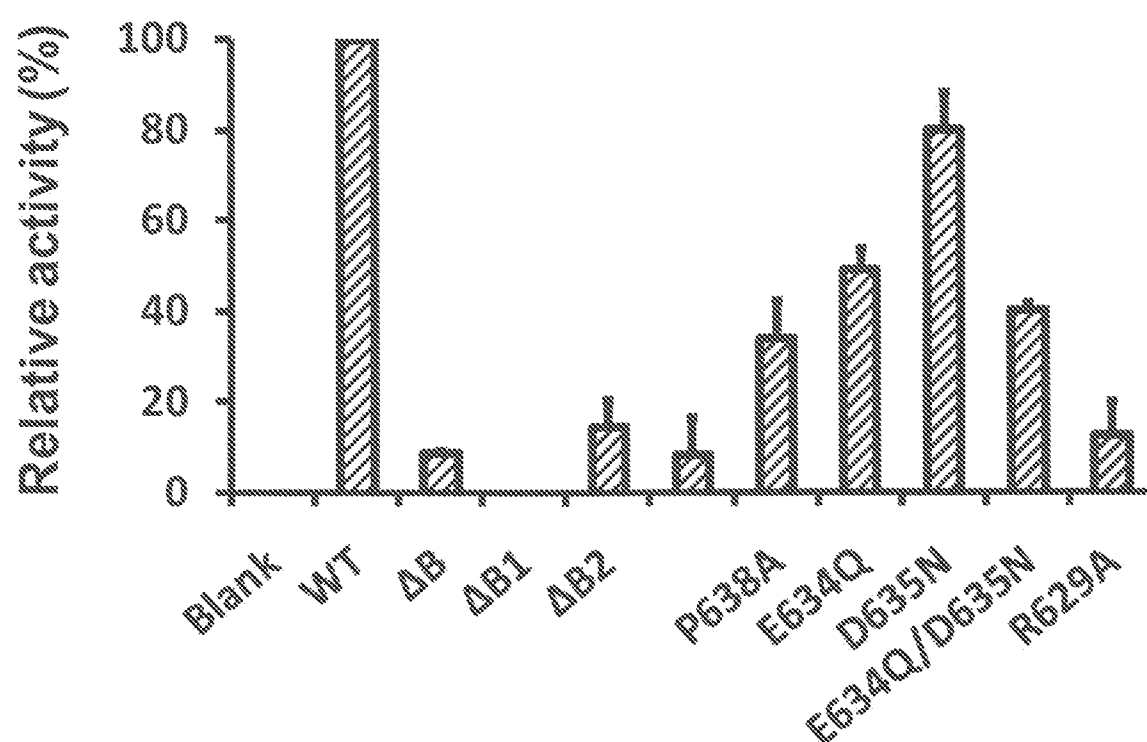


Figure 9B

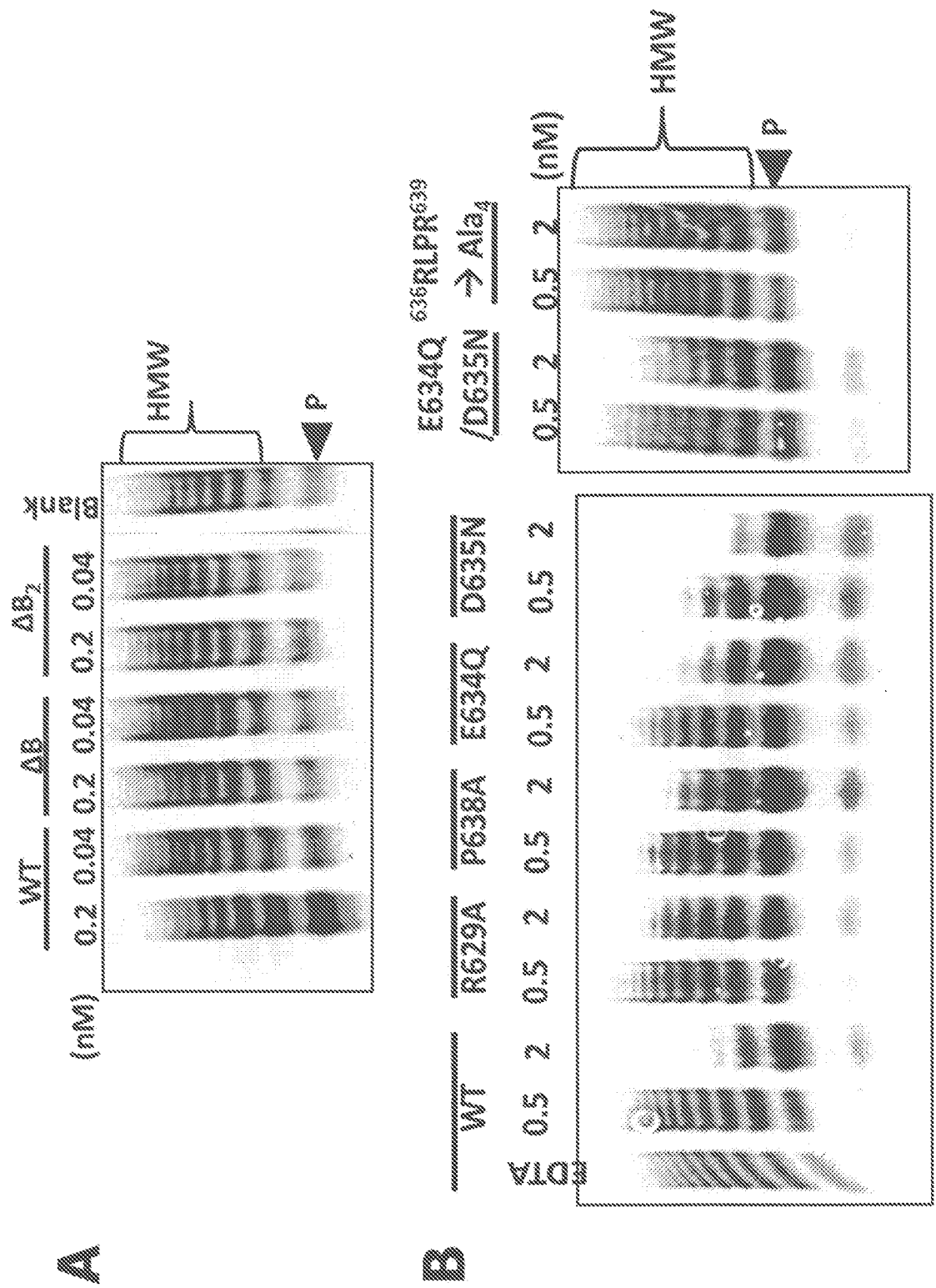


Figure 10

Residues	Domain	# unique peptides	Hydrogens exchanged with scFV 4-20: w/o scFV 4-20
28-228	M	25	1.3
229-428	M/D/T	7	1.4
429-628	T/C/S	10	0.9
629-642	S	6	0.4†
643-685	S	10	1.0

Figure 11A

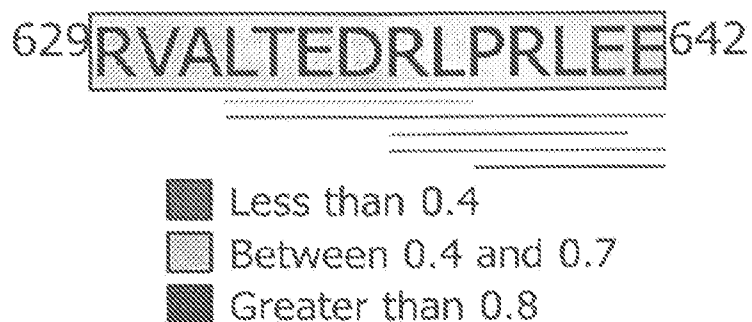


Figure 11B

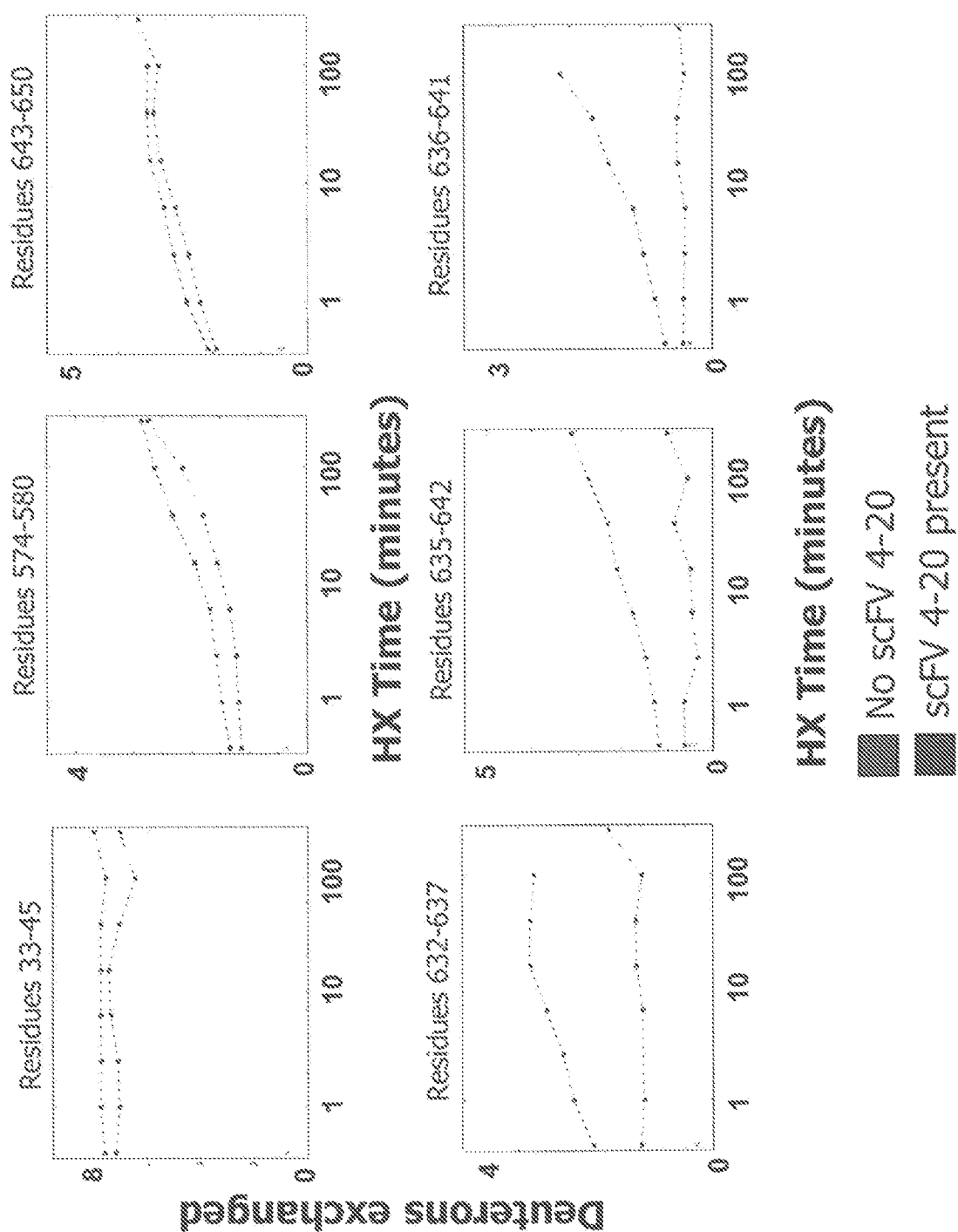


Figure 11C

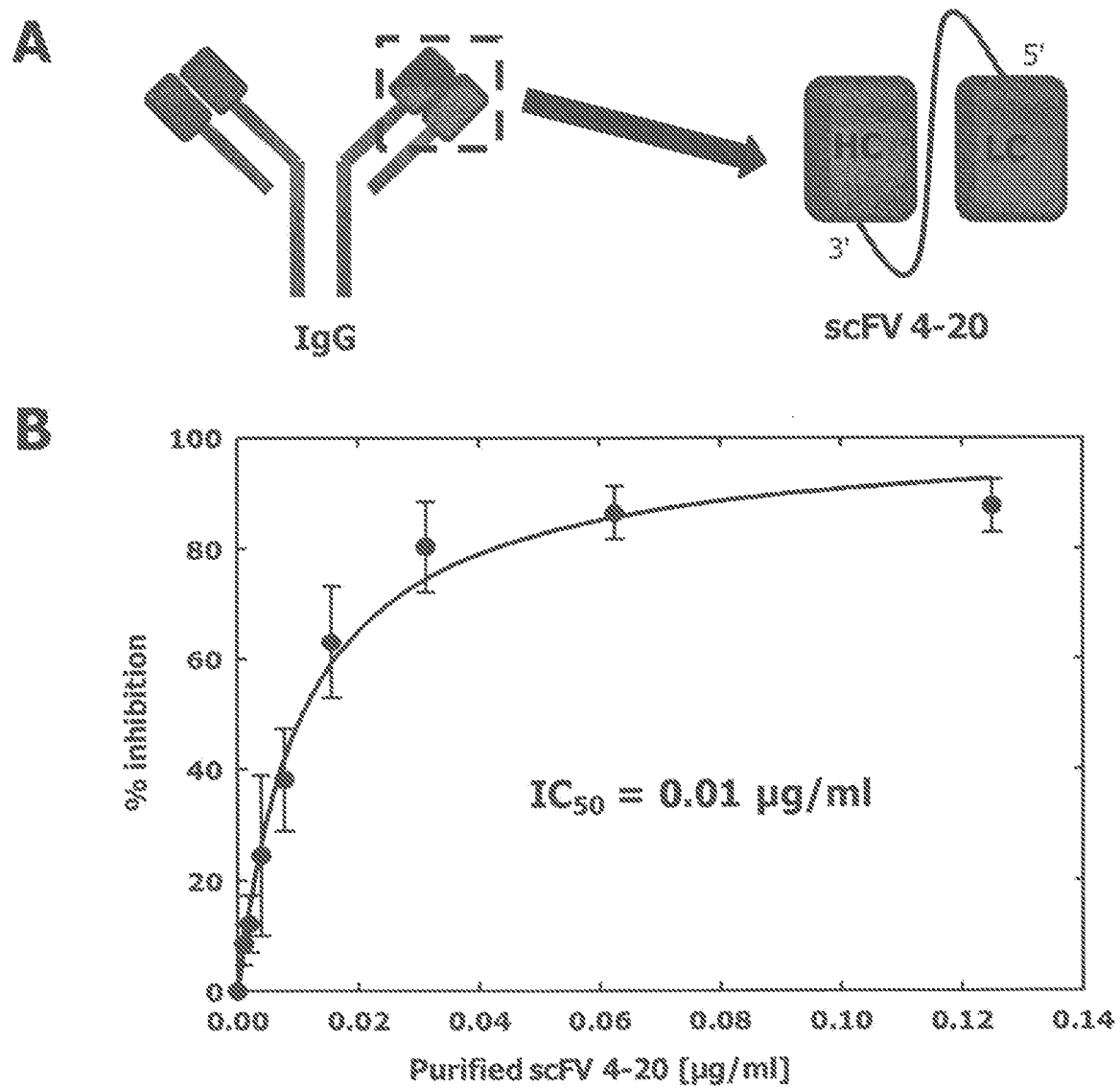


Figure 12

Inhibition of murine ADAMTS13 activity and alternation of VWF multimers by scFV 4-20

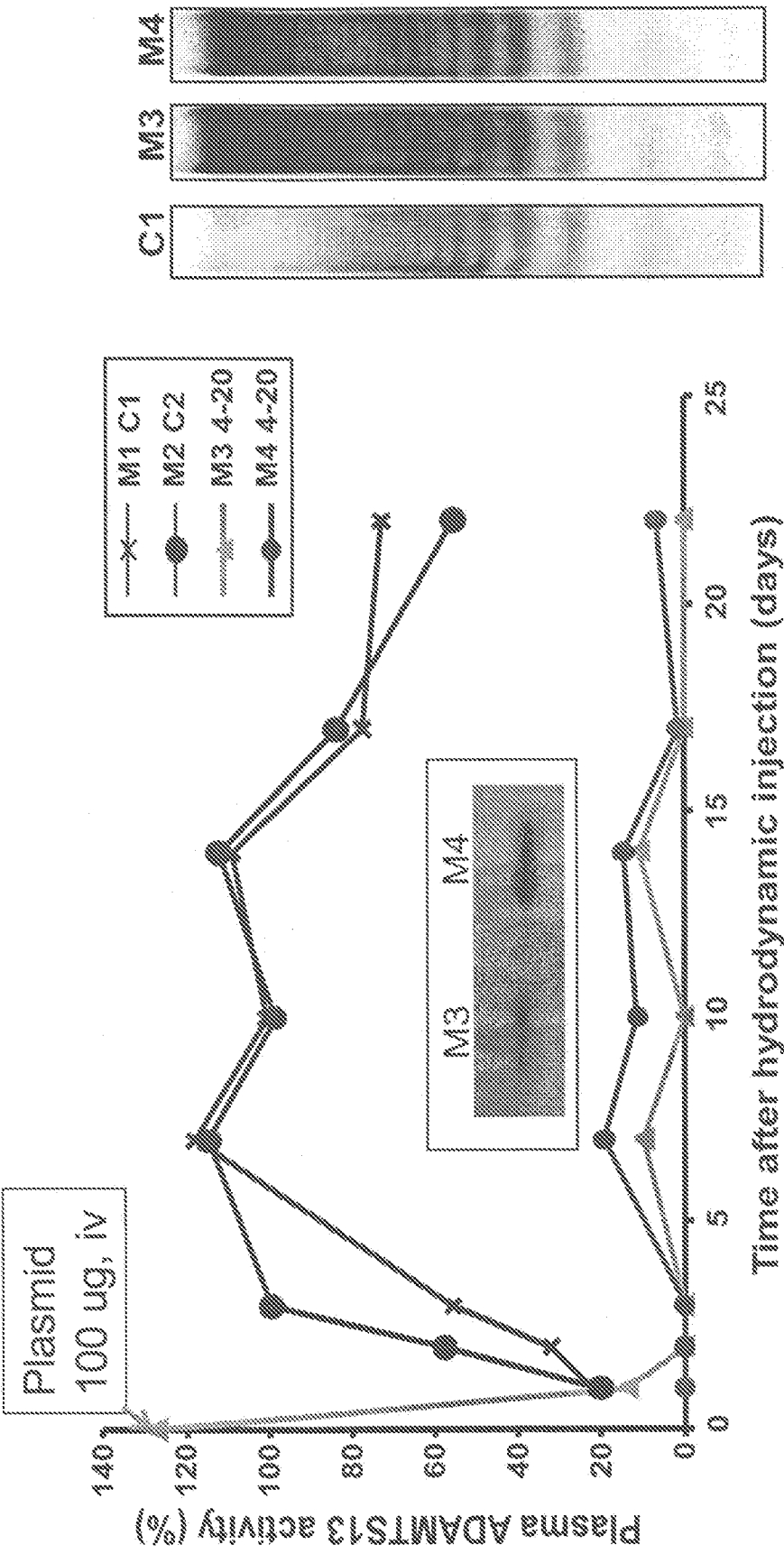


Figure 13

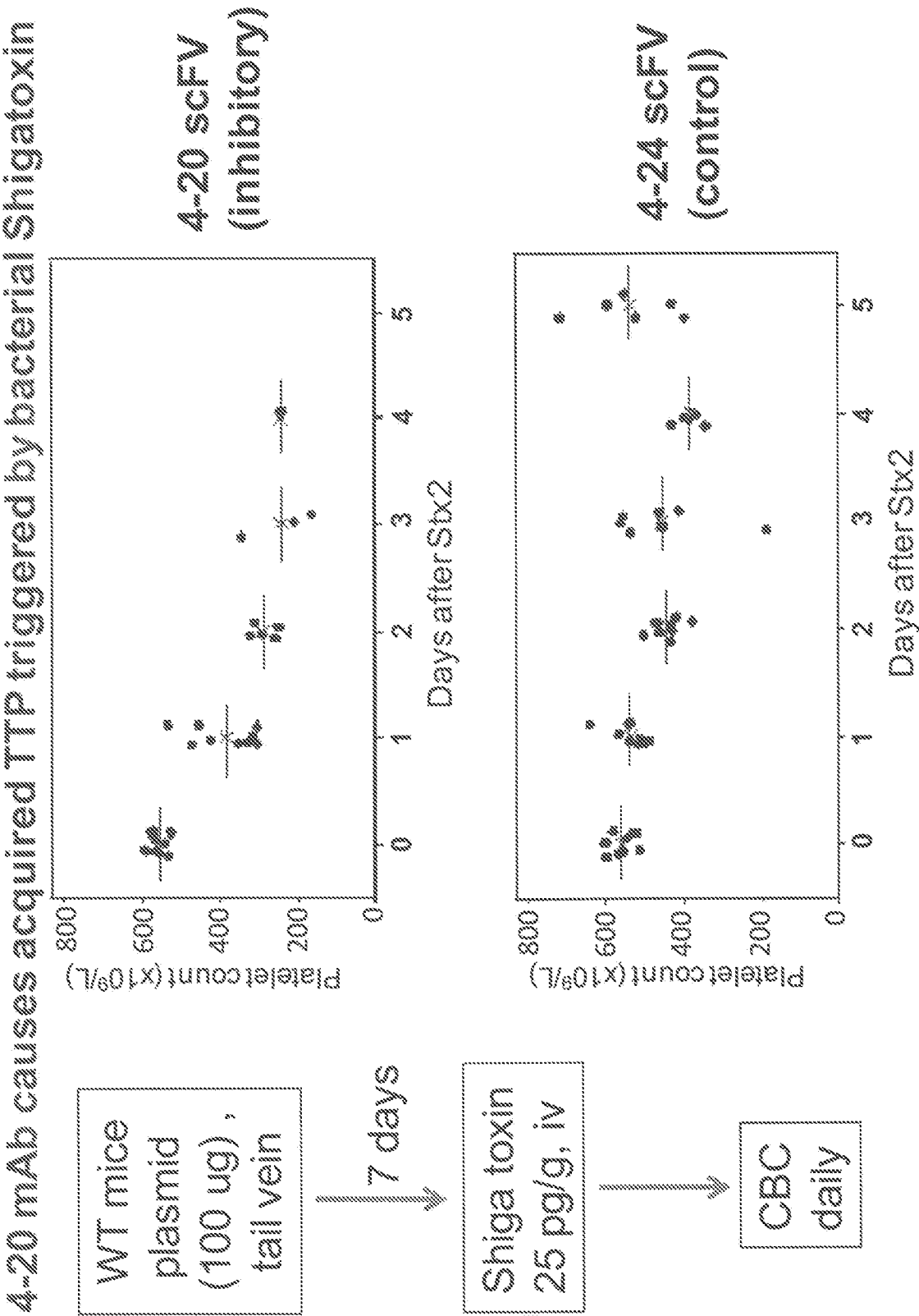


Figure 14

Survival rates in mice expressing control and inhibitory scFV after being challenged with Shigatoxin

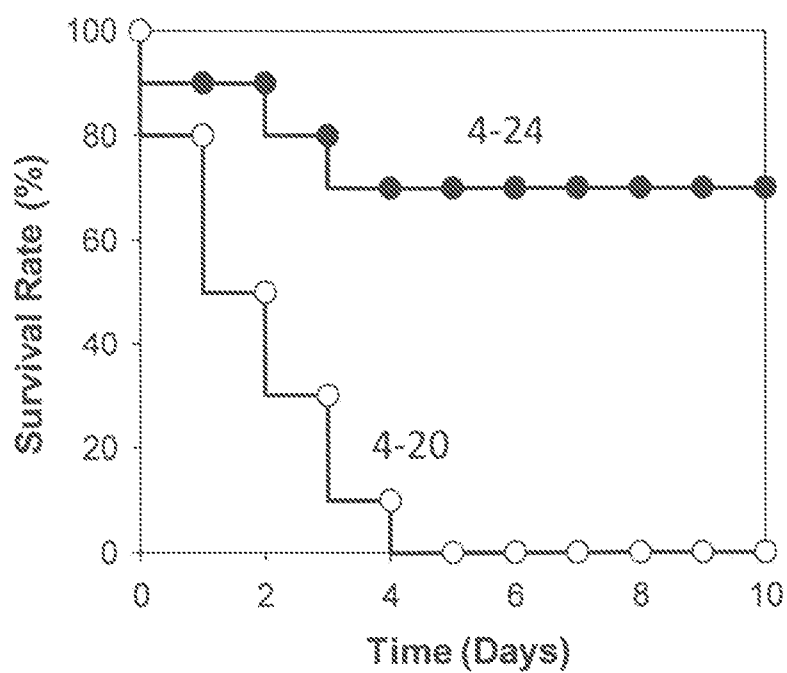


Figure 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/71289

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61 K 38/36; A61 K 38/00 (2013.01)**USPC - 514/13.7; 514/15.3**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 38/36; A61K 38/00

USPC - 514/13.7; 514/15.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
514/21.2; 514/14.9; 435/440; 435/320.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST; Google Scholar; PatBase

search terms: ADAMTS13, Disintegrin and metalloprotease with thrombospondin type 1, 13, ADAM metallopeptidase with thrombospondin type 1, C9orf8, vWF-CP, vWFCP, ADAM-TS13, ADAMTS-13, ADAM-TS 13, von Willebrand factor-cleaving protease,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	WO 2009/001743 A1 (SOEJIMA et al.) 31 December 2008 (31.12.2008) (The english translation of the WO 2009/001743 A1 description used for the opinion is from Espacenet, and can be found online at: < http://worldwide.espacenet.com/publicationDetails/description?CC=WO&NR=2009001743A1&KC=A1&FT=D&ND=3&date=20081231&DB=worldwide.espacenet.com&locale=en_EP >). para [0005]; [0011]; [0013]; [0016]; [0019]; [0026]; [0028]; [0040]-[0042].	1, 5, 6, 8, 9, 11, 14 ----- 7, 10
X	Ai et al., The Proximal Carboxyl-terminal Domains of ADAMTS13 Determine Substrate Specificity and Are All Required for Cleavage of von Willebrand Factor. The Journal of Biological Chemistry, 280, 29428-29434, August 19, 2005. abstract; Figs. 1 and 4; Table 1.	11-13
Y	Pos et al., Residues Arg568 and Phe592 contribute to an antigenic surface for anti-ADAMTS13 antibodies in the spacer domain. Haematol. November 1, 2011 vol. 96 no. 11 1670-1677 (Published online before print June 28, 2011, doi: 10.3324/haematol.2010.036327). abstract; Fig. 2; p. 1672, 2nd-3rd para; p. 1675, last para.	1-4, 7
Y	US 2009/0304672 A1 (GINSBURG et al.) 10 December 2009 (10.12.2009) para [0181]; [0203]-[0205].	1-4, 7
Y	US 2011/0177107 A1 (HOWARD T.) 21 July 2011 (21.07.2011) para [0024]; [0084]; [0120]-[0122].	10

☐ Further documents are listed in the continuation of Box C.☐

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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document member of the same patent family

Date of the actual completion of the international search

31 January 2013 (31.01.2013)

Date of mailing of the international search report

15 FEB 2013

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