Journal of Thrombosis and Haemostasis, **12**: 670–679 DOI: 10.1111/jth.12535

ORIGINAL ARTICLE

Identification of N-linked glycosylation and putative O-fucosylation, C-mannosylation sites in plasma derived ADAMTS13

N. SORVILLO,* P. H. KAIJEN,* M. MATSUMOTO,† Y. FUJIMURA,† C. VAN DER ZWAAN,* F. C. VERBIJ,* W. POS,*‡[1] R. FIJNHEER,§ J. VOORBERG*¶ and A. B. MEIJER***

**Department of Plasma Proteins, Sanquin-AMC Landsteiner Laboratory, Amsterdam, the Netherlands; †Department of Blood Transfusion Medicine, Nara Medical University, Kashihara City Nara, Japan; ‡Department of Cancer Immunology & AIDS, Dana-Farber Cancer Institute, Boston, MA, USA; §Department of Haematology, University Medical Center Utrecht, Utrecht; ¶Department of Vascular Medicine, University of Amsterdam, Amsterdam; and **Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands*

To cite this article: Sorvillo N, Kaijen PH, Matsumoto M, Fujimura Y, van der Zwaan C, Verbij FC, Pos W, Fijnheer R, Voorberg J, Meijer AB. Identification of N-linked glycosylation and putative O-fucosylation, C-mannosylation sites in plasma derived ADAMTS13. *J Thromb Haemost* 2014; **12**: 670–9

Summary. *Background:* Acquired deficiency of ADAMTS13 causes a rare and life-threatening disorder called thrombotic thrombocytopenic purpura (TTP). Several studies have shown that aberrant glycosylation can play an important role in the pathogenesis of autoimmune diseases. N-linked glycosylation and putative O-fucosylation sites have been predicted or identified in recombinant ADAMTS13. However, it is not known which of these sites are glycosylated in plasma derived ADAMTS13. *Objectives:* Here we investigated the presence of putative O-fucosylation, C-mannosylation and N-linked glycosylation sites on plasma derived ADAMTS13. *Methods/Results:* Sites of N-linked glycosylation were determined by the use of peptide N-glycosidase-F (PNGase F), which removes the entire carbohydrate from the side chain of asparagines. Nine of the 10 predicted N-linked glycosylation sites were identified in or near the metalloproteinase, spacer, thrombospondin type 1 repeat (TSR1) and the CUB domain of plasma ADAMTS13. Moreover, six putative O-fucosylated sites were identified in the TSR domains of plasma ADAMTS13 by performing searches of the tandem mass spectrometry (MS/MS) data for loss of hexose (162 Da), deoxyhexose (146 Da), or hexose-deoxyhexose (308 Da). The use of electron transfer dissociation (ETD) allowed for unambiguous identification of the modified sites. In addition to putative O-fucosylation and N-linked glycosylation, two putative C-mannosylation sites were identified within the TSR1 and TSR4 domains of ADAMTS13. *Conclusions:* Our data identify several glycosylation sites on plasma derived ADAMTS13. We anticipate that our findings may be relevant for the initiation of autoimmune reactivity against ADAMTS13 in patients with acquired TTP.

Keywords: ADAMTS-13 protein, human; autoimmune diseases; glycosylation; mass spectrometry; thrombotic thrombocytopenic purpura.

Correspondence: Jan Voorberg, Department of Plasma Proteins, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, Plesmanlaan 125, 1066 CX Amsterdam, the Netherlands.
Tel.: +31 20 512 3122; fax: +31 20 512 3310.
E-mail: j.voorberg@sanquin.nl

[1]Current address: Department of Cancer Immunology & AIDS, Dana-Farber Cancer Institute, Boston, MA 02115, USA

Received 15 August 2013
Manuscript handled by: R. Camire
Final decision: P. H. Reitsma, 13 February 2014

Introduction

ADAMTS13 is a plasma metalloproteinase that regulates platelet adhesion and aggregation by its ability to process ultra-large von Willebrand factor (VWF) multimers on the surface of endothelial cells [1–3]. It is a member of the ADAMTS family (a disintegrinlike and metalloproteinase with thrombospondin type 1 repeats) and like most secreted proteins it is predicted to contain several N- and/or O-linked oligosaccharides [2]. Recent studies have shown that recombinant ADAMTS13 is highly glycosylated presenting both O-fucosylated and N-glycosylated residues. O-fucosylation (glucose-β (1,3)-fucose disaccharide linked to the hydroxyl group of Ser or Thr residues) occurs in at least 6 thrombospondin type 1 repeats (TSR) and is required for an appropriate secretion of the recombinant protease [4]. Several N-linked glycosylation sites (oligosaccharide chain linked through the N-acetylglucosamine group to an Asn residue) have been predicted throughout the entire protein:

two in the metalloproteinase domain (N142 and N146), four are in or near the spacer domain (N552, N579, N614 and N667), one in the second (N707) and fourth (N828) TSR, and two in the CUB domains (N1235 and N1354) [5]. N-linked glycans on recombinant ADAMTS13 play an important role in the secretion of ADAMTS13 but are not necessary for its VWF cleaving activity [5].

Deficiency of ADAMTS13 causes a rare and life-threatening disorder called thrombotic thrombocytopenic purpura (TTP). Patients affected by acquired TTP develop autoantibodies directed towards the metalloproteinase ADAMTS13 [6]. Reduced activity or absence of the metalloproteinase causes lack of cleavage of UL-VWF resulting in microvascular obstruction, low platelet counts (thrombocytopenia) and fragmentation of red blood cells (hemolytic anemia) [3,7,8]. Our current knowledge on the etiology of acquired TTP is limited. A large number of case reports suggest that microbial infections are linked to the onset or recurrence of this autoimmune disease [6].

Aberrant glycosylation of proteins can play an important role in the pathogenesis of autoimmune disorders [9]. Alterations in protein glycosylation may modify or create novel B-cell epitopes and may also influence the presentation of peptides to T-cells [10]. Previous work has shown that O-linked glycans can alter proteolytic processing and/or presentation of glycopeptides on MHC class II molecules and therefore interfere with activation of T-cells [11,12]. Glycosylated "self derived" peptides might not be presented efficiently by antigen presenting cells and might escape negative selection in the thymus [11]. However, in the periphery, modification of these peptides by glycosidases allows appropriate presentation of "self derived" peptides and activation of autoreactive T-cells leading to autoimmune reactivity towards self antigens [11].

Similarly, aberrant glycosylation of ADAMTS13 might contribute to the onset of acquired TTP by exposure of cryptic T-cell epitopes that are shielded by glycans under quiescent, non-inflammatory conditions. In this study we employed collision induced dissociation (CID) and electron transfer dissociation (ETD) to identify glycosylation sites in plasma derived ADAMTS13. Glycopeptide analysis by CID/ETD MS/MS identified nine N-linked sites in or near the metalloproteinase, spacer, thrombospondin type 1 repeat (TSR) and the CUB domain of plasma ADAMTS13. Six potential O-fucosylated sites were identified in the TSR domains of the protease. In addition to N-linked and potential O-fucosylated modifications, two putative C-mannosylation sites were identified within the TSR domains of ADAMTS13.

Materials and methods

Purification and deglycosylation of ADAMTS13

Full length recombinant ADAMTS13 was produced in stable HEK293 cells and purified as described previously [13]. Plasma ADAMTS13 was purified as described in the Supplemental Material and Method section using a specific monoclonal antibody A10 directed against the disintegrin domain of ADAMTS13 (Figure S1) [13,14]. Purified plasma and recombinant ADAMTS13 (200 nM) were incubated with PNGase F (2 U; Roche Diagnostics, Almere, the Netherlands) in a final volume of 50 μL, overnight at 37 °C in order to enzymatically remove N-linked sugar moieties from the protein. Removal of the glycans was assessed by SDS-PAGE on NuPAGE Bis-Tris Gel System 4–12% gel (Invitrogen, Breda, the Netherlands) followed by silver staining.

Generation of peptides and mass spectrometry sample preparation

Purified recombinant and plasma ADAMTS13 (200 nM) treated and non-treated with PNGase F (volume of 50 μL) was denatured by the addition of 6 M of urea (final concentration 2.4 M urea) for 15 min at room temperature. Urea concentration was reduced to 1 M by addition of 50 mM ammonium bicarbonate buffer (ABC). Disulfide bonds of the samples were reduced and alkylated by adding 10 μL of 100 mM 1.4-dithiothreitol freshly prepared in 50 mM ABC buffer for 30 min at room temperature following incubation with 25 μL of freshly prepared 55 mM iodoacetamide stock solution for 30 min at room temperature. Trypsin and chymotrypsin (Promega, Promega Benelux, the Netherlands) digestion was performed by incubation of the samples with 0.1 μg of the appropriate enzyme at room temperature. For chymotrypsin digestions 2.5 mM of $CaCl_2$ was added to the samples prior to addition of the enzyme. After overnight digestion the peptide mixtures were acidified with formic acid (Biosolve, Valkenswaard, the Netherlands) to stop the enzymatic reaction and to allow for concentration of the peptides using C18 zip tips (Millipore, Amsterdam, the Netherlands) as per the manufacturer's instructions. Peptides were eluted with acetonitrile that was subsequently removed from the samples before mass spectrometry analysis by evaporation under vacuum by speedvac centrifugation (Thermo Fisher Scientific, Bremen, Germany).

Mass spectrometry analysis of peptides

Peptides were separated using a reverse phase C18 column (50 μm × 20 cm, 5 μm particles; Nanoseparations, Nieuwkoop, the Netherlands) at a flow rate of 100 nl/min with a gradient from 0 to 35% (v/v) acetonitrile in 0.1 M hydrogen acetate. Eluted peptides were then sprayed directly into a LTQ XL Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Bremen, Germany) using a nanoelectrospray source with a spray voltage of 1.9 kV. Full mass spectrometry (MS) scans were performed and the five most intense precursor ions

from each full scan in the Orbitrap were selected for collision induced dissociation (CID; 300–2000 m/z, resolving power 30.000). Under CID fragmentation the peptide precursor ion undergoes one or more collisions by interactions with neutral gas molecules. The generated vibrational energy results in ion dissociation at the amide bonds along the peptide backbone generating b- and y-type fragment ions. Subsequently the product ions are ejected for detection, generating CID fragmentation spectra (MS/MS). Alternatively, the product ions can be kept and selected for another CID fragmentation, which can be repeated several times (MS^n were n indicates the number of CID reactions). MS/MS/MS was in fact triggered upon detection of neutral loss of the top 10 peptides in the MS/MS spectrum. For appropriate identification of the glycosylated sites several peptides were selected manually for electron transfer dissociation (ETD) fragmentation. This leads to the cleavage of N-Cα backbone bonds and generates c- and z- type fragment ions. In contrast to CID fragmentation, ETD fragmentation does not result in removal of intact oligosaccharide moieties from the peptide. The LTQ Orbitrap was calibrated on a monthly basis as recommended by the manufacturer in order to ensure a high mass accuracy.

Data analysis

Peptides were identified by screening each SEQUEST output file against the UniprotKB non-redundant protein 25.H_sapiens.fasta database using Proteome Discoverer version 1.2 software (Thermo Scientific, Bremen, Germany). Data were also analyzed against a decoy database from the 25.H_sapiens.fasta database comprising the reverse of all protein sequences. A false discovery rate (FDR) of 5% (which indicates that the probability that a peptide are false positive is <5%) was used as a cut-off for this study. The identification of oxidation on methionine (+15.994 Da; dynamic modification) and modification on cysteine groups (carbamidomethyl groups +57.0214 Da; static modification) was included during the search. Peptides modified by N-linked glycosylation were identified by the conversion of asparagine to aspartic acid at the site of carbohydrate attachment, resulting in an increase of the peptide mass of 1 Da (Asn → Asp; +0.9840). Identification of putative O-fucosylated sites was determined by performing searches for the hexose-deoxyhexose group (+308 Da) on serine residues. Tryptophans were monitored for the presence of a putative C-mannosylated group (+162.058). Individual MS/MS fragmentation spectra were manually analyzed in order to identify the sequential loss of hexose (162 Da), deoxyhexose (146 Da), or hexose-deoxyhexose (308 Da) from O-fucosylated sites and the loss of 120 Da in MS/MS spectra of peptides containing the modified tryptophan, a characteristic cross-ring fragmentation product of aromatic C-glycosides.

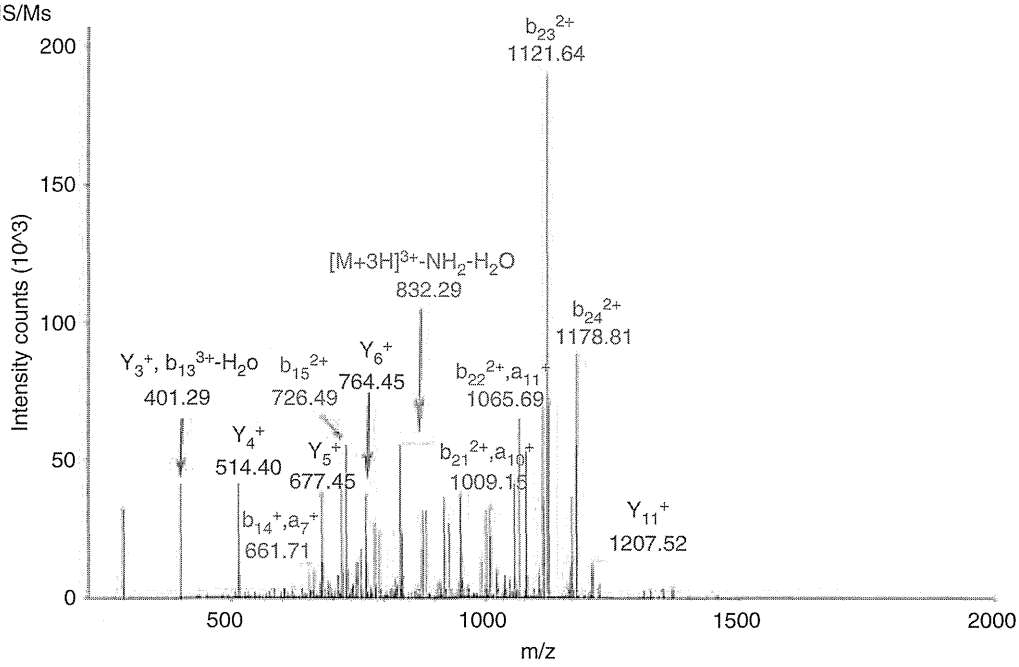
Results

Mapping of the N-linked glycosylation sites in plasma ADAMTS13

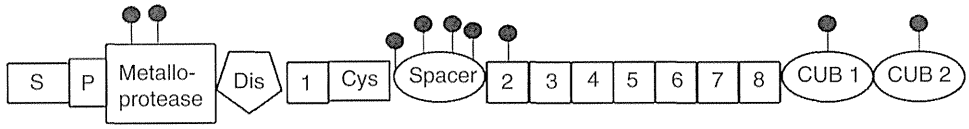
To identify N-linked glycosylation sites in plasma ADAMTS13 we used tandem MS to acquire mass/charge (m/z) values of peptides ions derived from in-solution tryptic and chymotryptic digestion of purified plasma ADAMTS13 after treatment with PNGase F. The enzyme is able to release the entire carbohydrate from the side chain of asparagine and in this process converts the amino acid asparagine to aspartic acid resulting in an increase of peptide mass of 1 Da. Treatment of purified plasma ADAMTS13 with PNGase F resulted in an increase of the sequence coverage from 72% towards 81% (Figure S2). Although PNGase F treatment increases ADAMTS13 sequence coverage not all peptides were detected. This is most probably due to the length of the peptides. Too long or extremely short peptides are out of range and not detectable by mass spectrometry. Full MS scans were performed and the five most intense precursor ions from each full scan were selected for CID and analyzed by MS/MS. Figure 1 illustrates the mass spectral data for the glycosylated peptide IAIHALATNMGAGTEGANASYILIR derived from the CUB2 domain of ADAMTS13. The series of b- and y-ions from the peptide confirm the presence of a glycan attached to N1354 for peptide I1337-R1361. Nine N-linked glycosylated peptides were identified throughout ADAMTS13 (Fig. 2). Mass spectra of the other N-linked peptides are provided in Figure S3. As shown in Fig. 2 two N-linked sites were identified in the metalloproteinase domain (N142 and N146), four in or near the spacer domain (N552, N579, N614 and N667), one in the second TSR domain (N707) and two in the CUB domains (N1235 and N1354). All peptides contained the N-glyco-

Fig. 1. Mapping of N-linked glycosylation sites in plasma ADAMTS13. Fragmentation spectra of deglycosylated peptide IAIHALATNMGAGTEGANASYILIR, derived from the CUB2 domain of ADAMTS13. Upper panel represents the MS/MS spectrum of the peptide. The peak corresponding to the parent ion is indicated in green; selected peaks corresponding to b- and y-ions are indicated in red and blue. The lower panel depicts the complete list of b- and y-ions. Theoretically predicted peptide masses are indicated in black. Y-ions that were found in the spectrum are indicated in blue whereas the b-ions that are indicated in red. Peptides masses highlighted in the spectrum are marked in yellow. Mass differences between Y_8^+ (950.53059) and Y_7^+ (835.50364) allows the identification of an Asp (monoisotopic mass: 115.02695) at position N1354 that is obtained after cleavage of N-linked glycans by PNGase F. Cleavage is obtained only when an Asn is modified by the addition of N-linked sugars. Cleavage by PNGase F converts an Asn to Asp resulting in an increase of peptide mass of 1 Da (from 114.04293 to 115.02695).

m/z:843.77850 (z = +3)



#1	b+	b2+	b3+	Seq.	y+	y2+	y3+	#2
1	114.09135	57.54931	38.70197	I				25
2	185.12847	93.06787	62.38101	A	2416.22904	1208.61816	806.08120	24
3	298.21254	149.60991	100.07570	I	2345.19192	1173.09960	782.40216	23
4	435.27145	218.13936	145.76200	H	2232.10785	1116.55756	744.70747	22
5	506.30857	253.65792	169.44104	A	2095.04894	1048.02811	699.02116	21
6	619.39264	310.19996	207.13573	L	2024.01182	1012.50955	675.34212	20
7	690.42976	345.71852	230.81477	A	1910.92775	955.96751	637.64743	19
8	791.47744	396.24236	264.49733	T	1839.89063	920.44895	613.96839	18
9	905.52037	453.26382	302.51164	N	1738.84295	869.92511	580.28583	17
10	1036.56087	518.78407	346.19181	M	1624.80002	812.90365	542.27152	16
11	1093.58234	547.29481	365.19896	G	1493.75952	747.38340	498.59136	15
12	1164.61946	582.81337	388.87800	A	1436.73805	718.87266	479.58420	14
13	1221.64093	611.32410	407.88516	G	1365.70093	683.35410	455.90516	13
14	1322.68861	661.84794	441.56772	T	1308.67946	654.84337	436.89800	12
15	1451.73121	726.36924	484.58192	E	1207.63178	604.31953	403.21544	11
16	1508.75268	754.87998	503.58908	G	1078.58918	539.79823	360.20124	10
17	1579.78980	790.39854	527.26812	A	1021.56771	511.28749	341.19409	9
18	1694.81674	847.91201	565.61043	N-Asn->Asp	950.53059	475.76893	317.51505	8
19	1765.85386	883.43057	589.28947	A	835.50364	418.25546	279.17273	7
20	1852.88589	926.94658	618.30015	S	764.46652	382.73690	255.49369	6
21	2015.94921	1008.47824	672.65459	Y	677.43449	339.22088	226.48301	5
22	2129.03328	1065.02028	710.34928	I	514.37117	257.68922	172.12857	4
23	2242.11735	1121.56231	748.04397	L	401.28710	201.14719	134.43388	3
24	2355.20142	1178.10435	785.73866	I	288.20303	144.60515	96.73919	2
25				R	175.11896	88.06312	59.04450	1



Glycosylation	Peptide sequence
N142+N146	MVILTEPEGAPNITANLTSSLVCGWSQTINPEDDTDPGHADLVLYSTR
N552	CQVCGGDNSTCSPR
N579	AREYVTFLTVTPNLTSVYIANHRPLFTHLAVR
N614	MSISPNTTYPSLEEDGR
N667	YGEEYGNLTRPDITFTYFQPKPR
N707	WVNYSCLDQARK
N1235	VLESSLNCSAGDMLLLWGR
N1354	IAIHALATNMGAGTEGANASYILIR

Fig. 2. Schematic representation of the confirmed N-linked glycosylation sites in plasma derived ADAMTS13 measured by tandem MS.

sylation consensus sequence N-X-S/T and had an increase of mass of 1 Da. Interestingly, ADAMTS13 derived peptides containing the N-linked sites were identified only in samples treated with PNGase F with the exception of peptides containing N142, N146, N707 and N1354 that were also detected in non-PNGase F treated ADAMTS13. This observation suggests that N-glycosylation at these sites is not complete. We were not able to identify whether a glycan was attached to N828 since peptides containing this amino acid were not detected in our analysis. All nine N-linked glycosylation sites were also identified in recombinant ADAMTS13 that was analysed in parallel (Figure S4).

Detection of terminal sugars on both plasma and recombinant ADAMTS13 was carried out by assessing the binding of digoxygenin-labelled lectins. Lectins specifically recognizing terminal α-D-mannose (GNA), β-D-galactose (DSA) and terminal sialic acid (SNA and MAA) bound to both recombinant and plasma ADAMTS13 confirming the presence of complex and hybrid N-glycan structures on both proteins (Figure S5). The lectin PNA, that binds to the disaccharide galactose-β(1–3)-N-acetylgalactosamine, recognized recombinant but not plasma derived ADAMTS13. The disaccharide might not be accessible due to the presence of one or two sialic acids or by the addition of fucose, that needs to be removed enzymatically to allow binding of the PNA lectin. Absence of binding of this lectin suggests the presence of complex glycans structures on plasma ADAMTS13.

CID/ETD MS/MS analysis of putative O-fucosylation sites in plasma ADAMTS13

Chromatography and tandem mass spectrometry analysis have shown that the thrombospondin type 1 repeats (TSRs) of recombinant ADAMTS13 and from several other proteins are modified by the addition of the disaccharide glucose-β-(1–3)-fucose-α1-O linked to serine or threonine residues [4,15–18]. To determine whether the same modification occurs in plasma ADAMTS13 we examined tryptic and chymotryptic peptides derived from purified plasma ADAMTS13 through mass spectral analysis using two different fragmentation approaches: collision induced dissociation (CID) and electron transfer dissociation (ETD). CID fragmentation of the glycosylated peptides resulted in a characteristic pattern in accordance with the sequential neutral losses of hexose (Hex, 162 Da), deoxyhexose (dHex, 142 Da) and of the disaccharide hexose-deoxyhexose (Hex-dHex, 308 Da) (Fig. 3, Figure S6). As shown in Fig. 3, for TSR5-derived peptide TGAQAAHVWTPAAGSCSVSCGR (charge z = +2), the mass of the most intense ions in the spectrum (m/z 1116.72) corresponds to the loss of Hex-dHex disaccharide) with respect to the mass of the parent peptide ion (m/z 1270.06). The lower ions (m/z 1189 for TGAQAAHVWTPAAGSCSVSCGR) corresponds to the loss of the single Hex. MS/MS/MS fragmentation of the TGAQAAHVWTPAAGSCSVSCGR peptide without the Hex and dHex yields extensive peptide backbone fragments and provides sufficient information to appropriately identify the peptide (Fig. 3; right panel). Results from CID analysis suggested the presence of six putative O-fucosylated peptides within 6 of the 8 TSR domains of ADAMTS13 (Fig. 4 and Suppl. Figure S6). Formally, the observed neutral loss of Hex (162 Da) and dHex (142 Da) does not allow for identification of the attached glycan moiety. The identified glycosylation sites within the TSRs domain of ADAMTS13 fall within the consensus sequence of O-fucosylation depicted as WX5C1X2-3 (S/T)C2X2G. This suggests that the neutral losses correspond to the loss of fucose (162 Da), glucose (142 Da) and the disaccharide glucose-β(1–3)-fucose (Hex-dHex, 308 Da). Despite the fact that the TSR1 domain of ADAMTS13 contains a consensus sequence for O-fucosylation (CSRSCG) the peptide containing such modification was not retrieved in the mass spectrom-

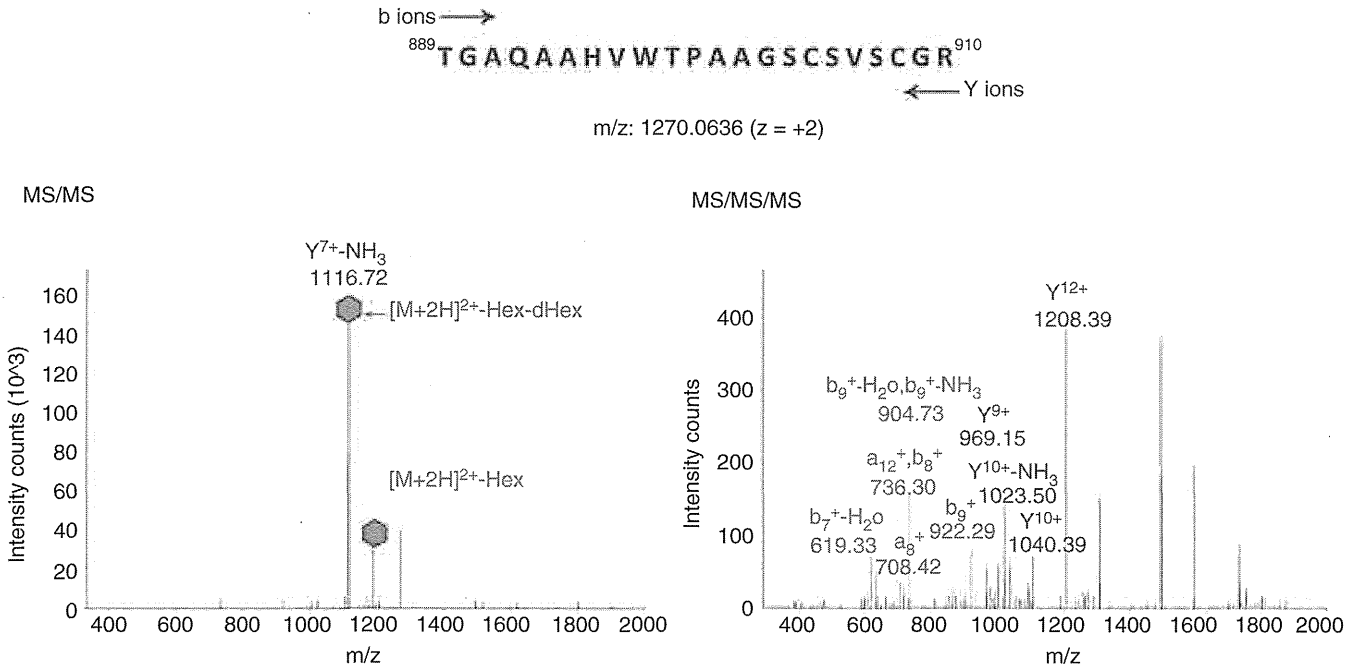
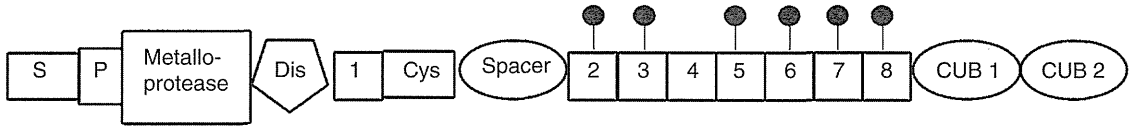


Fig. 3. Identification of putative O-fucosylation sites in TSR domains of ADAMTS13. Left panel represents the MS/MS spectrum of a tryptic peptide derived from the TSR5 domain of ADAMTS13. The major ions correspond to the sequential neutral loss of a hexose (81 Da; corresponding to loss of glucose from a doubly charged peptide; [M + 2H-Hex]2+, m/z 1.189) and of a hexose-deoxyhexose (154 Da loss of the glucose-fucose disaccharide from a double charged peptide; [M + 2H-Hex-dHex]2+, m/z 1116.72). Peaks corresponding to neutral losses are indicated by a red hexagon. Right panel shows the MS/MS/MS fragmentation confirming the assignment of the peptide. Selected peaks corresponding to b- and y-ions are indicated in red and blue.



Glycosylation	Peptide sequence	Parent mass +H	Charge	Product mass +H	Parent - Observed
TSR 2	GPCSV**S**CGAGLR	1528.66	2	1220	308
TSR 3	ELVETVQCQGSQQPPAWPEACVLEPCPPYWAVGDFGPCSA**S**CGGGLR	5512.46	4	5207.48	305
TSR 5	TGAQAAHVWTPAAGSCSV**S**CGR	2539.12	2	2233.44	305.56
TSR 6	LAACSV**S**CGR	1288.6	2	1081.6	307
TSR 7	VMSLGPCSA**S**CGLGTAR	2047.29	2	1740.9	306.39
TSR 8	WHVGTWMECSV**S**CGDGIQR	2573.07	3	2267.28	305.79

Fig. 4. Schematic representation of potential O-linked fucosylation sites of plasma derived ADAMTS13.

etry analysis for both plasma and recombinant ADAMTS13. (Figure S6 and S7) [4]. No consensus site for putative O-fucosylation is present within TSR4. In accordance with the lack of a consensus site we did not obtain evidence for the presence of a potential O-fucosylation site in TSR4. MS/MS/MS spectra of Hex-dHex containing peptides derived from TSR2, TSR3 and TSR6 did not contain sufficient information to allow for appropriate identification of the modified amino acid (Figure S6; panel C, D and E). The same potential O-fucosylation sites were also identified for recombinant ADAMTS13 (Figure S7).

ETD fragmentation of glycosylated peptides produces several c- and z-ions (nomenclature of Zubarev and co-workers [19]), resulting from the cleavage of the N-Cα bonds, and allows the intact oligosaccharide moieties to remain attached to the fragment ions containing the glycans. The ETD process represents an excellent tool to allow the localization of sites of modification in glycopeptides. Therefore, the same putative O-linked fucosylated peptides identified by CID were analyzed by ETD. Figure 5 shows the ETD fragmentation of the Hex-dHex modified peptide GPCSVSCGAGLR derived from the TSR2 domain. Both c- and z- ions were observed

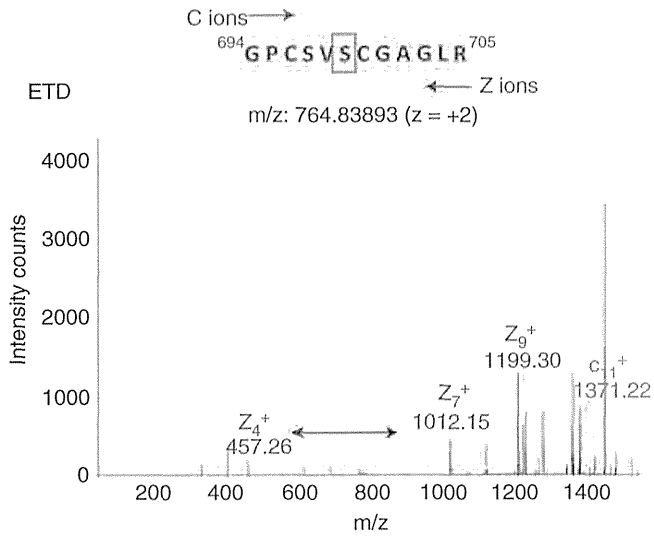


Fig. 5. Analysis of potential O-fucosylation of TSR domains of ADAMTS13 using ETD mass spectrometry. ETD spectrum of putative O-fucosylated peptide GPCSVSCGAGLR belonging to the TSR2 domain. The difference between z7+ and the z4+ is consistent with putative O-fucosylation of Ser698.

allowing sequence determination of the peptide. The glycosylation site was determined by the mass differences between z + 7 to z + 4 (indicated by the arrow). The ETD spectra obtained for peptides TGAQAAHVWTPAAGSCSVSCGR and WHVGTWMECSVSCGDGIR are depicted in Figure S8. Although the putative O-fucosylated modification sites were recognized as S698, S907 and S1087 respectively, ETD fragmentation of the glycosylated peptides of TSR5 and TSR8 suggested that these peptides can be modified at different sites: S691 and S695 for TGAQAAHVWTPAAGSCSVSCGR, S905 for WHVGTWMECSVSCGDGIR. This suggests the possibility of two positional isomers of the same peptide with putative O-fucosylation modifications on different residues. The ETD spectrum fragmentation for the remaining peptides derived from TSR3, TSR6 and TSR7 did not show extensive sequence coverage (data not shown) and therefore it was not possible to identify the exact site of modification. A consensus sequence for O-fucosylation has been defined previously as WX5C1X2-3(S/T)C2X2G [4,16]. Based on this consensus sequence the glycosylation sites for ELVETVQCQGSQQPAWPEACVLEPCPPYWAVGDFGPCSASCGGLR (TSR3), LAACSVSCGR (TSR6) and VMSLGPCSASCGLGTAR (TSR7) are most likely at S757, S965 and S1027, respectively.

C-mannosylation of plasma ADAMTS13

In addition to O-fucosylation TSRs often contain a consensus motif for C-mannosylation which has been defined as WXXW [15,16,18]. Examination of the sequence of ADAMTS13 suggests the presence of several potential C-mannosylation sites. We therefore analyzed ADAMTS13 derived peptides, obtained as described above, employing CID and ETD tandem mass spectrometry. Peptides carrying an additional 162 Da mass (suggested to correspond to a single mannosyl residue) were subjected to ETD fragmentation. Figure 6 and Figure S10 show the MS/MS fragmentation of respectively plasma and recombinant ADAMTS13 derived peptide: APSPWGSIR belonging to the TSR4 domain. Evidence for putative C-mannosylation of Trp884 was further suggested by the loss of 120 Da in the MS/MS spectra (Fig. 6; left panel), a characteristic cross-ring fragmentation product of aromatic C-glycosides. In addition, ETD-fragmentation was performed yielding a series of c- and z-ions that were consistent with the presence of C-mannose on Trp884 in the APSPWGSIR peptide (Fig. 6; right panel). Mass differences between z + 5 and z + 4 provided evidence for Trp884 as site of glycosylation. Evidence for a second putative C-mannosylation site at Trp390 in the WSSWGPR peptide in the TSR 1 domain was obtained (Figure S9 and S10) for both plasma and recombinant ADAMTS13.

Discussion

Like most secreted proteins ADAMTS13 undergoes post-translational modifications and is predicted to contain several N-linked oligosaccharides. In our current study we were able to demonstrate by means of tandem mass spectrometry that plasma ADAMTS13 is a highly glycosylated protein presenting several N-, O- and C-linked glycosylation sites. Treatment of plasma ADAMTS13 with PNGase F, which removes the N-linked glycan moieties, allowed for the identification of 9 out of 10 predicted N-linked glycosylation sites; two in the metalloproteinase domain (N142 and N146), four in or near the spacer domain (N552, N579, N614 and N667), one in the second TSR domain (N707) and two in the CUB domains (N1235 and N1354). Surprisingly, plasma ADAMTS13 derived peptides containing the N-linked sites N142, N146, N707 and N1354 were also detected in the non-PNGase F treated ADAMTS13 samples, suggesting that N-glycosylation at these residues is not complete. Interestingly, for recombinant ADAMTS13 all N-glycosylation peptides were detected only in the PNGase F treated samples. These findings suggest that N-glycosylation of plasma ADAMTS13 is more heterogenous when compared to recombinant ADAMTS13. The tryptic peptide containing the predicted N-linked glycosylation site at N828 was not identified in both plasma and recombinant ADAMTS13. Therefore we were unable to determine whether a glycan is attached to this residue. Mammalian N-linked moieties are highly versatile in nature. Lectin binding analysis of both plasma and recombinant ADAMTS13 revealed that both proteins contain high mannose structures, sialic acid and galactose residues (Figure S5). Absence of binding of peanut agglutinin (PNA), a lectin that binds the disaccharide galactose-β(1–

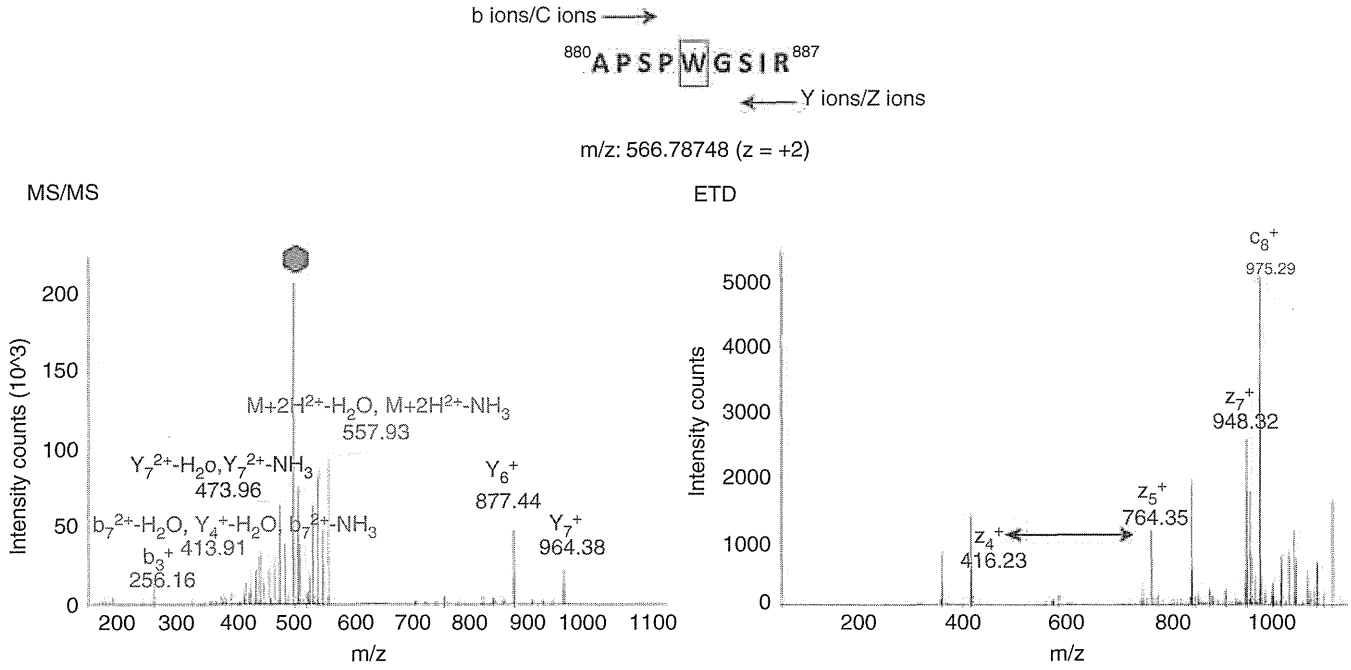


Fig. 6. Putative C-mannosylation of TSR domains of ADAMTS13. CID and ETD fragmentation spectrum of ADAMTS13 derived peptides. Left panel show the MS/MS spectrum of the APSPWGSIR peptide belonging to the TSR4 domain of ADAMTS13. The major peak in the spectrum corresponds to the neutral loss (120 Da) of a cross-ring fragmentation product potentially derived from a C-mannosylated Trp (indicated by the red hexagon). The peak corresponding to the intact peptide is indicated in green; selected peaks corresponding to b- and y-ions are indicated in red and blue. Right panel shows the ETD spectrum of the APSPWGSIR peptide. Differences between z5+ and z4+ ions allowed for identification of the modified Trp at position 884.

3)-N-acetylgalactosamine, to plasma derived ADAMTS13 might be due to the modification of the core structure by the addition of sugars such as sialic acid and/or fucose (Figure S5). Removal of sialic acid and/or fucose might therefore be necessary in order to allow binding of the PNA lectin to plasma derived ADAMTS13. Overall our findings suggest that plasma ADAMTS13 contains different terminal glycan structures when compared to recombinant ADAMTS13 (Figure S5) and suggests the presence of complex glycan structures on plasma ADAMTS13.

O-fucosylation is a different post translational modification in which fucose or the disaccharide glucose-β1,3-fucose is covalently attached to Ser or Thr residues within a putative consensus sequence CSX(S/T)CG [4,20]. It is well established that O-fucosylation takes place in TSRs [4,16,20]. Previous studies have identified several putative O-fucosylation moieties in the TSR domains of recombinant ADAMTS13 [4,21]. Through a combination of CID/ETD MS/MS analysis we provide evidence for neutral loss of Hex, dHex and Hex-dHex sugar moieties suggesting the presence of six putative O-fucosylation sites within the TSR2, TSR3, TSR5, TSR6, TSR7 and TSR8 domains of plasma ADAMTS13. As expected no putative O-fucosylation site was identified in the TSR1 since that region does not contain a consensus sequence. We were unable to identify the peptide containing the putative O-fucosylation site from the TSR4 domain of ADAMTS13 and therefore cannot exclude the possibility of such modification in this domain. MS/MS fragmentation spectra (Fig. 3; Figure S6 and S7) showed a characteristic neutral loss of a hexose-deoxyhexose disaccharide (308 Da) confirming the presence of the glycan within the TSR derived peptides. To further identify the site of glycosylation we performed an ETD analysis. Unlike CID analysis, ETD fragmentation does not remove the glycan from the peptide allowing for assignment of the modified residue [22–24]. Using this approach we were able to assign the exact site of modification for three of the TSRs glycosylated peptides: S698 for GPCSVSCGAGLR, S907 for TGAQAAHVWTPAAG SCSVSCGR and S1087 for WHVGTWMECSVSCGD-GIQR (Fig. 5; Figure S8). Unfortunately the precise site of glycosylation for the remaining peptides could not be identified due to low sequence coverage. Taking into account previous findings of putative O-fucosylated sites in recombinant ADAMTS13 [4] and the consensus sequence of the modification we suggest that O-fucosylation takes place at S757, S965 and S1027.

In addition to N- and O-linked oligosaccharides, proteins are often modified by C-mannosylation. C-mannosylation is a common post-translation modification of TSR [16,18]. In this study we show that Trp884 and Trp390 in the TSR 4 and TSR 1 domain of both plasma and recombinant are potentially modified by C-mannosylation, suggesting that this modification might not only occur within the consensus sequence (WXXW) but also takes place at other sites [18]. Recently, Akiyama and co-workers showed that Trp387 in TSR1 was C-mannosylated in a recombinant ADAMTS13 variant [25]. Our data suggest that

Trp390 in plasma derived ADAMTS13 contains a putative C-mannosylation site. Interestingly, ions corresponding to putative C-mannosylated WSSWGPR peptide were identified that did not contain the mannose-residue at Trp390. This suggest that Trp387 might also be modified in plasma derived ADAMTS13. However, the fragmentation spectrum did not allow for accurate assignment of the putative C-mannosylation at Trp387 (data not shown). In a recent study, Ling and co-workers showed that replacement of Trp387 by Ala resulted in a reduced expression and processing activity of ADAMTS13 suggesting that C-mannosylation might be required for optimal folding [26].

Protein glycosylation has not only been considered to play an important role in cell-cell communication and adhesion, protein structural stability, membrane structure and cellular signaling. Several studies have shown that protein glycans may be crucial also for T-cell recognition of autoantigens in autoimmune disorders [10]. Previously we have shown that antigen presenting cells derived from HLA-DRB1*11-positive individuals present a CUB2 domain derived peptide FINVAPHARIA (residues 1327–1338) [27]. Since HLA-DRB1*11 is considered to be a risk factor for the development of acquired TTP [28–30] functional presentation of this CUB2 domain-derived peptide might contribute to the onset of acquired TTP [27]. Presentation of the FINVAPHARIA peptide (residues 1327–1338) may potentially be affected by the presence of an N-linked glycan at N1354. Moreover, non-DRB1*11 donors present a second CUB domain peptide ASYILIRDTHSLRTTA (residues 1355–1370) which is right adjacent to N1354 [27]. This raises the possibility that differential glycosylation at N1354 might contribute to the onset of acquired TTP in patients capable of presenting such peptides.

Addendum

N. Sorvillo performed experiments, analyzed data, made the figures and wrote the paper. P. Kaijen, C. van der Zwaan, F. Verbij, W. Pos, M. Matsumoto performed experiments. Y. Fujimura and M. Matusmoto provided protocols and prepared large amounts of antibody for purification of ADAMTS13 from plasma. A. B. Meijer and J. Voorberg designed research, analyzed the data, wrote and reviewed the paper. R. Fijnheer designed research and reviewed the paper.

Acknowledgements

Dr. Hendrik Feys is kindly acknowledged for providing anti-TSR5-8 monoclonal antibody 20D2.

Funding

This study was supported by grant PPOC-08-021. Grant support from Takeda Medical Foundation is gratefully acknowledged.

Disclosure of Conflict of Interest

The authors state that they have no conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Purification of pADAMTS13.
Fig. S2. Sequence coverage of ADAMTS13.
Fig. S3. Mapping of N-linked glycosylation sites in plasma ADAMTS13.
Fig. S4. Mapping of N-linked glycosylation sites in recombinant ADAMTS13.
Fig. S5. Identification of terminal N-linked glycans in both plasma and recombinant ADAMTS13.
Fig. S6. Identification of putative O-fucosylation sites in TSR domains of plasma derived ADAMTS13.
Fig. S7. Identification of putative O-fucosylation sites in TSR domains of recombinant ADAMTS13.
Fig. S8. Identification of putative O-fucosylation sites in TSR domains of plasma derived ADAMTS13.
Fig. S9. Putative C-mannosylation of TSR1 domain of plasma ADAMTS13.
Fig. S10. Putative C-mannosylation of TSR1 and TSR4 domains of recombinant ADAMTS13.

References

1 Moake JL. Thrombotic thrombocytopenic purpura: the systemic clumping "plague". *Annu Rev Med* 2002; **53**: 75–88.
2 Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *J Biol Chem* 2001; **276**: 41059–63.
3 Dong JF. Cleavage of ultra-large von Willebrand factor by ADAMTS-13 under flow conditions. *J Thromb Haemost* 2005; **3**: 1710–6.
4 Ricketts LM, Dlugosz M, Luther KB, Haltiwanger RS, Majerus EM. O-fucosylation is required for ADAMTS13 secretion. *J Biol Chem* 2007; **282**: 17014–23.
5 Zhou W, Tsai HM. N-Glycans of ADAMTS13 modulate its secretion and von Willebrand factor cleaving activity. *Blood* 2009; **113**: 929–35.
6 Pos W, Luken BM, Sorvillo N, Kremer Hovinga JA, Voorberg J. Humoral immune response to ADAMTS13 in acquired thrombotic thrombocytopenic purpura. *J Thromb Haemost JTH* 2011; **9**: 1285–91.
7 Motto DG, Chauhan AK, Zhu G, Homeister J, Lamb CB, Desch KC, Zhang W, Tsai HM, Wagner DD, Ginsburg D. Shigatoxin triggers thrombotic thrombocytopenic purpura in genetically susceptible ADAMTS13-deficient mice. *J Clin Invest* 2005; **115**: 2752–61.
8 Kremer Hovingas JA, Lämmle B. Role of ADAMTS13 in the pathogenesis, diagnosis, and treatment of thrombotic thrombocytopenic purpura. *Hematology Am Soc Hematol Educ Program* 2012; **2012**: 610–6.
9 Purcell AW, van Driel IR, Gleeson PA. Impact of glycans on T-cell tolerance to glycosylated self-antigens. *Immunol Cell Biol* 2008; **86**: 574–9.

10 Szabo TG, Palotai R, Antal P, Tokatly I, Tothfalusi L, Lund O, Nagy G, Falus A, Buzas EI. Critical role of glycosylation in determining the length and structure of T cell epitopes. *Immunome Res* 2009; **5**: 4.
11 Hanisch F-G, Schwientek T, von Bergwelt-Baildon MS, Schultze JL, Finn O. O-Linked glycans control glycoprotein processing by antigen-presenting cells: a biochemical approach to the molecular aspects of MUC1 processing by dendritic cells. *Eur J Immunol* 2003; **33**: 3242–54.
12 Ishioka GY, Lamont AG, Thomson D, Bulbow N, Gaeta FC, Sette A, Grey HM. MHC interaction and T cell recognition of carbohydrates and glycopeptides. *J Immunol* 1992; **148**: 2446–51.
13 Pos W, Crawley JT, Fijnheer R, Voorberg J, Lane DA, Luken BM. 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**: 1640–9.
14 Hiura H, Matsui T, Matsumoto M, Hori Y, Isonishi A, Kato S, Iwamoto T, Mori T, Fujimura Y. Proteolytic fragmentation and sugar chains of plasma ADAMTS13 purified by a conformation-dependent monoclonal antibody. *J Biochem (Tokyo)* 2010; **148**: 403–11.
15 Hofsteenge J, Huwiler KG, Macek B, Hess D, Lawler J, Mosher DF, Peter-Katalinic J. C-Mannosylation and O-Fucosylation of the Thrombospondin Type 1 Module. *J Biol Chem* 2001; **276**: 6485–98.
16 Gonzalez de Peredo A, Klein D, Macek B, Hess D, Peter-Katalinic J, Hofsteenge J. C-mannosylation and o-fucosylation of thrombospondin type 1 repeats. *Mol Cell Proteomics* 2002; **1**: 11–8.
17 Wang LW, Dlugosz M, Somerville RP, Raed M, Haltiwanger RS, Apte SS. O-fucosylation of thrombospondin type 1 repeats in ADAMTS-like-1/punctin-1 regulates secretion: implications for the ADAMTS superfamily. *J Biol Chem* 2007; **282**: 17024–31.
18 Wang LW, Leonhard-Melief C, Haltiwanger RS, Apte SS. Post-translational modification of thrombospondin type-1 repeats in ADAMTS-like 1/punctin-1 by C-mannosylation of tryptophan. *J Biol Chem* 2009; **284**: 30004–15.
19 Zubarev RA, Kelleher NL, McLafferty FW. Electron capture dissociation of multiply charged protein cations. A nonergodic process. *J Am Chem Soc* 1998; **120**: 3265–6.
20 Luo Y, Nita-Lazar A, Haltiwanger RS. Two distinct pathways for O-fucosylation of epidermal growth factor-like or thrombospondin type 1 repeats. *J Biol Chem* 2006; **281**: 9385–92.
21 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**: 19274–9.
22 Chalkley RJ, Thalhammer A, Schoepfer R, Burlingame AL. Identification of protein O-GlcNAcylation sites using electron transfer dissociation mass spectrometry on native peptides. *Proc Natl Acad Sci U S A* 2009; **106**: 8894–9.
23 Perdivara I, Deterding LJ, Cozma C, Tomer KB, Przybylski M. Glycosylation profiles of epitope-specific anti-β-amyloid antibodies revealed by liquid chromatography-mass spectrometry. *Glycobiology* 2009; **19**: 958–70.
24 Lin Z, Lo A, Simeone DM, Ruffin MT, Lubman DM. An N-glycosylation Analysis of Human Alpha-2-Macroglobulin Using an Integrated Approach. *J Proteomics Bioinform* 2012; **5**: 127–34.
25 Akiyama M, Nakayama D, Takeda S, Kokame K, Takagi J, Miyata T. Crystal structure and enzymatic activity of an ADAMTS13 mutant with the East Asian-specific P475S polymorphism. *J Thromb Haemost* 2013; **11**: 1399–406.
26 Ling J, Su J, Ma Z, Ruan C. The WXXW motif in the TSR1 of ADAMTS13 is important for its secretion and proteolytic activity. *Thromb Res* 2013; **131**: 529–34.
27 Sorvillo N, van Haren SD, Kaijen PH, Ten Brinke A, Fijnheer R, Meijer AB, Voorberg J. Preferential HLA-DRB1*11 dependent presentation of CUB2 derived peptides by ADAMTS13 pulsed dendritic cells. *Blood* 2013; **121**: 3502–10.
28 Scully M, Brown J, Patel R, McDonald V, Brown CJ, Machin S. Human leukocyte antigen association in idiopathic thrombotic thrombocytopenic purpura: evidence for an immunogenetic link. *J Thromb Haemost* 2010; **8**: 257–62.
29 Coppo P, Busson M, Veyradier A, Wynckel A, Poullin P, Azoulay E, Galicier L, Loiseau P. HLA-DRB1*11: a strong risk factor for acquired severe ADAMTS13 deficiency-related idiopathic thrombotic thrombocytopenic purpura in Caucasians. *J Thromb Haemost* 2010; **8**: 856–9.
30 John ML, Hitzler W, Scharrer I. The role of human leukocyte antigens as predisposing and/or protective factors in patients with idiopathic thrombotic thrombocytopenic purpura. *Ann Hematol* 2012; **91**: 507–10.

特 集
造血器疾患 —最新の治療戦略（血小板・凝固・線溶系疾患）—

血栓性血小板減少性紫斑病

藤村吉博[1]，松本雅則[1]，石西綾美[1]，八木秀男[1]，
小亀浩市[2]，宮田敏行[2]

Key words : TTP, ADAMTS13, VWF, USS

はじめに

1924年，米国のMoschcowitz[1]によって肺を除く全身諸臓器の細動脈に出来たヒアリン膜血栓症として最初に報告された重篤疾患は，1966年にAmorosi & Ultmann[2]による自他験例の計271例の解析にて，細血管障害性溶血性貧血，破壊性血小板減少，血小板血栓による臓器機能障害（特に腎機能不全），発熱，そして動揺性精神神経障害の5徴候（pentad）を特徴とする事が示され，血栓性血小板減少性紫斑病（thrombotic thrombocytopenic purpura, TTP）と命名された。

一方，血管内皮細胞で産生されるvon Willebrand因子（VWF）は巨大分子構造を持つ止血因子であるが，2001年にこのVWFの特異的切断酵素であるADAMTS13（a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13）が同定された[3~8]。今日ではTTP症例の2/3以上が，このADAMTS13活性欠損により，血中に未消化の超巨大VWF多重体（unusually large VWF multimers, UL-VWFM）が蓄積し，これにより細血管内で生ずる高ずり応力下に過剰な血小板凝集が生じ，全身性重篤疾患が引き起こされる事が広く認識されるようになった。この結論に至る迄の約90年間の歴史を振り返ると，その後半期の渦中にいた筆者等は改めて基礎と臨床の研究者間の緊密な情報交換の重要性を痛感するのである。本項では，このTTPの病態解明への集約過程と治療について紹介し，最後に現在直面している問題点について述べる。

[1]奈良県立医科大学　輸血部
[2]国立循環器病研究センター研究所　分子病態部

表裏一体のVWD-TTP研究歴史

von Willebrand病（VWD）は1924年フィンランド人医師Erik Adolf von Willebrandによって同国Bothnia湾内のÅland島で，血族結婚を繰り返す家系で男女両性に出現する先天性出血性素因として最初に報告された[9]。この背景からVWD研究の初期は北欧中心に発展し，1950年代にスウェーデンのInga Marie Nilsson等は[10]VWD患者では凝固VIII因子活性が低下している事，VWD患者にVIII因子活性を欠く血友病A血漿を輸血すると，VIII因子活性が出現し，しかもその血中半減期が著しく長いという発見をした。1964年に米国のPool & Shannon[11]がクリオ沈殿の分離技術を確立し，これにはVIII因子活性が豊富に含まれる事を示し，この製剤が血友病Aの治療に用いられるようになった。1970年前後にアガロース ゲルクロマトグラフィーでクリオ沈澱からVIII因子活性を持つ蛋白が高純度に精製された。この分離方法にてオランダのvan Mourik等は[12]精製VIII因子分画にはVWDに見られる血小板粘着能低下を補正する作用がある事を見出した。また，米国のZimmerman等は[13]，精製VIII因子関連蛋白に対する家兎免疫沈降抗体の作成を通じて血友病AとVWDは免疫学的に鑑別しうる事を示した。しかし，これらVIII因子と血小板粘着能補正活性は常にその挙動を共にする事から[14]，これらは同一分子上に存在すると考えられた。以後，VIII因子活性を示す蛋白は第VIII因子関連抗原（VIII-related antigen: VIIIR:AG）と呼ばれるようになった。結果的にはこの表記は誤りで，VIIIR:AGと称されたものはVWF抗原であった。1985年前後にVIIIとVWFの両蛋白質のcDNAクローニングが行われ，両者が異なった蛋白である事が明瞭となり，VIIIR:AGの名称は1986年に国際血栓止血学会の国際標準化委員会

(Scientific and Standardization Committee, SSC) で正式に廃止された[15~20]。

van Mourik らは[21] 1974 年に VIIIR:AG について，一つの不可思議な現象を報告していた。それは，精製した VIIIR:AG は巨大分子構造を持つ事が示唆され，ポリアクリルアミドゲル電気泳動ではゲル先端からわずかにしか内部に移動しないが，このサンプルを低イオン強度緩衝液（具体的には低 NaCl 濃度環境）に一昼夜透析しておくと低分子化し，「ゲル内を早く進む蛋白バンド（fast-moving component: FMC）と，遅く進むバンド（slow-moving component: SMC）の二つ（Fig. 1）が出現し，しかも各々のバンドは抗 VIIIR:AG 抗体と一本の免疫沈降線を形成するが，お互いのバンドは融合しない事から免疫原性は異なる」というものであった。この後，同研究グループからはこの現象を説明できる新たな研究の進捗はなかった。

1980 年，Scripps 研究所の Ruggeri & Zimmerman[22] により SDS アガロースゲル電気泳動による VWF 蛋白の解析法が確立され，正常血漿中の VWF は分子量 500 kD～15,000 kD の多岐にわたる多重体（multimer, M）構造を持つ事が示された。一方，個々の VWF バンドは 1 本の主バンドとその上下に各々 1 本のサテライトバンドからなる三連符構造を示す事から，健常人に於いても *in vivo* proteolysis がある事，また type 2 VWD では高分子量（high molecular weight, HMW)-VWFM が欠失している事から，かかる患者では前記の proteolysis が亢進していると考えられるようになった。実際，1987 年に同研究所の Berkowitz 等は[23] 抗 VWF モノクローナル抗体を用いて，還元条件下での SDS ポリアクリルアミドゲル電気泳動後の western blot にて本来の VWF サブユニット分子量 250 kD に対して，type 2A VWD 患者血漿中には分解を受け低分子化した 176 kD と 140 kD のサブユニットバンドがコントロールに比して，より多く存在する事を報告している。さらに同ラボの Dent 等は[24] type 2A-VWF を精製し，患者に見られる特異的 VWF サブユニット断片の解析にて，その切断部位は成熟 VWF サブユニットを構成する 2050 アミノ酸残基の Tyr842-Met843（全長 cDNA レベルのアミノ酸残基では

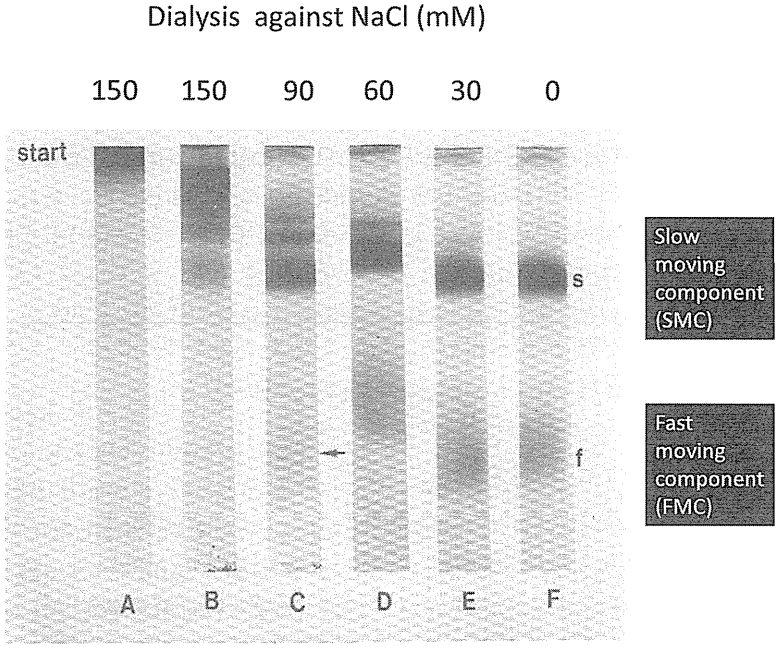


Fig. 1 Large-pore polyacrylamide gel electrophoresis of the "purified factor VIII-related antigen (VIIIR:AG)" (later shown as VWF). Human cryoprecipitate was separated by agarose gel chromatography, and the "purified VIII:RG" (VWF) was eluted at the void volume. Because of the high molecular weight nature of "purified VIIIR:AG" (VWF), it did not enter the gel. However, after dialysis against the low ionic strength buffer (pH7.4) over night, the "purified VIIIR: AG" (VWF) turned to two protein bands, namely slow-moving component (SMC) and fast-moving component (FMC). This phenomenon was a clue to discover ADAMTS13.[21]

Tyr1605-Met1606に相当）結合である事を見出した。しかしながら，このペプチド結合切断がVWF-CP（ADAMTS13）という酵素によってなされている事を示したのは，後述のように1996年のFurlan等[25]，そしてTsai等[26]である。彼らはVWF-CP（ADAMTS13）活性測定法を確立して，TTPの多くの症例ではこの活性が著減しているというブレークスルーを見出した。即ち，VWF機能が弱すぎると出血症状が表面に出てVWDとなり，逆に強すぎると血栓症状が現れてTTPとなる。換言すると，VWD-TTPは両面貨幣（two-sided coin）の間柄にある。これより，真のVWF-CP研究はこの年から始まったと考えられる。前記のvan Mourik等の観察は，彼等が精製したVIIIR:AGはその質量の99%がVWFで，真のVIII因子は1%に満たない程度であった。そして，このVWFに微量ながらVWF-CP/ADAMTS13が混在（接着）しており，これが低イオン強度下で酵素機能を発現したと説明できる。

ADAMTS13活性測定法の進歩

HMW-VWFMは抗生物質リストセチン存在下に強い血小板凝集を惹起する。一方，HMW-VWFMは固相化コラーゲンへの結合能も高く，さらに細コイル灌流，cone plate，そしてparallel plate flow chamberなどで，高ずり応力環境下に暴露すると，ADAMTS13による分解反応を受けて低分子化し，酵素反応が完結すると，VWFはN末端とC末端の2種類のダイマーに転じる事になる。これより，初期のADAMTS13活性測定法には様々なものが考案されたが，酵素消化後の測定原理では，1)リストセチン血小板凝集法[27]，2)VWFサブユニットのN末端とC末端を認識する抗VWF抗体を用いたenzyme-linked immunosorbent assay（ELISA），3)コラーゲン結合能ELISA[28]，4)SDS-アガロース電気泳動[29]，5)SDS-ポリアクリルアミド電気泳動[30]，等に分類される。今日，HMW-VWFMは高ずり応力下では速やかにADAMTS13で分解される事が知られているが，この高ずり応力下での測定系では高感度定量が難しい。それ故，静止系での測定が主に行われているが，この場合，基質は精製HMW-VWFMを用いているため，長時間の反応を要する事と，またVWFが消化され易いように1.5 mol/*l*濃度の尿素やグアニジン塩酸などの蛋白変性剤を加える必要があった。

1996年にスイスのFurlan等は[25] VWF-CP活性測定法の確立を報告し，TTPではこれが著減している事を示した。この知見はブレークスルーであったが，測定原理の着想は前記van Mourik等[21]の報告から得たもので，実施は，精製VWF，正常血漿希釈または患者血漿，終濃度1.5 mol/*l*尿素，10 mM Pefabloc（セリンプロテアーゼ阻害剤）を混和し，低イオン強度（0 mol/*l* NaCl）緩衝液で37℃下に透析しながら24時間酵素消化反応を行うと云うものであった。この反応物はその後VWFM解析を行なって判定すると云う時間浪費性のものであった。一方，ほぼ同時期にアメリカのTsai等は酵素消化を1.5 mol/*l*グアニジン塩酸下に行い，反応物をSDSポリアクリルアミドゲル電気泳動で解析するすると云う方法を報告している。以後，Furlan等[29]とTsai等[30]の二つの研究グループの論文が1998年のNEJMに同時掲載され，TTPが世界の注目を引くようになった。

Cruz等[31]は，ADAMTS13での切断部位はVWF-A2ドメイン内（Tyr1605-Met1606）で，またこの結合の切断を受け易いtype 2 VWDの遺伝子変異がこのドメイン内にある事から，まずHis標識VWF-A2ドメイン（Gy1481-Arg1668）の遺伝子発現蛋白を作成し，この基質が静止系で蛋白変性剤非存在下にADAMTS13で切断される事を見いだし，ELISAによる酵素活性測定系を確立した。Kokame等[32]はVWF-A2ドメイン内での基質狭小化を計り，最終的にAsp1596-Arg1668の73アミノ酸残基が最小基質となる事を示し，このGln1599に蛍光基Nmaを，またAsn1610に消光基Dnpを導入することにより，FRETS-VWF73という蛍光測定法を確立した[33]。本法は，今日ADAMTS13活性測定のgold standard法として世界的に普及している。しかし，本法は被検血漿中の高濃度ビリルビンやヘモグロビンの影響を受ける事が指摘されてきた。これより，最近，蛍光強度を増加させ，前記物質の干渉作用を無くし，また生理的pH条件下で測定可能なFRETS-rVWF71と名付けられた改良型測定法がMuia等[34]によって報告された。

筆者らはルーチンラボでの利便性を考え，chromogenic ADAMTS13 act-ELISAを開発した[35]。これはADAMTS13切断で生じるVWF73の断端アミノ酸残基Tyr1605を特異的に認識するマウスモノクロナール抗体N10（IgG）を得たことにより達成された。本法の測定感度は<0.5%（正常100%）と鋭敏で，前記阻害物質の影響を受けない。一方，他の高感度測定法としてGST-VWF73-Hisを基質に用い，SELDI-TOF-MASで測定する方法[36]が報告されているが，器材が大掛かりである事から汎用性については問題が残る。最近，国内外でADAMTS13活性測定の臨床現場でのリアルタイム測定を視野に入れ，全自動測定法が開発されつつある。一つは化学発光法，他は金コロイド凝集法で，これら2方法は共に前記N10抗体を利用したものである。測定時間はいずれも10分前後で，且つ極めて高感度と伝えられており，今後の進展が待たれる。本邦ではADAMTS13活性とそのインヒビター測定は現在，薬事承認に向けて準備されつつある。

本邦における血栓性微小血管障害症（TMA）患者登録と解析状況の update

筆者らは 1998 年に Furlan 等の VWFM 法による VWF-CP/ADAMTS13 活性測定法を本邦で最初に立ち上げ[37]，この後，全国の医療施設から依頼された検体についても，その測定を受諾する事になった。2001 年以降は測定検体数が飛躍的に増え，当ラボのホームページ〈http://www.naramed-u.ac.jp/~trans/〉を開設し，検体受け入れをシステム化した。また 2005 年 4 月からは前記の新規開発した chromogenic ADAMTS13 act-ELISA による活性測定を取り入れたため，平日に検体を受け取ればその日の内に結果を返却できるシステムも構築できた。

原則として ADAMTS13 活性が著減（通常 <5%）し，同インヒビターが陰性で，後述の患者背景と合わせて先天性 TTP（下記 USS）と診断できたものは，倫理委員会規定に従って国立循環器病研究センターで *ADAMTS13* 遺伝子解析を実施している。一方，ADAMTS13 活性が著減しておらず，また同インヒビターも陰性であるが，TMA 発作の反復性，家族性が認められるものは従来，原因不詳の先天性 TMA（congenial TMA with the etiology unknown）と分類していたが，本年，徳島大学小児科の香美先生や東京大学腎臓・内分泌内科の南学先生が中心となり，非典型溶血性尿毒症症候群（atypical HUS, aHUS）の診断基準が作成された[38]。これによると，我々が原因不詳の先天性 TMA と分類していた患者の殆どが先天性 aHUS のカテゴリーに入る事が示された。先天性 aHUS は補体や補体調節因子の遺伝子異常にて生じ，遺伝形式は通常，常染色体優性である。これに対し，先天性 TTP（USS）の遺伝形式は常染色体劣性なので，最近の患者数は aHUS が USS を遥かにしのぐ状況となっている。過去 15 年間に本邦の医療機関から VWF-CP/ADAMTS13 活性測定を依頼され，実施した検体数は膨大で，集積された TMA 患者数は 2012 年 12 月末で 1,149 例であった（Fig. 2）。これらの内訳を Table 1 に示す。

先天性 TTP（Upshaw-Schulman syndrome, USS）：診断と治療の問題点

小児血液専門医にとって，USS の hallmark とは，「新生児期に Coombs 試験陰性の重症黄疸があり，これは交換輸血で救命されるが，その後も貧血と血小板減少が慢性に経過し，これらの症状が血漿輸血にて劇的に改善

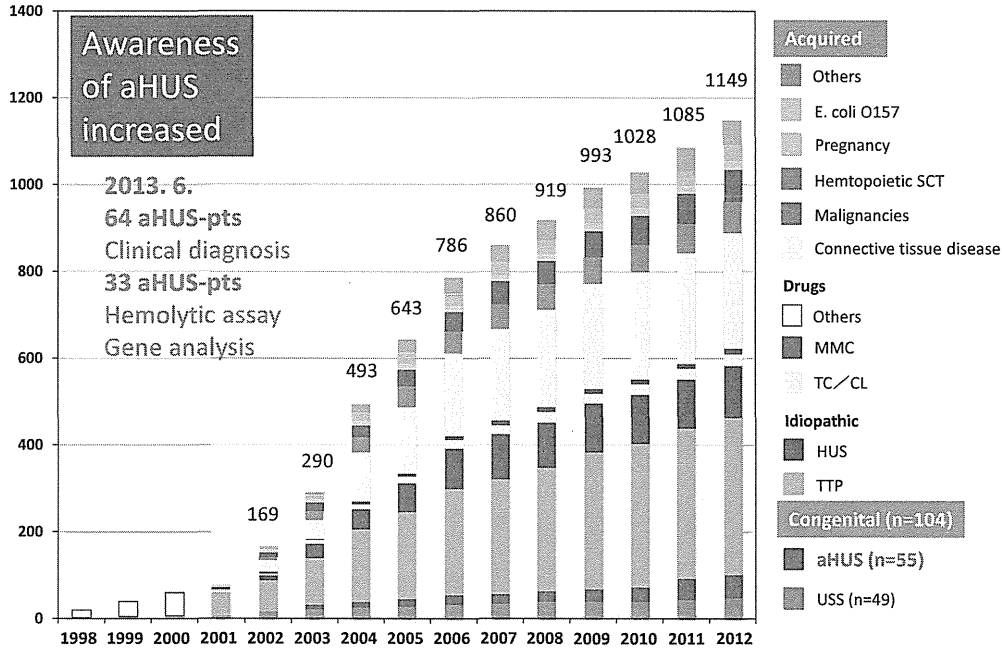


Fig. 2　Cumulative number of TMA patients in NMU registry.
Since 1998, our laboratory of Nara Medical university (NMU) has been functioning as a TMA referral center in Japan through analyzing VWF-cleaving protease/ADAMTS13 activity. Until the end of 2012, the cumulative number reached to 1149, which included 104 patients of congenital TMA (49 with USS and 55 with aHUS). Abbreviations: SCT, stem cell transplantation; MMC, mitomycin C, TC/CL, ticlopidine/clopidogrel.

Table 1 Plasma levels of ADAMTS13 activity and ADAMTS13 inhibitor in 1149 patients with TMA, registered to our laboratory of NMU between 1998 and 2012.

	Congenital TMAs		Acquired TMAs											Total
			Idiopathic		Secondary									
	Upshaw-Schulman syndrome (USS) (n=49)	aHUS (n=55)	Thrombotic thrombocyto-penic purpura (TTP) (n=361)	Hemolytic-uremic syndroime (HUS) (n=119)	Drug-induced (n=42)			Conective tissue diseases and Autoimmune diseases (CTDs/ADs) (n=263)	Malignancies (n=73)	Hematopoietic stem celltransplant-ation (HSCT) (n=75)	Pregnancy (n=19)	Stx-E. coliinfection (n=37)	Others (Liver cirrhosis, etc) (n=56)	(n=1149)
					Ticlopidine (n=25) / Clopidogrel (n=2)	Mitomycin C (n=12)	Others (n=3)							
ADAMTS13:AC (%)	(n=48)	(n=55)	(n=361)	(n=119)	(n=25/n=2)	(n=12)	(n=3)	(n=263)	(n=73)	(n=75)	(n=19)	(n=37)	(n=56)	(n=1148)
<3	46	0	269	0	23	0	3	57	8	0	7	0	19	432
3−<25	2	2	70	22	2	2	0	76	24	23	5	6	17	251
25−<50	0	14	19	58	1	6	0	79	25	34	4	21	7	268
≧50	0	39	3	39	1	4	0	51	16	18	3	10	13	197
ADAMTS13:INH (U/ml)	(n=48)	(n=43)	(n=313)	(n=50)	(n=24/n=2)	(n=9)	(n=3)	(n=206)	(n=32)	(n=26)	(n=9)	(n=22)	(n=30)	(n=817)
≧2	0	0	139	0	18	0	0	31	5	0	4	0	12	209
0.5−<2	0	7	140	3	6	1	3	84	10	5	2	3	10	274
<0.5	48	36	34	47	2	8	0	91	17	21	3	19	8	334

( ) Sample number determined

する疾患」であった。この歴史の詳細については拙著で報告しているので割愛する[39]。実際，USS の病態解明には多くの研究者の貢献があったが，Upshaw と Schulman の両名の名前が今日も残っているのは，この両者のみが 1960 年と 1978 年に，溶血性貧血と慢性血小板減少を示すそれぞれの患者に対して，血小板輸血ではなく血漿輸血により，血小板数が著しく増加する事を報告した事による[40, 41]。当時は血漿中に血小板産生刺激因子（platelet stimulating factor）が存在する事を仮想していたが，この考えは，後年 thrombopoietin（TPO）が同定され，USS との関連は完全に否定された。

筆者等は 1998 年以降，本邦 USS 患者 3 家系の VWF-CP 活性を調べ，患者は活性が著減し，同両親は活性が半減を示すが無症状であることから，本疾患が常染色体劣性遺伝形式であることを報告したが[42]，その 6 ヶ月後 Levy 等[7]により *ADAMTS13* 遺伝子がクローニングされ，USS が *ADAMTS13* 変異遺伝子の劣性遺伝による事が示された。現在，世界で USS 患者は約 150 名同定されているが，筆者等は本邦で 2012 年末迄に 49 名の同患者を同定した。USS 患者で同定された *ADAMTS13* 遺伝子変異を Fig. 3 に示す[43]。注目すべきは欧米で発見された変異と本邦とでは全く異なる点である。また，本邦患者の自然史（natural history）を要約すると Table 2 のようになる。ここで特筆すべきは，前記の USS hallmark である交換輸血を必要とするような新生児重症黄疸を示す early-onset type（早期発症型）は僅か 39％の患者にしか見られない点と，USS の遺伝は常染色体劣性なので，患者数は本来，男女ほぼ同数と想定されるが，登録症例は圧倒的に女性優位である。これは USS 女性の場合，妊娠により TTP 発作が誘導されるので，妊娠適齢期である 20～40 才間に発見され易いことによる。一方，USS 男性にはこの年齢層で正確に診断されている例はなく，40～45 才を超えて初めて TTP 発作を起こして診断されている例が散見される。かかる例は late-onset type（晩期発症型）と表現されている。

Taguchi 等[44]が 2012 年に報告した男性症例を late-onset type の典型例として Fig. 4 に紹介する。症例は 1931 年生で，来院時は 63 才であった。男性はこの年令で初めて TTP 発作を起こして来院したが，直後には血漿交換療法の対応が出来ず，同日は血漿輸注だけを行った。しかし翌日に臨床症状と検査値の双方に著しい改善がみられ，以後は TTP 発作時に血漿輸注のみで治療していた。しかし加齢と共に TTP 発作回数も増加し，77 才時（2007 年）には小脳梗塞を起こした。この時点で

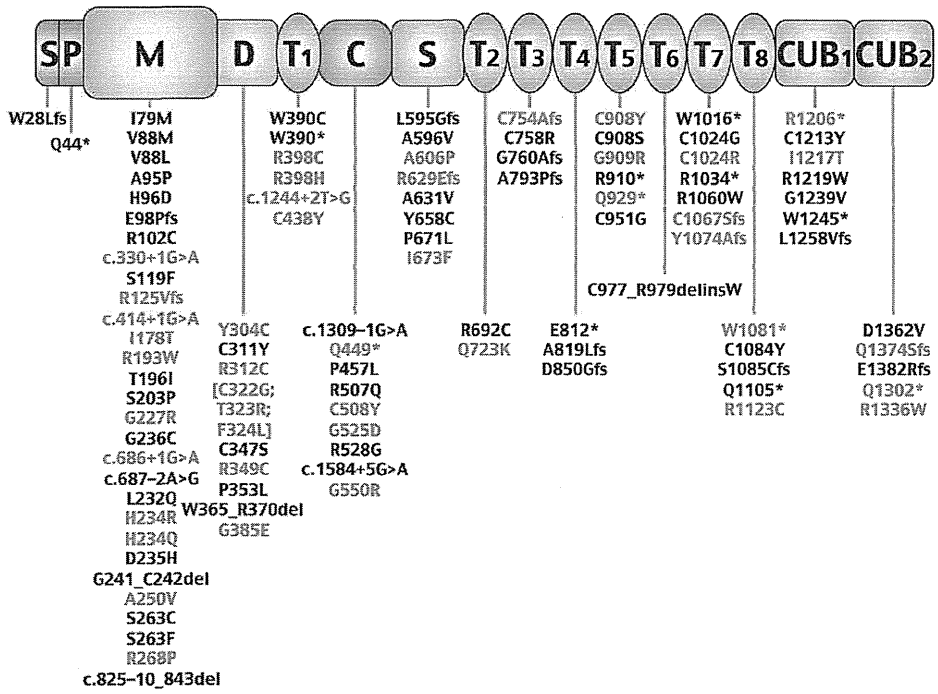


Fig. 3 *ADAMTS13* gene mutations responsible for congenital TTP (Upshaw-Schulman syndrome, USS).
The description of protein sequence mutation follows the recommendation of the Human Genome Society (WWW.hgvs.org/mutnomen/recs-prot.html). Mutations in red were identified in Japanese patients[43].

Table 2 Summary of natural history in 49 patients with Upshaw-Schulman syndrome in Japan.

Family:
- **43** families
- Female predominance (**31** F and **18** M)
- Up to **79** years of age

EXBT to severe newborn jaundice: **19** pts/**49** (**39**%)
Hemodialysis for CKD: **5** pts (**3** pts were dead)

Aggravating factors:
- Severe infections such as FLU
- **Pregnancy**: **23** occasions in **13** female pts (**1** pt was diagnosed after death)
- DDAVP
- interferon
- **heavily drinking alcohol**
- **Aging**

ADAMTS13 gene analysis in 47 pts: **9** homo. and **38** compound hetero.

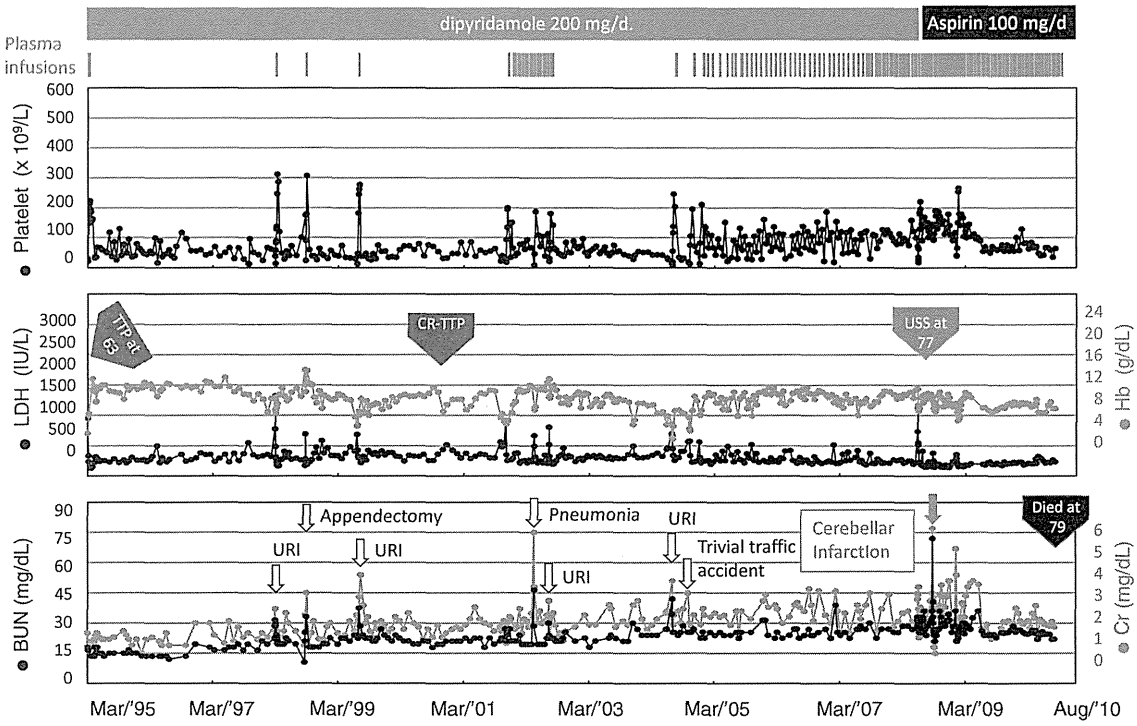


Fig. 4 Clinical course of a representative case of Upshaw-Schulman syndrome (USS) with the late-onset phenotype. This Japanese male patient first developed a TTP-bout at the age of 63 years and was admitted to a local hospital, where plasma exchange was not ready available, and so he was treated with plasma infusion, which dramatically improved his clinical conditions on the following day. Since then, he had been treated with plasma infusion on demand at TTP-bouts, but its frequency became quite often with aging. At age of 77 years, he had cerebellar infarct, and on that occasion ADAMTS13 was analysed, that disclosed a moderate deficiency of ADAMTS13 activity (2.4-3.4% of the normal) and a homozygous *ADAMTS13* gene mutation of p.C1024R. His parents were indeed the first cousins. As for his natural history, he worked as a business man until the age of 60 years and received health examination annually, but he never had appreciable abnormalities in both labolatory and clinical findings[44].

ADAMTS13 解析を実施し，ADAMTS13 活性は 2.4～3.4％と著減し，また *ADAMTS13* 遺伝子解析では p.C1024R のホモ接合型変異が同定された。これより，家族歴を詳細に再調査すると患者の御両親はいとこ婚であった。本患者は会社員として定年迄勤務し，その間毎年定期健康診断を受けていたが，特に異常を指摘された事はなかったという。本患者はこの後 79 才で小脳梗塞の関連後遺症で逝去されたが，本症例が典型的な late-onset phenotype の経過模様を示した理由として，(1)男性，(2)ADAMTS13 活性は 2.4～3.4％と僅かながら検出される例であった，の 2 点があげられる。これは TTP 発作の契機は前記のように，血管内皮細胞からの UL-VWFM 放出が過剰に起こる時と考えられており，女性では妊娠，特に妊娠後期がこれにあてはまるが，男女を問わず，40 才を超えると VWF 量は青年期のほぼ 1.5 倍に増えてくる事が知られているので，男性ではこの年齢層（40～60 才）で TTP 発作が発来し，USS 体質が発見され易いと説明される。

【治療】 USS 患者の約半数は診断確定後，約 2 週間に 1 回の頻度で定期的 FFP 輸注（5～10 m*l*/kg BW）を受けている。これは ADAMTS13 活性と抗原の血中半減期が略 2.5 日である事と，輸血後に血小板数は 7～10 日目でピークを迎えると云うデータに基づいている。懸念される ADAMTS13 インヒビター発生の報告例は現在のところ皆無であるが，我々のデータでは[45] 2011 年に検索した USS 患者 43 例中 7 例（16.2％）に IgG 型の非中和抗体が検出されているので，今後，中和抗体の発生についても慎重に見守ってゆく必要性があると考えている。一方，日本赤十字社の血液製剤については 1988 年以降から HCV 検査が導入されているが，それ以前から血漿輸注療法を受けている USS 患者では HCV 既感染者もいる。現在，遺伝子発現 ADAMTS13 製剤の Phase I 臨床治験が欧米で開始されたと伝えられているので，その成果と同製剤の速やかな臨床への導入が期待されている。

後天性 TTP：診断と治療の問題点

TTP 患者の 90％以上は後天性 TTP で，この診断は，ADAMTS13 活性著減と ADAMTS13 に対する活性中和抗体（IgG 型インヒビター）あるいは非中和抗体（主に IgG 型）が陽性の結果で診断される群と，ADAMTS13 活性は略正常であっても古典的 5 徴候（pentad）でなされる群の 2 種類がある。筆者等は前者を仮に定型 TTP，後者を非定型 TTP と呼んでいるが，非定型 TTP は非典型溶血性尿毒症症候群（atypical hemolytic uremic syndrome, aHUS）との鑑別が困難で，また aHUS には先天性と後天性の双方があるため，後天性 TTP で定型と非定型の患者割合は報告者によって大きく異なる。筆者らが 1998～2012 年の間に奈良医大輸血部で登録した 1,149 名の TMA 患者の解析結果では TTP は 361 名で TMA 全体の 31.4％（361/1,149）であり，このうち 74.5％（269/361）は定型 TTP であった。TTP の発生頻度については ADAMTS13 発見以前に，人口 100 万人当たり 4.7 人という数字が出されているが，現在ではこれよりも遥かに高い頻度と推定される。実際，今日では血小板減少と貧血があり，ADAMTS13 活性著減が確認されれば，これで定型 TTP と診断されている。後天性・定型 TTP では再発率が高い事，また非定型 TTP ではこれが少ない事も近年報告されている。一方，後天性の定型及び非定型 TTP のいずれもが，一次性（特発性）に生じるものと，基礎疾患，薬物，移植等に関連して二次性に生じるものがある。また，後天性・定型 TTP に発生する IgG 型 ADAMTS13 インヒビターのエピトープは共通して Spacer ドメイン内にある。このドメインは VWF 切断時に，この基質を捕捉するに重要な部位と考えられており，Pos 等[46, 47] はこのエピトープの詳細な解析にて Spacer ドメイン内の 5 アミノ酸残基（Arg568-Phe592-Arg660- Tyr661-Tyr665）が特に重要である事を示した。

【治療】(1)血漿交換（plasma exchange, PE）：後天性定型的 TTP に対する first-line 治療は PE である。PE の効用については，① ADAMTS13 インヒビター除去，② UL-VWFM の除去，③ ADAMTS13 の補充，④止血に必要な正常サイズの VWFM の補充，そして⑤炎症性サイトカインの除去，などで説明されている[48]。実施は 1 回 40～60 m*l*/kg（1 日当たり循環血液〔血漿〕量の 1～1.5 容）を輸注する。開始して 3 日間は 1 日当たり循環血液〔血漿〕量の 1.5 容/回で連日行う。殆どの症例でステロイド使用やステロイドパルス療法（後述）が併用される。PE の効果は血小板数，神経症状，LDH などの溶血モニタ—を観察しながら判断し，反応が悪ければ 5 日間連日行う事もしばしばある。現行 PE に対する保険適応は週 3 回，1 ヶ月に 12 回，期間は 3 ヶ月以内という制限がある。しかし，後述のように難治性・後天性 TTP においては様々な免疫療法と PE の併用が長期に渡って求められる事がしばしばあり，保険適応の期間と回数については，早急に見直しが必要と考えている。**(2)ステロイド療法**：多くの症例で，ステロイドパルス療法として PE 開始初期から実施されている。その方法は，PE 終了直後にメチルプレドニゾロン 1,000 mg を生理食塩水 100 m*l* に溶解し，1 日 1 回約 1 時間かけて点滴静注する。これを 3 日間連続して行う。4 日目からはプレドニン錠（5 mg）を 1 mg/kg，分 1 で投与開始する。その後，臨床症状を見ながら急速に減量する。**(3)抗血小板薬**：PE にて血小板数が回復し始めた時期（英国ガイドラインでは 50,000/u*l* 以上）に使用が考慮され，1)

低用量アスピリン（100 mg）1錠 分1（保険適用外），の使用が推奨されている。その他として，2)ペルサンチン（100 mg）3錠 分3（保険適用外）があるが，出血時間延長が懸念される。最近，出血副作用が少ない為に，TTP治療の新しい抗血小板療法剤として注目されているのが，3)シロスタゾール（100 mg）2錠 分2（保険適用外）である。しかし，これら全ての抗血小板剤については，その効果検証の課題が残っている。**(4)難治性TTPに対する治療**：PE治療に抵抗性を示す症例に対し，保険適応外であるが，1)シクロスポリン経口療法（保険適用外）〔処方例〕ネオーラルカプセル 6 mg/kg 分3（保険適用外），2)シクロホスファミド経口療法〔処

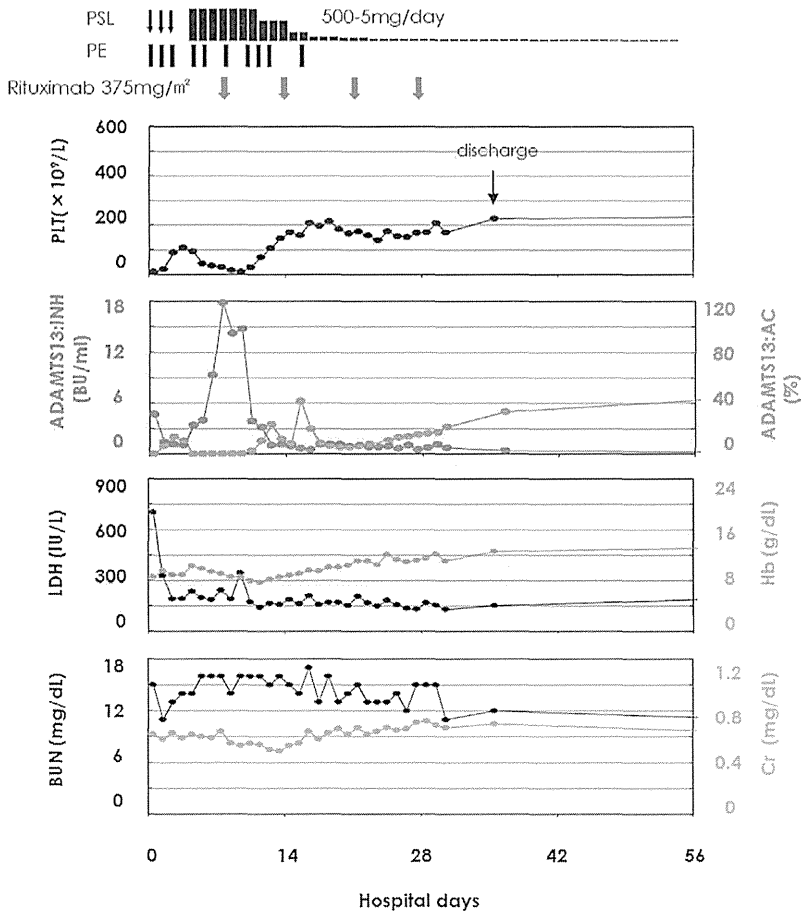


Fig. 5 The clinical course of ai-TTP patient with ADAMTS13 inhibitor boosting by plasma exchange.
A 67-year female was admitted to our hospital with complaints of thrombocytopenia (platelet count $10\times10^9/l$), hemolytic anemia (hemoglobin 8.6 g/d*l* and lactate dehydrogenase 703 IU/*l*), unconsciousness, and fever (38°C), without any known etiology. At that time, plasma levels of ADAMTS13 activity and inhibitor were <0.5% (of the normal). and 4.7Bethesda U/m*l*, respectively. Plasma exchange was performed for the consecutive 3 days with a steroid pulse therapy (methylpredonisolone 1 g/day×3) that resulted in an increase of ADAMTS13 activity (13%) and/or platelet count ($100\times10^9/l$). However, soon after this, both platelet count and ADAMTS13 activity dropped again, and the inhibitor titer was re-increased up to 18 Bethesda U/m*l* (inhibitor boosting). Rituximab was then administered from the day 7 after plasma exchange initiation, with a dose of 375 mg/m^2 per week for 4 times. Then, the clinical and laboratory findings rapidly improved to remission.

方例〕エンドキサン錠（50 mg）2錠 分2（保険適用外），3)ビンクリスチン（VCR）注 初回1～2 mg 静注。1週間後1 mg 追加静注（保険適用外），4)リツキシマブ注1回 375 mg/m^2，1週1回，点滴静注，4～8回（保険適用外）（後に詳述），5)脾摘などがある。**(5)次世代のTTP 治療薬**：最近，欧米では recombinant (r) ADAMTS13 製剤の Phase I 治験が開始された。一方，ADAMTS13 は UL-VWFM の構造・機能モジュレーターで，作用物質は UL-VWFM である。UL-VWFM 依存性高ずり応力惹起血小板凝集においては，VWF-血小板膜糖蛋白 GPIb 軸の反応制御が最も効果的な抗血栓作用を示すと考えられている。このような分子標的療法として，AWJ-2 に代表される VWF-A1 ドメイン認識キメラ抗体，南米ラマで産生された nanobody（AKX-0681, Abylynx NV 社，Belgium），VWF-A1 ドメインアプタマー（ARC 1779，Archmix 社，Boston）等がある。これらの多くが，海外では臨床治験対象と考えられている。

【リツキシマブと Inhibitor boosting】リツキシマブは本来 B 細胞性リンパ腫の治療薬であるが，本剤が後天性・定型 TTP に対しても極めて有効である[49]との結果が 2002 年以降，多数報告されている。これより，2012 年にフランスの TMA レファレンスセンターグループによって PE と併用したリツキシマブ治療効果について，22 例の TTP に対して前方視的研究がなされた[50]。この時のリツキシマブ投与量は 375 mg/m^2 で，回数は計4回と従来法に準じているが，投与日は TTP 診断日を X とすると，(X, X+3, X+7, X+14) 日と最初の2週間以内に4回投与する形になっている。結果は，1)血小板数の回復が速やかで，寛解に至るまでの期間が短縮した，2)寛解後1年以内の再発が皆無であった，3)この1年期間中，ADAMTS13 活性はリツキシマブ使用群でより高値，また同結合 IgG 抗体はより低値との結果が示された。しかし予想に反して，4)PE の血漿使用量については両群で差を認めず，また，5)末梢の B リンパ球枯渇期間9ヶ月は B-リンパ腫での使用と同じであった。これを裏付けるように，実際，この期間中の再発は皆無であった。しかし，6)寛解後1年を超えると再発例が 14%（3/21，1例は早期死亡）に見られた。

我々は最近，難治性 TTP の一表現型として，PE 後に IgG 型の ADAMTS13 インヒビター力価が急上昇（inhibitor boosting）するものがある事を確認した（Fig. 5）。自験例の後天性・定型 TTP では約 40%がこの型を示した。この inhibitor boosting が確認されると，通常の PE 単独療法やステロイドパルス療法の併用も殆ど効果がなくなる。かかる例には PE とリツキシマブの併用は必須と考えている。我々が経験した典型例では，PE 治療前に高いインヒビター力価（>2 Bethesda U/m*l*）を示した TTP 症例は，PE 治療後，数日間は反応して血小板数も増大するが，1週間以内に血小板数の再低下が見られ，この時点で inhibitor boosting が確認された。かかる例にはリツキシマブの効用はより顕著であった。このような事実を受け，ごく最近，後天性定型的 TTP に対するリツキサンの医師主導型治験（代表：埼玉医科大学総合診療内科　宮川義隆）の実施が厚生労働省から認可された。該当症例は ADAMTS13 活性著減例で，PE 連続5日間実施するも臨床症状の改善のないもの，あるいは PE 治療前の ADAMTS13 インヒビター力価が2 Bethesda U/m*l* 以上と高いものとの条件がある。興味のある方は宮川先生までご連絡下さい。

謝　辞

本研究は，1999～2000 年間は厚生省特定疾患：血液系疾患調査研究班—血液凝固異常症分科会（分科会会長：中川雅夫　京都府立医科大学教授），2001～2006 年間は厚生労働科学研究費補助金の難治性疾患克服研究事業：血液凝固異常症に関する調査研究（主任研究者：池田康夫　慶応義塾大学医学部教授），そして 2007 年以降は継続同班（主任研究者：村田満　慶応義塾大学医学部教授）からの研究費，文部科学省科学研究助成費，そして武田医学特別研究助成費にて行った。ここに謝辞を記す。

著者の COI（conflicts of interest）開示：藤村吉博；特許使用料（アルフレッサファーマ株式会社），講演料（旭化成株式会社，アレクシオンファーマ株式会社），研究費（アレクシオンファーマ株式会社，武田医学特定研究助成）

文　献

1) Moschcowitz E. Hyaline thrombosis of the terminal arterioles and capillaries: a hitherto undescribed disease. Proc NY Pathol Soc. 1924; **24**: 21-24.
2) Amorosi EL, Ultmann JE. Thrombotic thrombocytopenic purpura: report of 16 cases and review of the literature. Medicine (Baltimore). 1966; **45**: 139-159.
3) Gerritsen HE, Robles R, Lämmle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. Blood. 2001; **98**: 1654-1661.
4) Plaimauer B, Zimmerman K, Völkel D, et al. Cloning, expression, and functional characterization of the von Willebrand factor-cleaving protease (ADAMTS13). Blood. 2002; **100**: 3626-3632.
5) Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. Blood. 2001; **98**: 1662-1666.