# ADAMTS13 safeguards the myocardium in a mouse model of acute myocardial infarction

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Dear Sirs,

The adhesive protein von Willebrand factor (VWF) plays an essential role on haemostasis (1–3). However, excessive functions of VWF could trigger thrombotic complications. To prevent this, the VWF-cleaving protease ADAMTS13 negatively regulates VWF function by reducing the size of VWF multimers, thereby decreasing their thrombogenic potential (1–3). Since the VWF function is dependent on shear stress (1–4), the relevance of ADAMTS13 may be more pronounced in the microcirculation (5), which is characterised by high shear stress created by blood flow. Indeed, functional deficiencies of ADAMTS13 cause thrombotic occlusion of the microvasculature, e.g. arterial capillaries, resulting in thrombotic thrombocytopenic purpura (3, 6).

Previously, we (7) and others (8, 9) reported that ADAMTS13 deficiency aggravates the extent of brain ischaemic stroke in a mouse model of ischaemia/reperfusion injury by middle cerebral arterial occlusion, suggesting that ADAMTS13 is neuroprotective. These studies demonstrated that ADAMTS13 plays a beneficial role in the microcirculation, which is critical for the preservation of organ functions, raising the possibility that ADAMTS13 might also play a role in coronary ischaemic events such as myocardial infarction. We investigated this possibility in an experimental model of acute myocardial infarction in ADAMTS13 gene deleted (*Adamts13* -/-) mice.

*Adamts13*-/- (KO) mice were generated on C57BL/6 background by our study group, as described (7, 10). All mice were 12–14 weeks of age, healthy, fertile, and had body weights of 25–30 grams. Mouse experiments were done according to protocols approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University. Researchers were blinded to the genotype of each animal until all studies were completed. Experimental acute myocardial infarction (AMI) in mice was induced as previously described (11). Briefly, following anesthesia by diethyl ether inhalation and insertion of a polyethylene tube into trachea, the left anterior descending coronary artery was ligated with a polyamide suture 2 mm from the tip of the left auricle, under thoracotomy with ventilator-assisted respiration. The same procedure without coronary artery ligation was performed in sham operations. In some experiments, recombinant human ADAMTS13 (3 μg/mouse, equivalent to 2,800 U/kg) was injected intravenously in 30 minutes (min) after the operation. This recombinant protein (designated as MDTCS) used was previously described (12). In brief, MDTCS spans from the metalloproteinase (M) domain to spacer (S) domain (amino acid residues 75–685); it possesses VWF-cleaving activity equivalent to whole ADAMTS13 molecule, as evaluated by the *in vitro* FRETS-VWF73 assay (12, 13)

Seven days after the coronary artery ligation, mouse cardiac (left-ventricular) function was evaluated by M-mode echocardiography. Subsequently, mice were sacrificed and their hearts were excised for histological analysis of myocardial infarction, as previously described (11). In brief, the ventricles of excised hearts were cut into 1-mm transverse slices and subjected to 2,3,5-triphenyltetrasolium chloride (TTC) and Azan staining. After inspection of the TTC specimens confirmed that myocardial infarction was successfully induced in mice, the "infarction ratio" was calculated from the Azan specimens by computer-assisted image analysis (analySIS software-version 2007; Olympus Soft Imaging Solutions). Infarction ratio was defined as the ratio of the area with fibrin deposition, corresponding to the infarct, to the total area of left ventricle.

Echocardiography revealed significantly increased end-diastolic diameter of left ventricle and reduced ejection fraction in knock-out (KO) mice, compared to wild-type mice, indicating that cardiac functions are relatively poor in KO mice (▶Fig. 1A). In addition, histological studies revealed significantly larger infarctions in myocardia of KO mice (▶Fig. 1B). Intravenous administration of recombinant human ADAMTS13 rescued the myocardial symptoms in KO mice (▶Fig. 1). Thus, our results clearly indicate that as in brain ischaemic stroke, ADAMTS13 plays a role in safeguarding the myocardium from coronary artery ischaemia.

During the preparation of this manuscript, a similar study by De Meyer et al. (14) appeared, demonstrating a protective effect of ADAMTS13 in mouse myocardial infarction. Those authors used a protocol for AMI induction somewhat different from ours: their study (14) and all previous brain stroke studies (7–9) employed a transient ischaemia/reperfusion model to experimentally induce ischaemia. By contrast, our approach to AMI induction, a persistent coronary artery ligation, represents a greater challenge regarding recovery of organ function following ischaemic damage, further highlighting the favorable effects of ADAMTS13. The successful res-

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cue by recombinant ADAMTS13 in our more stringent system, in which it was administered just after the AMI induction, may imply the therapeutic potential for patients with acute coronary syndrome. Interestingly, our truncated recombinant molecule (MDTCS) was found to be fully effective *in vivo*, although the functional relevance of carboxyl-terminal domains of ADAMTS13, lacking in MDTCS, was controversial under flow conditions (15, 16).

The mechanisms underlying the beneficial effects of ADAMTS13 on myocardium remain poorly understood. As discussed in the previous brain stroke study (7), ADAMTS13 possibly prevents the thrombotic occlusion of microvasculature at the post-ischaemic reperfusion stage. In light of close associations between AMI and inflammation, the regulation of inflammatory mechanisms (17) could be critically involved in this regard. Indeed, De Meyer et al. (14) demonstrated that the recombinant ADAMTS13 infusion effectively reduced the neutrophil accumulation within infarct area, underscoring anti-inflammatory effects of ADAMTS13.

Since the activity of VWF (1–4) as well as ADAMTS13 (5) accelerates in a shear stress-dependent manner, the down-regulation of VWF-dependent inflammatory responses by ADAMTS13, such as leukocyte recruitment (17), is assumed to be more crucial in the microcirculation system, where blood flow creates a typical high shear stress. The small vessels of the microvasculature, such as arterial capillaries, can be plugged even by a single leukocyte. Such blockage could cause ischemic damage in vital organs even in the absence of thrombotic vessel occlusion by platelet aggregate formation. In fact, our histological examination did not reveal any increase in the incidence of thrombotic lesions in the microvessels in heart tissues of KO (results not shown).

Our results demonstrate that proper functional regulation of von Willebrand factor-dependent thrombotic or inflammatory responses by ADAMTS13 could contribute to better local microcirculation, which is crucial for healthy organ function. These findings suggest that ADAMTS13 may have therapeutic potential against acute coronary syndromes.

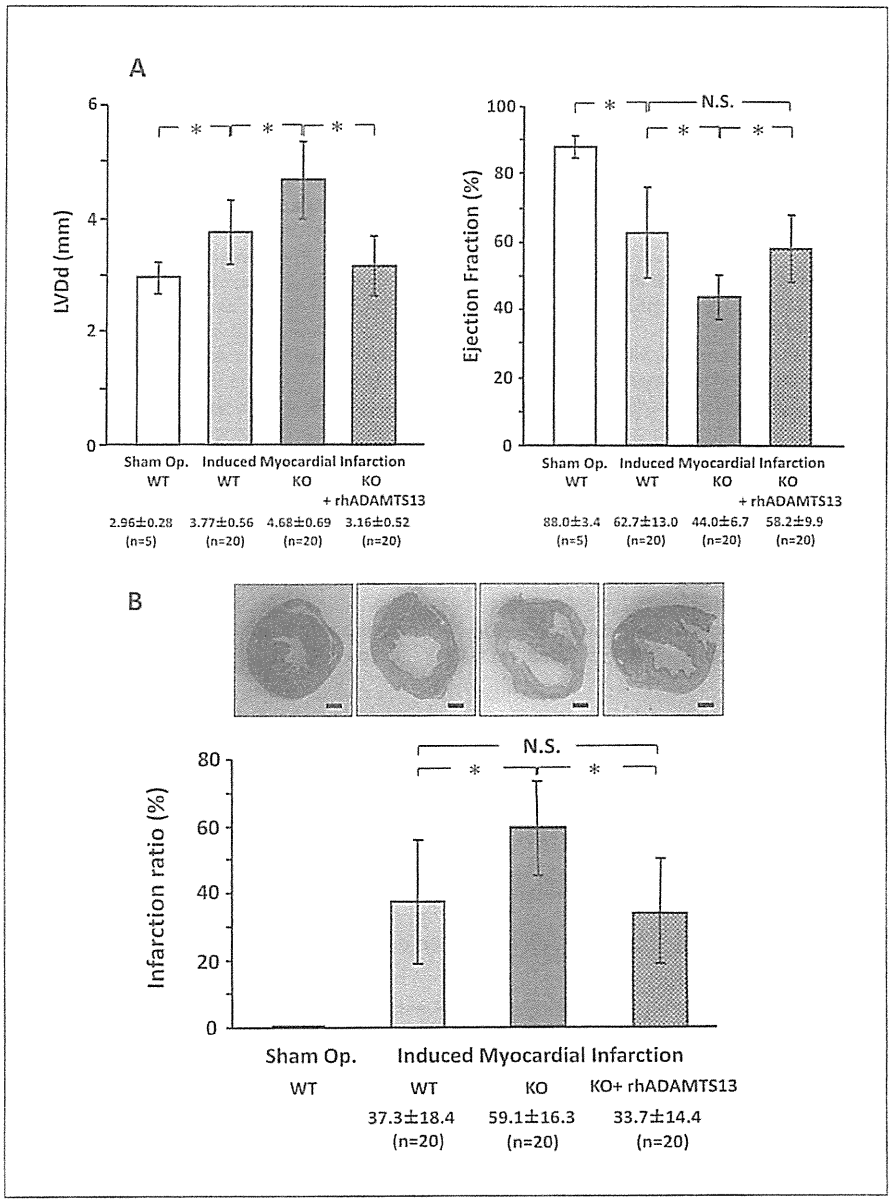


**Figure 1: Evaluation of cardiac functions by echocardiography and histological evaluation in wild-type (WT) or ADAMTS13 KO mice with induced myocardial infarction.** Acute myocardial infarction (AMI) was successfully induced in 20 WT mice (9 male, 11 female) and 20 KO mice (10 male, 10 female). In another 20 KO mice (8 male, 12 female), recombinant human ADAMTS13 (3 μg/mouse) was injected intravenously in 30 min after induction of AMI (KO+rhADAMTS13). Results of sham operation in five WT mice (2 male, 3 female) are also included in the figure. A) Statistical analysis of M-mode echocardiography indicates that KO mice exhibited significantly (*$p < 0.01$) increased left ventricular end-diastolic diameter (LVDd; left panel) and decreased ejection fraction (right panel) compared to WT. Note that the reduced cardiac functions observed in KO mice were improved by rhADAMTS13 injection, to become comparable (N.S.; not significant) with those of WT mice. All data are expressed as mean ± standard deviation. Differences between two groups of data were evaluated by Student's t-test. P-values < 0.05 were considered to denote statistical significance. B) Upper panels: representative microscopic images of transverse sections of ventricle subjected to Azan staining (original magnification; 20X, scale bars, 1 mm). Vital heart tissue is indicted in red; fibrin deposition, corresponding to the infarct area, is indicated in blue. In agreement with results of echocardiography, the infarction ratios (lower panel), corresponding to the upper images, indicated that myocardial infarctions were significantly (*$p < 0.01$) larger in KO mice than in WT mice, but were reduced by rhADAMTS13 injection, to become comparable (N.S.) with those of WT mice.

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**Conflicts of interest**
None declared.

## References

1. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. J Thromb Haemost 2003; 1: 1335–1342.
2. Sugimoto M, Matsui H, Mizuno T, et al. Mural thrombus generation in type 2A and 2B von Willebrand disease under flow conditions. Blood 2003; 101: 915–920.
3. Lenting PJ, Pegon JN, Groot E, et al. Regulation of von Willebrand factor-platelet interactions. Thromb Haemost 2010; 104: 449–455.
4. Matsui H, Sugimoto M, Mizuno T, et al. Distinct and concerted functions of von Willebrand factor and fibrinogen in mural thrombus growth under high shear flow. Blood 2002; 100: 3604–3610.
5. Shida Y, Nishio K, Sugimoto M, et al. Functional imaging of shear-dependent activity of ADAMTS13 in regulating mural thrombus growth under whole blood flow conditions. Blood 2008; 111: 1295–1298.
6. Levy GG, Nichols WC, Lian EC, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature 2001; 413: 488–494.
7. Fujioka M, Hayakawa K, Mishima K, et al. ADAMTS13 gene deletion aggravates ischemic brain damage: a possible neuroprotective role of ADAMTS13 by ameliorating postischemic hypoperfusion. Blood 2010; 115: 1650–1653.
8. Zhao B-Q, Chauhan AK, Canault M, et al. von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic brain injury in experimental stroke. Blood 2009; 114: 3329–3334.
9. De Meyer SF, Schwarz T, Deckmyn H, et al. Binding of von Willebrand factor to collagen and glycoprotein Ib alpha, but not to glycoprotein IIb/IIIa, contributes to ischemic stroke in mice. Arterioscler Thromb Vasc Biol 2010; 30: 1949–1951.
10. Banno F, Kokame K, Okuda T, et al. Complete deficiency in ADAMTS13 is prothrombotic, but it alone is not sufficient to cause thrombotic thrombocytopenic purpura. Blood 2006; 107: 3161–3166.
11. Takeda Y, Uemura S, Iwama H, et.al. Treatment with recombinant placental growth factor (PIGF) enhances both angiogenesis and arteriogenesis and improves survival after myocardial infarction. Circ J 2009; 73: 1674–1682.
12. Akiyama M, Takeda S, Kokome K, et al. Crystal structures of the noncatalytic domains of ADAMTS13 reveal multiple discontinuous exosites for von Willebrand factor. Proc Natl Acad Sci USA 2009; 106: 19274–19279.
13. Kokame K, Nobe Y, Kokubo Y, et al. FRETS-VWF73. A first fluorogenic substrate for ADAMTS13 assay. Br J Haematol 2005; 129: 93–100.
14. De Meyer SF, Savchenco AS, Haas MS, et al: Protective anti-inflammatory effect of ADAMTS13 on myocardial ischemia/reperfusion injury in mice. Blood 2012; Epub ahead of print. doi:10.1182/blood-2012–06–439935.
15. Tao Z, Wang Y, Choi H, et al: Cleavage of ultralarge multimers of von Willebrand factor by C-terminal-truncated mutants of ADAMTS-13 under flow. Blood 2005; 106: 141–143.
16. Zhang P, Pan W, Rux AH, et al. The cooperative activity between the carboxyl-terminal TSP1 repeats and the CUB domains of ADAMTS13 is crucial for recognition of von Willebrand factor under flow. Blood 2007; 110: 1887–1894.
17. Chauhan AK, Kisucka J, Brill A, et al. ADAMTS13: a new link between thrombosis and inflammation. J Exp Med 2008; 205: 2065–2074.

# Phenprocoumon and acenocoumarol treatment in paediatric patients

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Dear Sirs,

Vitamin K antagonists (VKAs) are increasingly used in children. Data on dosing, safety and efficacy in paediatric patients are limited and mostly concern warfarin (1–3). Only one study prospectively investigated the initial and maintenance dosages of acenocoumarol in children, and no study about phenprocoumon is available (2). As a consequence, treatment recommendations are usually based on adult guidelines. We performed this multicenter retrospective cohort study to evaluate initial and maintenance dosages of phenprocoumon and acenocoumarol in various paediatric age groups, and to describe the treatment quality, efficacy and safety of both VKAs.

We studied 163 consecutive children aged 0 to 18 years treated with phenprocoumon or acenocoumarol in the Leiden University Medical Center (LUMC), Academic Medical Center (AMC) in Amsterdam and the anticoagulation clinics in Leiden, Amsterdam and Rotterdam between January 1998 and May 2009. Data were collected from medical records and databases. To study the pharmacodynamics, two therapeutic international normalised ratio (INR) ranges (therapeutic range, TR) were used: INR 2.0 to 3.0 and INR 2.5 to 3.5. These ranges were based on international recommendations for children, which did not change during the ten-year study period (4, 5). To achieve and maintain these TRs, the anticoagulation clinics aimed for a slightly higher INR range (2.0 to 3.5 and 2.5 to 4.0, respectively). Treatment quality included: i) time to achieve TR, ii) number of INR tests and iii) dose changes, and (4) time spent within the TR. The percentage time spent within the TR was measured by linear interpolation, assuming that the INR values vary linearly between two INR control moments (6–8). This is considered from 14 days before an INR measurement up to 14 days after the INR measurement. If the interval between two INR measurements exceeded 28 days, the INR was considered not predictable for the middle part of this interval. The number of days that exceeded the interval of 28 days was considered as per-

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ORIGINAL ARTICLE

# Global haemostasis and point of care testing

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**Summary.** The evaluation of the coagulation profile has used so far either clotting-based or chromogenic assays with different endpoints. Clotting-based techniques are the most used worldwide, and they certainly are useful for diagnosis of clotting factor deficiencies. However, the information provided is relatively limited, and therefore the individual profile of coagulation is poorly assessed. This is reflected by the weak correlation between the results of these assays and the clinical phenotype. Among the assays that benefited from technological advances, thrombin generation and thromboelastography are probably the most actively investigated, but they require specific instruments and are not fully automated. Their standardisation level is rapidly progressing, and they are progressively entering the clinical scene, with the attempt to provide additional information on the coagulation process and a meaningful clinical correlation. These inherited bleeding disorders frequently require replacement therapy using clotting factor concentrates that increase the plasma level of the missing clotting factor. The classical adjustment of the therapy is mainly based on the measurement of the plasma clotting activity of the protein administered. If one considers that a certain level of thrombin generated would predict clinical efficacy, monitoring of thrombin formation might offer new possibilities to individually predict the bleeding phenotype, select the most adapted therapeutic product and tailor the dose. The same holds true for thromboelastography/thromboelastometry which evaluate fibrin formation as well as clot resistance to fibrinolytic challenge, one step further down in the coagulation process. In this regard, these 2 assays could be seen as complementary in terms of information provided on the coagulation profile at the individual level.

Keywords: aggregation, bleeding disorders, dense granule release, platelet function

## Introduction

Clot waveform analysis represents another assay for assessing global clotting function. It is based on the continuous monitoring of light transmittance or absorbance during routine coagulation tests such as the activated thromboplastin time and the prothrombin time. During clot formation in these assays, changes in light transmittance are analyzed by continuous measurements and are designated the clot waveform. This assay has been applied not only for diagnosis and evolution of sepsis, but also in the field of inherited bleeding disorders. Among its interests is the fact that it could be used on several coagulation instruments with a dedicated software.

However, the correlation of the parameters deduced from these various global clotting assays with *in vivo* bleeding phenotype and the clinical response to therapeutic agents still requires further clinical studies on larger cohorts. If haemophilia has been mainly studied so far, relatively little is known for the rare inherited coagulation disorders. In this regard, specific information should be provided in the near future.

Platelet inherited function defects also contribute to the aetiologies of bleeding disorders, with usually a slightly different clinical phenotype. The investigation of these platelet defects mainly uses light transmittance aggregometry in response to various agonists and dense granule release assays. They are relatively commonly performed, but the standardisation of the concentrations of each agonist is important for establishing proper diagnosis of the platelet defect. In addition, careful evaluation of the modification of the aggregation curves in response to one or multiple agonists should be considered for detection of common platelet function defects. The detection of platelet release abnormalities uses dense granule adenosine triphosphate evaluation. Because ATP release shows significant variability, abnormalities in platelet function should be confirmed

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on another sample. Implementation of recommended guidelines using validated reference intervals for maximal aggregation and quality assurance should ensure an improved diagnosis of platelet function disorders which should limit the risk of false positive or negative findings.

## Thrombin generation assay

Haemostasis is a dynamic process that involves both several plasma proteins and cellular components interacting in a highly complex system that leads to fibrin clot formation. The complexity of the haemostatic system is associated with highly variable responses of patients to haemostatic challenges. To reliably determine the haemostatic profile of a patient may be highly relevant for tailoring therapies to the individual needs of each given patient with bleeding disorders. Routine coagulation assays, which use the formation of detectable fibrin as end point, have limited usefulness in evaluating the clinical outcome of patients with bleeding risk. The reasons of the phenotypic heterogeneity are not fully understood. The co-inheritance of thrombophilia factors or increased levels of other coagulation proteins might be responsible for variations of the bleeding phenotype [1–3]. Thrombin generation tests measuring the final enzymatic product of the coagulation system, thus taking into account not only factor VIII/IX levels but also the activity of other coagulation factors, inhibitors and the effect of platelets may better correlate with the clinical haemostatic profile of patients [4,5]. A correlation between thrombin generation test results and clinical bleeding phenotype has already been reported by several groups. It has been reported that patients with haaemophilia and severe clinical bleeding tendency have a low thrombin generating capacity (endogenous thrombin potential, ETP < 50% of normal), independently of their fVIII/fIX levels [6–8]. A recent case-control study showed that ETP measurement in platelet-rich plasma was able to identify patients with severe haemophilia but with a mild clinical phenotype [8].

The development of an inhibitor in patients with haemophilia renders treatment and prevention of bleeding episodes more challenging. The optimal use of bypassing agents is hampered by a lack of laboratory assays to evaluate and monitor therapeutic efficacy of these drugs and determine adequate dosing. The capability of determining most effective therapeutic option and the optimal individual dose of bypassing agents for a given patient would represent a major advance [9–12]. A recent prospective assessment of the thrombin generation test for monitoring the coagulation induced by rfVIIa and activated prothrombin complex concentrate (aPCC) showed a correlation between thrombin generating capacity and clinical outcome of patients with inhibitors in ten elective surgeries [13]. In this study, dose tailoring of bypassing agents was performed using a standardized three-step-protocol including (i) *in vitro* spiking experiments evaluating the thrombin generation ability of increasing concentrations of rfVIIa and aPCC in order to determine the minimal dose of each bypassing agent that normalizes thrombin generation capacity (Fig. 1A); (ii) *ex vivo* confirmation step where thrombin generation is measured before and after the administration of the bypassing agent which fully normalized *in-vitro* thrombin generation (Fig. 1B) and (iii) monitoring of the chosen dose of the bypassing agent during the surgery and postoperative period (Fig. 1C).

Another potential interest of thrombin generation measurement in haemophilia might be represented by individual tailoring of prophylaxis regimens. Two pilot studies reported promising results showing that 24 h after factor replacement therapy, patients having similar fVIII levels might have significantly different thrombin generation capacity [6,14]. Furthermore, pilot data have illustrated that the three step protocol previously used in surgical setting might be helpful to individually tailor prophylaxis regimen of patients with severe haemophilia and inhibitors [15]. However, these hypotheses need to be prospectively investigated.

Evaluation of the clinical bleeding risk of patients with hereditary factor XI deficiency is another challenge for haematologists. Most patients with fXI deficiency are mild bleeders, but it has been recognized that patients with similar fXI activity may exhibit different bleeding phenotypes. Routine laboratory assays such as measurement of fXI clotting activity is crucial for establishing the diagnosis, but does not correlate with the individual bleeding risk of patients. A recent study assessing thrombin generation capacity of patients with fXI deficiency reported a dramatic impairment of thrombin generation in patients exhibiting severe bleeding tendency and patients having unusually good thrombin generation profiles were associated with a less severe bleeding phenotype, independently of their fXI level [16].

Over the last decade, a large number of pilot studies assessing thrombin generation tests have reported promising data in the field of bleeding disorders. There is now sufficient translational research data demonstrating the potential interest of the assay in clinical settings, as well as in clinical trials to test its correlation with the clinical outcome of patients. A working party of the ISTH fVIII/fIX SSC is currently expending tremendous efforts to standardize the assay [17,18] and will be making recommendations for the use of thrombin generation assays in bleeding disorders, which is a crucial step before bringing the test into clinical haematology laboratories.

## Whole blood thromboelastometry

### *The method*

As of today, the traditional thromboelastographic principle introduced by Hartert [19] has been adopted in computerized version of the TEG® apparatus manufactured by Haemoscope® and a modified version was introduced in 1996 by Calatzis today named thromboelastometry (ROTEM®) [20] in which the pin oscillates instead of the cup. A ball bearing focusing the pin apparently makes the ROTEM® less sensible for movements. Both the TEG® and the ROTEM® provide a digital signal allowing for additional computation of the continuous coagulation signal leading to the derivation of several quantifiable parameters (Figs 1A and B).

TEG or ROTEM allows a continuous assessment of the viscoelastic properties of a forming clot. Both devices consist of a cup into which the sample (whole blood, platelet-rich or -poor plasma) and reagents are placed, and a pin which sits in the center of the cup when the device is running. In the ROTEM® device the pin is oscillating, whereas the cup is the moving part in the TEG®. Clot formation reduces movement of the pin and this is electronically registered and visualized on a computer providing a coagulation signal similar to that of the traditional thromboelastography (Fig. 1A).

Table 1 lists currently recommended pre-analytical and analytical procedures for performing thromboelastometry measurements.

## Complementary additional information on overall haemostatic capacity

Each of the currently available assays reflects a part of the haemostatic process. Thrombin generation measurements are excellent in providing detailed information on the kinetic pattern of thrombin generation. In contrast, whole blood thromboelastometry reflects process downstream of thrombin generation, thus the integrated action of fibrin polymerisation and platelet activation and their contributions to the establishment of a three dimensional clot structure. Furthermore, the formed clot can be challenged with tissue plasminogen activator and clot stability can be tested for resistance to accelerated fibrinolysis. Hence, whole blood viscoelastic measurement is seen as a complementary test to the elsewhere described thrombin generation methods.

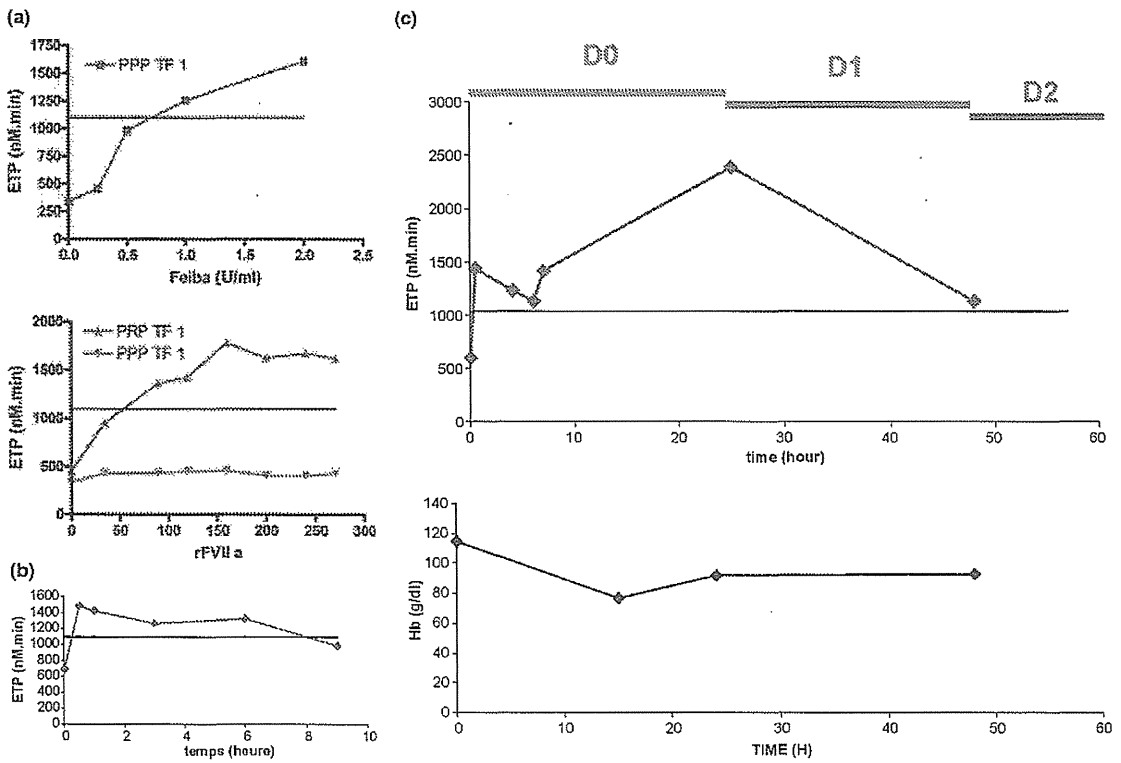


Fig. 1. A representative case illustrating the use of the three-step protocol.

### *Abnormalities in clot stability and effect of clot stabilizing intervention*

Thromboelastometry complements thrombin generation measurements by being able to provide information about clot firmness and clot stability. Clot stability and resistance toward facilitated fibrinolysis can be investigated in assays containing TF + tissue plasminogen activator. Adopting such assays, thromboelastometry studies have shown effect of tranexamic acid [21] and also recently factor XIII supplementation [22].

### *Correlation of thromboelastography with clinical phenotype*

A number of studies have demonstrated considerable heterogeneity in the baseline whole blood coagulation patterns amongst patients with verified factor VIII levels < 1% [23]. Furthermore, data have illustrated that patients diagnosed with severe haemophilia A (fVIII:C < 1%) but having unusually good whole blood clotting profiles are associated with a less severe bleeding phenotype [24].

### *Prediction of response to by-passing agents*

The low tissue factor assay has also been used to illustrate different response patterns to various levels of coagulation factor VIII concentrate. In addition, both *in vitro* and *in vivo* studies have demonstrated the ability of thromboelastography to predict the clinical response to bypassing agents in patients with inhibitors [25–27]. A small clinical study has shown that thromboelastography may be used to individualize therapy and provide more judicious use of bypassing agents as well as more convenient treatment regimens [28]. Recently, thromboelastometry has been utilised to correct the haemostatic performance of recombinant factor VIIa during surgery by showing need for fresh platelet concentrate to secure effect of recombinant factor VIIa [29].

### *Future perspectives*

Ongoing scientific activities aim to further standardize the use of thrombin generation and thromboelastometry for use in haemophilia. Important future questions will include source and concentration of tissue factor for the global assays.

A series of additional by-passing agents are in development [30], thus further emphasizing the need for global assay to monitoring and provide theranostic guidance.

## Disclosures

The authors stated that they have no interests which may be perceived as posing a conflict or bias.

## References


1 Beltra'n-Miranda CP, Khan A, Jaloma-Cruz AR, Laffan MA. Thrombin generation and phenotypic correlation in haemophilia A. *Haemophilia* 2005; 11(4): 326–34.

2 Nichols WC, Amano K, Cacheris PM, *et al.* Moderation of hemophilia A phenotype by the factor V R506Q mutation. *Blood* 1996; 88(4): 1183–7.

3 Ninivaggi M, Dargaud Y, van Oerle R, De Laat B, Hemker HC, Lindhout T. Thrombin generation assay using FIXa as a trigger to quantify accurately factor VIII levels in haemophilia A. *J Thromb Haemost* 2011; 9: 1549–55.

4 Hemker HC, Beguin S. Phenotyping the clotting system. *Thromb Haemost* 2000; 84: 747–51.

5 Hemker HC, Giesen P, AlDieri R, et al. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiol Haemost Thromb* 2002; 32: 249–53.

6 Dargaud Y, Beguin S, Lienhart A, *et al.* Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B. *Thromb Haemost* 2005; 93: 475–80.

7 Trossaert M, Regnault V, Sigaud M, Boisseau P, Lecompte T, Fressinaud E. Mild hemophilia A with factor VIII assay discrepancy: potential utility of thrombin generation assays. *J Thromb Haemost* 2008; 6: 486–93.

8 Santagostino E, Mancuso ME, Tripodi A, *et al.* Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile. *J Thromb Haemost* 2010; 8: 737–43.

9 Va'radi K, Negrier C, Berntorp E, *et al.* Monitoring the bioavailability of FEIBA with a thrombin generation assay. *J Thromb Haemost* 2003; 1: 2374–80.

10 Dargaud Y, Lambert T, Trossaert M. New advances in the therapeutic and laboratory management of patients with haemophilia and inhibitors. *Haemophilia* 2008; 14(Suppl 4): 20–7.

11 Dargaud Y, Negrier C. Thrombin generation testing in haemophilia comprehensive care centres. *Haemophilia* 2010; 16: 223–30.

12 Dargaud Y, Lienhart A, Meunier S, *et al.* Major surgery in a severe haemophilia A patient with high titre inhibitor: use of the thrombin generation test in the therapeutic decision. *Haemophilia* 2005; 11: 552–8.

13 Dargaud Y, Lienhart A, Negrier C. Prospective assessment of thrombin generation test for dose monitoring of bypassing therapy in hemophilia patients with inhibitors undergoing elective surgery. *Blood* 2010; 116: 5734–7.

14 Lewis SJ, Stefens E, Florou G, *et al.* Measurement of global haemostasis in severe haemophilia A following FVIII infusion. *Br J Haematol* 2007; 138: 775–82.

15 Dargaud Y, Negrier C. Individually tailored prophylaxis in patients with severe hemophilia. *J Coagulation Disord* 2010; 2: 11–7.

16 Rugeri L, Quelin F, Chatard B, De Mazancourt P, Negrier C, Daragud Y. Thrombin generation in patients with FXI deficiency and clinical bleeding risk. *Haemophilia* 2010; 16: 771–7.

17 Dargaud Y, Luddington R, Gray E, Negrier C, Lecompte T, Petros S, Hogwood J, Bordet JC, Regnault V, Siegemund A, Baglin T. Effect of standardization and normalization on imprecision of calibrated automated thrombography: an international multicentre study. *Br J Haematol* 2007; 139: 303–9.

18 Dargaud Y, Luddington R, Gray E, Lecompte T, Siegemund T, Baglin T, Hogwood J, Regnault V, Siegemund A, Negrier C. Standardisation of thrombin generation test – which reference plasma for TGT? An international multicentre study. *Thromb Res* 2010; 125: 353–6.

19 Hartert H. Blutgerinnungsstudien mit der thrombelastographie, einem neuen untersuchungsverfahren. *Klinische Wochenschrift* 1948; 26: 577–83.

20 Calatzis A, et al. A comparison of the technical principle of the roTEG coagulation analyser and conventional thrombelastographic systems. *Ann Haematol* 1996; 72(Suppl. 1): P90 (abstract).

21 Hvas AM, *et al.* Tranexamic acid combined with recombinant factor VIII increases clot resistance to accelerated fibrinolysis in severe hemophilia A. *J Thromb Haemost* 2007; 5: 2408–14.

22 Rea CJ, *et al.* Factor XIII combined with recombinant factor VIIa: a new means of treating severe haemophilia A. *J Thromb Haemost*, 2011; 9: 510–6.
23 Ingerslev J, Poulsen LH, Sorensen B. Potential role of the dynamic properties of whole blood coagulation in assessment of dosage requirements in haemophilia. *Haemophilia* 2003; 9: 348–52.
24 Chitlur M, *et al.* Thromboelastography in children with coagulation factor deficiencies. *Br J Haematol* 2008; 142: 250–6.
25 Sorensen B, Ingerslev J. Whole blood clot formation phenotypes in hemophilia A and rare coagulation disorders. Patterns of response to recombinant factor VIIa. *J Thromb Haemost* 2004; 2: 102–10.
26 Sorensen B, Ingerslev J. Thromboelastography and recombinant factor VIIa-hemophilia and beyond. *Semin Hematol* 2004; 41(Suppl 1): 140–4.
27 Sorensen B, Persson E, Ingerslev J. Factor VIIa analogue (V158D/E296V/M298Q-FVIIa) normalises clot formation in whole blood from patients with severe haemophilia A. *Br J Haematol* 2007; 137: 158–65.
28 Young G, *et al.* Individualization of bypassing agent treatment for haemophilic patients with inhibitors utilizing thromboelastography. *Haemophilia* 2006; 12: 598–604.
29 Sorensen B, Ingerslev J. Platelet infusion supports recombinant factor VIIa in a patient with severe haemophilia A and inhibitor–clinical outcome and laboratory observations. *Thromb Haemost* 2010; 103: 1275–6.
30 Sorensen B. Future pharmacological strategies in management of Haemophilia. *Thromb Res* 2010; Oct; 126(4): 259–61. Epub 2010 Feb 12

## Clot waveform analysis

Clot waveform analysis (CWA) is a convenient method for assessing global clotting function. It is based on the continuous monitoring of light transmittance or absorbance during routine coagulation tests such as the activated thromboplastin time (aPTT) and the prothrombin time (PT). Numerous automated instruments are capable of performing CWA. Principally, CWA can be conveniently performed simultaneously with routine coagulation tests [1]. Changes in light transmittance are determined by continuous measurements and are designated the clot waveform (CW). This complete clotting process recorded in the CWA is categorized into three parts, e.g. the pre-coagulation phase, the coagulation phase and the post-coagulation phase. After the onset of coagulation, light transmittance is decreased in association with the formation of fibrin and is defined by a slope in the waveform. The advantages of utilizing CWA are provided by the quantitative assessment of various parameters derived by mathematically processing the waveform data. Early reports suggested, however, that |min1| and |min2| are promising parameters for quantitative evaluation of clotting function [2]. Observations of CWA patterns during routine aPTT and PT assays can provide supportive and novel data in a variety of coagulation disorders and during monitoring of anti-coagulant therapy such as heparin. Characteristic CW patterns are observed in specific coagulation abnormalities compared with normal reference plasma, and two components, the duration of pre-coagulation phase and the steepness of the slope of the coagulation phase, appear to be especially informative. A further advantage offered by the application of CWA is the possibility of assessing fibrin deficiency and fibrinolytic activity. Furthermore, modification to a "biphasic" pattern is a useful tool for diagnosis of sepsis and disseminated intravascular coagulation (DIC) [3]. CWA could discriminate between different levels of fVIII:C in this critical category of severe HA defined as having <1.0 IU $dl^{-1}$ fVIII:C by conventional assays [2]. Furthermore, the CWA parameter, |min2|, appeared to be more directly correlated with both the degree of abnormality of the CW and the fVIII:C level [4]. Similarly, in experiments in 36 patients with severe HA, significant correlations between |min2| and fVIII:C were confirmed, and the parameters correlated well with those of thrombin generation [4,5]. It is evident that since the distinction between severe and non-severe haemophilia cannot be determined precisely by the level of fVIII or fIX activity alone, the influence of other plasma components should be considered. It may be especially important that CWA can clearly discriminate between severe and non-severe groups. Defective clotting function in haemophilia can be assessed using CWA, and this method may be applicable to monitor the haemostatic and prophylactic effects of regular infusions of fVIII concentrate during ITI therapy in patients with inhibitor. Our previous results suggested that fVIII infusions may be continued with clinical benefit in some haemophilia patients with high responding inhibitors in whom the haemostatic response may be monitored effectively using CWA [6]. Lastly, CWA is also useful for assessment of clotting function in acquired haemophilia A, since this refractory and severe bleeding disorder cannot be estimated by the level of fVIII activity [7]. Thus, CWA has greater versatility and considerable potential for the evaluation of overall clotting function in various disorders of haemostasis. Internationally recognized standardization of methods and test parameters are required, however, for optimization of the technique.

## References


1 Braun PJ, Givens TB, Stead AG *et al.* Properties of optical data from activated partial thromboplastin time and prothrombin time assays. *Thromb Haemost* 1997; 78: 1079–87.

2 Shima M, Matsumoto T, Fukuda K *et al.* The utility of activated partial thromboplastin time (aPTT) clot waveform analysis in the investigation of haemophiliaA patients with very low levels of factor VIII activity (FVIII:C). *Thromb Haemost* 2002; 87: 436–41.

3 Toh CH *et al.* Early identification of sepsis and mortality risks through simple, rapid clot-waveform analysis. Implications of lipoprotein-complexed C reactive protein formation. *Intensive Care Med* 2003; 29: 55–61.

4 Matsumoto T, Shima M, Takeyama M *et al.* The measurement of low levels of factor VIII or factor IX in haemophiliaA and haemophiliaB plasma by clot waveform analysis and thrombin generation assay. *J Thromb Haemost* 2006; 4: 377–84.

5 Shima M, Matsumoto T, Ogiwara K. New assays for monitoring haemophilia treatment. *Haemophilia* 2008; 14: 83–92.

6 Kasuda S, Tanaka I, Shima M *et al.* Effectiveness of factor VIII infusions in haemophilia A patients with high responding inhibitors. *Haemophilia* 2004; 10: 341–6.

7 Matsumoto T, Nogami K, Ogiwara K, Shima M. A putative inhibitory mechanism in the tenase complex responsible for loss of coagulation function in acquired haemophilia A patients with anti-C2 autoantibodies. *Thromb Haemost* 2012; 107: 288–301.

## Diagnostic usefulness of adenosine triphosphate release assays and aggregation tests with native or platelet count adjusted platelet rich plasma

Platelet function disorders are quite prevalent among individuals with bleeding problems [1–5]. At present, aggregation and dense granule release assays are the commonly performed, and the most useful tests to diagnose platelet function disorders [1–4,6,7]. Laboratories need to consider recent evidence on aggregation and dense granule release tests for platelet disorders [1–5,8], and the guideline recommendations on these assays [9–12] to optimize their diagnostic evaluation of platelet function disorders.

Light transmittance aggregometry (LTA) is considered the "gold standard" of platelet function tests, despite its lack of standardization [13,14]. The usefulness of LTA, for diagnosing impaired platelet function among individuals referred for bleeding disorder assessments, has been estimated in recent prospective studies [1,3]. A merit of these studies is that they tested LTA in accordance with guidelines [9,10], using validated reference intervals (RI) for maximal aggregation (MA) [15]. When LTA MA is abnormal with two or more panel agonists, there is a high likelihood (estimated as OR, odds ratio) of impaired function from a bleeding disorder (OR: ≥23), and an inherited secretion defect (OR: ≥91) which is the most common type of platelet function disorder [1,3]. In comparison, the bleeding time is much less useful (OR for bleeding disorders: 3.5) [1]. Most LTA abnormalities with single agonists are false positive results, not predictive of bleeding problems [1,3]. In general, LTA shows good reproducibility and less variability than dense granule release endpoints [2–4]. Receiver operator curves (ROC), which evaluate sensitivity and specificity, indicate LTA has high specificity and moderate sensitivity for inherited platelet disorders [1,3]. Abnormal findings can also reflect acquired disorders [1,3]. LTA agonists that are sensitive to common inherited platelet function defects include commonly tested agonists (i.e. Horm collagen, tested at 1.25 μg $mL^{-1}$; epinephrine; and arachidonic acid) and thromboxane analogue U46619 [1], which is used less frequently [7,14,16].

ontroversies have emerged about whether LTA should be performed using native platelet rich plasma (PRP) or PRP adjusted to a standardized platelet count as native samples show more aggregation with weak agonists [3,17–20] A recent prospective study was the first to rigorously compare these samples types for bleeding disorder diagnosis, using non-inferiority analysis of the areas under ROC for MA data, with predefined ROC area differences (<0.15 to define non-inferiority; >0 to define superiority) to evaluate detection of bleeding disorders and inherited platelet secretion defects [3]. Native and adjusted PRP show small differences in their mean MA responses to most agonists (ranges, controls: −3.3 to 5.8; patients referred for bleeding disorder assessments: −3.0 to 13.7), with native samples showing more variability with ristocetin [3]. For detecting reduced MA from bleeding disorders (with two or more agonists), native PRP were non-inferior, whereas adjusted PRP were superior, despite their wider RI with weak agonists [3]. While this study validates using either native or adjusted PRP for LTA assessments of bleeding disorders, adjusted PRP were superior to native PRP for detecting impaired LTA from bleeding disorders [3]. Furthermore, native PRP (which show more variable responses to ristocetin) have not been validated for ristocetin induced platelet aggregation assessments of von Willebrand disease [3].

North American guidelines recommend that laboratories consider a single abnormal agonist response by LTA as a potential false positive findings (except with collagen and ristocetin) as such abnormalities are not predictive of platelet function disorders [1,3,10]. On the other hand, evidence to date indicates that LTA abnormalities with multiple agonists are strongly associated with bleeding disorders (OR ≥23) and inherited platelet secretion defects (OR ≥91), which are the most common type of platelet function disorder [1,3]. Studies on the reproducibility of LTA indicate that most results (be they normal or abnormal) are confirmed on repeat testing [4]. Nonetheless, it is considered good practice to confirm abnormalities on another sample to exclude preexamination or analytical artifacts [10]. Abnormalities with multiple agonists should be considered suspicious of a platelet function disorder [10].

Like LTA, assays of dense granule adenosine triphosphate (ATP) release using Chronolume® (Chronolog Corporation, Haverston, PA, USA), a commercial luciferin–luciferase reagent containing magnesium, are helpful to detect impaired platelet function due to a bleeding disorder (OR: 17; diagnosis based on clinical opinion, not laboratory tests) or an inherited platelet disorder (OR: 128). ROC analyses indicate that like LTA, ATP release has high specificity and moderate sensitivity for inherited platelet disorders [2], with most function defects detected by the combination of: 6 μM epinephrine, 5.0 μg $mL^{-1}$ Horm collagen, and 1 μM thromboxane analogue U46619 [2]. ATP release abnormalities are predictive of platelet disorders, regardless of LTA findings (respective OR: if LTA abnormal: 261; if LTA normal: 105) [2]. However, the predictive power could been overestimated for subjects with normal LTA findings as ATP release was considered in the definition of platelet disorders [2]. Because ATP release findings show significant variability [2], abnormalities in platelet function should be confirmed on another sample. Several experts recommend performing aggregation and ATP release as separate tests because Chronolume® potentiates sub-maximal aggregation and falsely normalizes the aggregation findings for some platelet disorders (e.g. Quebec platelet disorder)

[5,21]. Furthermore, the agonists, and agonist concentrations, that are useful for LTA and ATP release differ [5]. There have not been any reported prospective studies on the diagnostic usefulness of whole blood ATP release, and ATP release assessed with native PRP or low platelet count samples. Laboratories should be aware that the sample platelet count influences how much platelet dense granule ATP is available for release.

To optimize platelet function testing, laboratories should consider the recent evidence, guidelines, and strategies that help detect common platelet function defects [1–5,8–12,22] including the use of properly determined RI (based on adequate numbers of control tests) and quality controls [14,16,23,24]. An improved diagnosis of platelet function disorders could limit the risk of false positive or negative findings worldwide.

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## Disclosures

The author has declared no conflict of interests.

## References


1 Hayward CP, Pai M, Liu Y, *et al.* Diagnostic utility of light transmission platelet aggregometry: results from a prospective study of individuals referred for bleeding disorder assessments. *J Thromb Haemost* 2009; 7: 676–84.

2 Pai M, Wang G, Moffat KA, *et al.* Diagnostic usefulness of a lumi-aggregometer adenosine triphosphate release assay for the assessment of platelet function disorders. *Am J Clin Pathol* 2011; 136: 350–8.

3 Castilloux JF, Moffat KA, Liu Y, Seecharan J, Pai M, Hayward CP. A prospective cohort study of light transmission platelet aggregometry for bleeding disorders: is testing native platelet-rich plasma non-inferior to testing platelet count adjusted samples? *Thromb Haemost* 2011; 106: 675–82.

4 Quiroga T, Goycoolea M, Matus V, *et al.* Diagnosis of mild platelet function disorders. Reliability and usefulness of light transmission platelet aggregation and serotonin secretion assays. *Br J Haematol* 2009; 147: 729–36.

5 Hayward CP, Moffat KA, Castilloux JF et al. Simultaneous measurement of adenosine triphosphate release and aggregation potentiates human platelet aggregation responses for some subjects, including persons with Quebec platelet disorder. *Thromb Haemost* 2011; 106: 675–82.

6 Pai M, Hayward CP. Diagnostic assessment of platelet disorders: what are the challenges to standardization? *Semin Thromb Hemost* 2009; 35: 131–8.

7 Hayward CP, Moffat KA, Plumhoff EA, Van Cott E. Approaches to investigating common bleeding disorders: An evaluation of North American coagulation laboratory practices. *Am J Hematol* 2012; 87 Suppl 1: S45–50.

8 Mezzano D, Quiroga T, Pereira J. The level of laboratory testing required for diagnosis or exclusion of a platelet function disorder using platelet aggregation and secretion assays. *Semin Thromb Hemost* 2009; 35: 242–54.

9 Christie D. J., Avari T., Carrington L. R., Cohen E., DeBiase B. A., Harrison P., Kickler T. S., Kottke-Marchant K., Ledford-Kraemer M., Rand M. L., Schmaier A. H., McCabe White M. Clinical and Laboratory Standards Institute (CLSI). *Platelet function testing by aggregometry; approved guideline.* CLSI document H58-A (ISBN 1-56238-683-2), available on-line. Wayne, PA, USA: Clinical and Laboratory Standards Institute, 2008. 28[31], 1–45.

10 Hayward CP, Moffat KA, Raby A, *et al.* Development of North American consensus guidelines for medical laboratories that perform and interpret platelet function testing using light transmission aggregometry. *Am J Clin Pathol* 2010; 134: 955–63.

11 Harrison P, Mackie I, Mumford A, *et al.* Guidelines for the laboratory investigation of heritable disorders of platelet function. *Br J Haematol* 2011; 155: 30–44.

12 Hayward CP, Harrison P, Cattaneo M, Ortel TL, Rao AK. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. *J Thromb Haemost* 2006; 4: 312–9.

13 Cattaneo M. Light transmission aggregometry and ATP release for the diagnostic assessment of platelet function. *Semin Thromb Hemost* 2009; 35: 158–67.

14 Cattaneo M, Hayward CP, Moffat KA, Pugliano MT, Liu Y, Michelson AD. Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report from the platelet physiology subcommittee of the SSC of the ISTH. *J Thromb Haemost* 2009; 7: 1029.

15 Hayward CP, Moffat KA, Pai M, *et al.* An evaluation of methods for determining reference intervals for light transmission platelet aggregation tests on samples with normal or reduced platelet counts. *Thromb Haemost* 2008; 100: 134–45.

16 Moffat KA, Ledford-Kraemer MR, Nichols WL, Hayward CP. Variability in clinical laboratory practice in testing for disorders of platelet function: results of two surveys of the North American Specialized Coagulation Laboratory Association. *Thromb Haemost* 2005; 93: 549–53.

17 Cattaneo M, Lecchi A, Zighetti ML, Lussana F. Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count. *Haematologica* 2007; 92: 694–7.

18 Linnemann B, Schwonberg J, Mani H, Prochnow S, Lindhoff-Last E. Standardization of light transmittance aggregometry for monitoring antiplatelet therapy: an adjustment for platelet count is not necessary. *J Thromb Haemost* 2008; 6: 677–83.

19 Mani H, Luxembourg B, Klaffling C, Erbe M, Lindhoff-Last E. Use of native or platelet count adjusted platelet rich plasma for platelet aggregation measurements. *J Clin Pathol* 2005; 58: 747–50.

20 Favaloro EJ, Mohammed S. Platelet function testing: auditing local practice and broader implications. *Clin Lab Sci* 2010; 23: 21–31.

21 Callan MB, Shofer FS, Wojenski C, Giger U. Chrono-lume and magnesium potentiate aggregation of canine but not human platelets in citrated platelet-rich plasma. *Thromb Haemost* 1998; 80: 176–80.

22 Hayward CP. Diagnostic evaluation of platelet function disorders. *Blood Rev* 2011; 25: 169–73.

23 Jennings I, Woods TA, Kitchen S, Walker ID. Platelet function testing: practice among UK National External Quality Assessment Scheme for Blood Coagulation participants, 2006. *J Clin Pathol* 2008; 61: 950–4.

24 Duncan EM, Bonar R, Rodgers SE, Favaloro EJ, Marsden K. Methodology and outcomes of platelet aggregation testing in Australia, New Zealand and the Asia-Pacific region: results of a survey from the Royal College of Pathologists of Australasia Haematology Quality Assurance Program. *Int J Lab Hematol* 2008; 31: 398–406.

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CASE REPORT

# Two newborn-onset patients of Upshaw–Schulman syndrome with distinct subsequent clinical courses

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**Abstract** Upshaw–Schulman syndrome (USS) is caused by a congenital deficit in ADAMTS13 activity owing to genetic mutations. USS is characterized by severe neonatal jaundice with a negative Coombs test and repeated childhood episodes of thrombocytopenia reversible by fresh frozen plasma (FFP) infusions. We present two patients with USS, both of whom underwent exchange blood transfusions as newborns, although the disease subsequently developed along different clinical courses. USS-CC5 initially received a diagnosis of neonatal jaundice due to fetomaternal ABO incompatibility with an indirect positive Coombs test, which masked the diagnosis of USS. Before prophylactic FFP infusions were initiated, USS-CC5 had chronic thrombocytopenia. In contrast, thrombocytopenia developed in USS-HH4 only in response to infections and spontaneously normalized without FFP infusions. Analyses of the ADAMTS13 genes in USS-CC5 and USS-HH4 revealed compound heterozygotes of p.R398C/p.Q723K and p.Q449X/p.Q1374Sfs, respectively. Analysis of von Willebrand factor (VWF) multimers in plasma samples taken from both patients in remission showed single symmetrical multimer bands, which differ from the triplet structure of bands observed in normal samples. These data suggested that plasma VWF multimers in the patients had not been proteolytically modified. Our results indicate the presence of a previously unknown regulatory mechanism for VWF-dependent high-shear stress-induced platelet aggregation.

**Keywords** Upshaw–Schulman syndrome · Fetomaternal ABO incompatibility · ADAMTS13 gene analysis · Von Willebrand factor multimers

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## Introduction

Upshaw–Schulman syndrome (USS) is caused by mutations in the ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motifs-13) gene that disrupt the activity of the encoded enzyme; the disorder is also referred to as congenital thrombotic thrombocytopenic purpura (TTP) [1–4]. Reduced ADAMTS13 activity results in increased circulating levels of unusually large von Willebrand factor multimers (UL-VWFMs), which cause microcirculatory platelet thrombi in response to high-shear stress. Although approximately 100 patients with USS have been identified in 80 families worldwide, the precise incidence of this rare disease is still unknown [5]. Kokame et al. [6] recently analyzed ADAMTS13 cDNA sequences and found rare non-synonymous ADAMTS13 gene mutations in 128 normal Japanese individuals, leading to estimates of 80–160 patients with USS among the 138 million individuals in the Japanese population.

USS typically results in severe neonatal jaundice with a negative Coombs test and a requirement for exchange blood transfusion, and repeated childhood episodes of thrombocytopenia that are reversed by infusing fresh

frozen plasma (FFP) [7]. More recently, however, two distinct clinical phenotypes have been identified in patients with USS: the major population exhibits the early-onset phenotype that includes newborn-onset disease, whereas the minor population shows the late-onset phenotype, in which overt TTP develops after adolescence [4, 8].

In this paper, we describe two young, unrelated patients with USS who both had severe neonatal jaundice that required exchange blood transfusions. The subsequent clinical courses of disease in these individuals, however, differed; one patient requires periodic prophylactic plasma infusions, whereas the other does not. Further, fetomaternal ABO incompatibility masked the diagnosis of USS in the first patient. We performed a comparative study of the two patients with USS and their family members, including ADAMTS13 genotyping, VWF multimer analysis during remission and the natural histories of disease.

## Materials and methods

### Patients

Clinical and laboratory data for two unrelated patients with USS (cases USS-CC5 and USS-HH4) and their family members are described in the "Results" section.

### Analyses of VWF antigen and VWF multimers

Plasma VWF antigen levels were measured using sandwich enzyme-linked immunosorbent assays (ELISAs) and rabbit anti-human VWF polyclonal antiserum (DAKO, Denmark) [9]. VWF antigen levels in 1 mL of pooled normal human plasma were defined as 100 %. VWF antigen levels in the 20 healthy control subjects were 102 ± 33 % (mean ± SD) [10].

Analysis of VWF multimers was performed according to the method described by Ruggeri and Zimmerman [11], with the following modifications. Briefly, plasma samples were separated by electrophoresis on sodium dodecyl sulfate (SDS)-1.2 % agarose gels, and samples were subjected to Western blotting with anti-VWF polyclonal antibodies and luminographic detection [12]. Blots were scanned and densitometric analyses were performed using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

### Assays of ADAMTS13 activity and ADAMTS13 inhibitor levels

Plasma levels of ADAMTS13 activity and ADAMTS13 inhibitor were measured using a chromogenic activity ELISA (ADAMTS13-act-ELISA; Kainos, Tokyo) [13]. ADAMTS13 activity in pooled normal plasma was defined as 100 %. The detection limit of the assay was 0.5 % of normal values. ADAMTS13 inhibitor titers are expressed as Bethesda units (BU); one unit was defined as the amount necessary to reduce ADAMTS13 activity to 50 % of control levels. A titer of <0.5 BU/mL in the assay was considered negative.

### Assays of ADAMTS13 antigen

Plasma levels of ADAMTS13 antigen were determined in quantitative sandwich ELISAs using two anti-ADAMTS13 monoclonal antibodies, as previously reported [14]. ADAMTS13 antigen levels in pooled normal plasma were defined as 100 %. The detection limit of the assay was 0.1 % of normal values. Plasma ADAMTS13 antigen levels were also analyzed quantitatively and qualitatively on Western blots under reducing conditions [15]. Two milliliters of diluted or undiluted plasma samples were added to each lane and separated using SDS-5 % polyacrylamide gel electrophoresis under reducing conditions. Proteins were electrophoretically blotted onto microporous polyvinylidene difluoride membranes. Blots were probed for ADAMTS13 antigen using WH2-11-1 as primary monoclonal antibodies, and horseradish peroxidase-conjugated goat anti-mouse IgG as secondary antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, MO, USA). The epitope for WH2-11-1 antibodies resides in the fourth TSP1 domain. After incubations with Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Shelton, CT), blots were exposed to X-ray films. Densitometric analysis of ADAMTS13 antigen was performed by examining the 190-kD band using ImageJ software. The detection limit of plasma ADAMTS13 antigen using this method was 3 % of normal values.

### ADAMTS13 gene analysis

All DNA analyses were performed with permission from the Ethics Committees of the hospitals at which the samples were collected and the institute where the genes were analyzed. Written informed consent was obtained from all subjects. Nucleotide sequences for all 29 exons of ADAMTS13, including intron–exon boundaries, were determined by directly sequencing polymerase chain reaction products, as previously described [16, 17]. All disease-causing ADAMTS13 mutations described in this paper were determined not to be common polymorphisms based on screening 96 individuals from the Japanese general population.

## Results

### Patients

*Case USS-CC5*

The propositus was born in 2004 in Nihonkai General Hospital, the last of three offspring of non-consanguineous parents. The delivery was natural after a gestation period of 37 weeks and 6 days and the newborn weighed 2,750 g. Eleven hours after delivery, the baby developed severe jaundice (total bilirubin 17.6 mg/dL at 12 h after delivery) and petechiae. The direct Coombs test was negative, whereas the indirect Coombs test was positive. Further, a weak anti-B antibody signal was detected in antibody dissociation experiments using the patient's red blood cells. His blood type was B-Rh(D), whereas that of his mother was O-Rh(D). Therefore, the patient was suspected of having newborn hemolytic anemia due to fetomaternal ABO incompatibility. Two exchange blood transfusions using mixed blood containing O-Rh(D) red blood cells and AB-Rh(D) FFP were performed followed by phototherapy. Subsequently, the patient's platelet count dropped to $7 \times 10^9$ platelets/L, and platelet concentrates (total of $8 \times 10^{10}$ platelets on two occasions) were infused without any notable adverse reactions. The jaundice gradually improved, although his platelet count remained low ($40–60 \times 10^9$ platelets/L). The patient was discharged 7 days after birth.

At 7 months of age, the patient was infected with influenza A and showed mild anemia (hemoglobin 7.8 g/dL) and thrombocytopenia ($10 \times 10^9$ platelets/L). Laboratory tests showed elevated serum levels of lactate dehydrogenase (LDH) (1,502 IU/L), low haptoglobin levels (<10 mg/dL), and negative Coombs tests. Examination of the bone marrow revealed no significant abnormality. Thus, a diagnosis of chronic idiopathic thrombocytopenic purpura was made, and high-dose intravenous IgG therapy was initiated, although no marked increase in platelet counts was noted ($20–50 \times 10^9$ platelets/L).

Since then, the patient has developed repeated episodes of thrombocytopenia, particularly together with febrile conditions. He received one more treatment with intravenous IgG and steroid, without any notable benefit. In fact, his plasma levels of LDH remained high (446–1,502 IU/L), and platelet counts were low ($20–50 \times 10^9$ platelets/L). Further, at the age of 2 years and 8 months, he suddenly developed a transient speech impediment and incomplete right hemiparesis, which spontaneously resolved within a few hours; head computed tomography scans revealed no notable abnormalities. Because of the unusual clinical history, the patient was referred to Nara Medical University at the age of 2 years and 9 months for ADAMTS13 analysis. A severe deficiency of ADAMTS13 activity (<0.5 % of normal values) and lack of ADAMTS13 inhibitor (< 0.5 BU/mL) were confirmed, suggesting a diagnosis of USS. The patient has since been receiving prophylactic FFP infusions every 2 weeks, which have produced transient increases in the platelet count (Fig. 1). Plasma ADAMTS13 activity levels in his father, mother and two older brothers were 46, 30, 40, and 23 % of normal values, respectively.

*ADAMTS13 gene analysis*

The patient was found to be a compound heterozygote for two mutations in the ADAMTS13 gene: p.R398C (c.1192 C > T, exon 10) and p.Q723K (c.2167 C > A, exon 18). The parents and two older brothers were heterozygous carriers of one of the two mutations. No single nucleotide polymorphisms that caused a missense mutation were found in the patient or his family members.

Plasma levels of ADAMTS13 based on antigen ELISAs were <0.1 % of normal values in the patient, and 24, 36, 23 and 36 % of normal values in the father, mother and two siblings, respectively. Further, plasma levels of ADAMTS13 antigen based on Western blotting were <3 % of normal values in the patient, and 36, 34, 38, and 38 % of normal values in the father, mother and two siblings, respectively (Fig. 2).

*Case USS-HH4*

In 2003, the proposita was born in a neighboring maternity clinic as the second of two offspring of non-consanguineous parents. Her delivery was assisted with a vacuum extractor after a gestation period of 40 weeks and 2 days. The newborn weighed 3,018 g. One day after birth, she was transferred to Nihonkai General Hospital because of severe jaundice (total bilirubin 23.7 mg/dL at 27 h after delivery), cyanosis, and thrombocytopenia ($10 \times 10^9$ platelets/L). Direct and indirect Coombs tests were negative. Her blood type was AB-Rh(D), whereas those of her father and mother were A-Rh(D) and B-Rh(D), respectively. Thus, the etiology of the severe jaundice was unclear. After admission, she was received a total of four exchange blood transfusions using a mixed blood containing O-Rh(D) red blood cells and AB-Rh(D) FFP. She also underwent four platelet transfusions (a total of $14 \times 10^{10}$ platelets). Subsequently, she developed transient renal insufficiency (blood urea nitrogen, 32.4 mg/dL; creatinine, 1.0 mg/dL). She also had patent ductus arteriosus, which was treated with surgical ligation 29 days after birth to prevent congestive heart failure. During the perioperative period, she received FFP infusions to replenish hemostatic factors, and was discharged 48 days after birth.

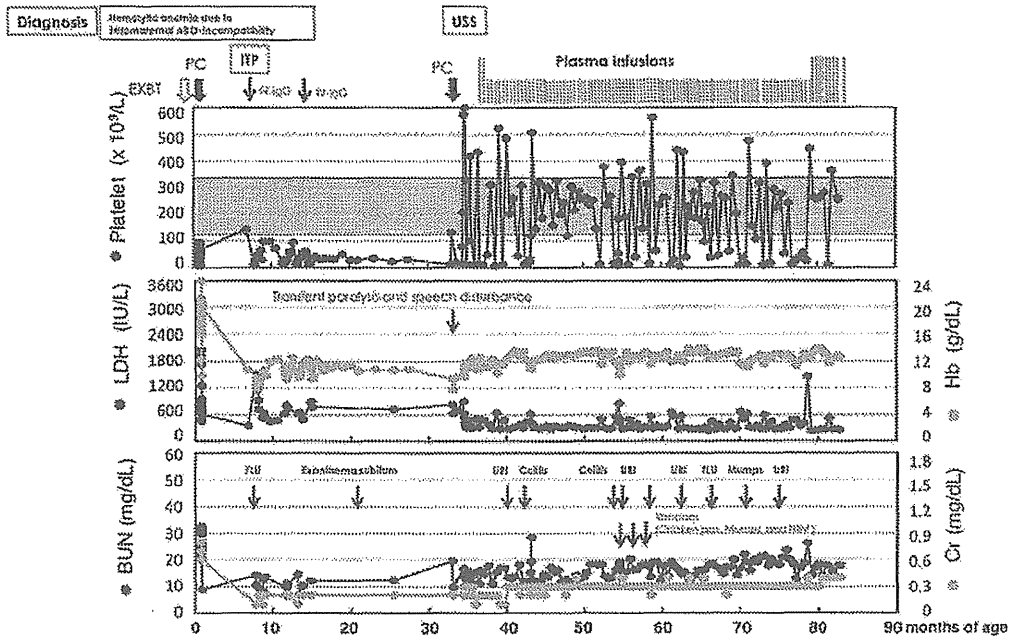


**Fig. 1** Laboratory data and the clinical course of USS in patient CC5. The patient was a male born in 2004 in Nihonkai General Hospital. Soon after birth, he developed severe jaundice and petechiae. He received two exchange blood transfusions and phototherapy. Platelet concentrates were infused twice to address thrombocytopenia of unknown etiology. At 7 months of age, he received a misdiagnosis of chronic idiopathic thrombocytopenic purpura. At the age of 2 years and 9 months, he was received a diagnosis of USS caused by severely deficient ADAMTS13 activity (<0.5 % of normal values) and no ADAMTS13 inhibitors (<0.5 BU/mL). Since the USS diagnosis, the patient has received prophylactic FFP infusions every 2 weeks. Marked, yet transient, increases in platelet counts and decreases in LDH levels were observed 2–3 days after the FFP infusions. *BUN* blood urea nitrogen, *Cr* creatinine, *Hb* hemoglobin, *EXBT* exchange blood transfusion, *PC* platelet concentrate, *IV-IgG* intravenous immunoglobulin, *ITP* idiopathic thrombocytopenic purpura, *USS* Upshaw–Schulman syndrome, *FLU* influenza A infection, *URI* upper respiratory infection

At 14 months of age, she developed chicken pox with mild thrombocytopenia, and a few weeks later presented with upper respiratory infection with a fever and cough, followed by severe thrombocytopenia ($17 \times 10^9$ platelets/L) and elevated serum levels of LDH (1,007 IU/L). A test for C-reactive protein was negative. Examining her bone marrow revealed hemophagocytosis in 3.8 % of the nucleated cells; subsequent fluid therapy increased her platelet count to $144 \times 10^9$ platelets/L, resulting in a preliminary diagnosis of viral infection-associated hemophagocytic syndrome. One month later, however, she developed mild anemia (hemoglobin, 9.7 g/dL) and moderate thrombocytopenia ($75 \times 10^9$ platelets/L), which was not specifically treated. She then developed several episodes of petechiae with fever due to upper respiratory infections. Of note, she was infected with influenza A at 2 years and 2 months old, which induced severe thrombocytopenia that gradually resolved after administration of the anti-influenza drug oseltamivir. After she became 3 years old, the incidence of petechiae decreased together with the frequency of febrile episodes. Because of the recurrent episodes of purpura, the patient was referred to Nara Medical University for ADAMTS13 analysis in 2008. She received a diagnosis of USS based on severe deficiency of ADAMTS13 activity (<0.5 % of normal values) and a lack of ADAMTS13 inhibitor. Plasma ADAMTS13 activity levels in her father, mother and older brother were 50, 44 and 38 % of normal values, respectively.

Although she had a history of severe neonatal jaundice followed by an exchange blood transfusion, the patient did not receive FFP infusions outside of the newborn period owing to the mild clinical signs and symptoms of her disease. She is now 8 years old and has not developed any major complications, such as renal insufficiency or neurologic abnormalities (Fig. 3).

*ADAMTS13 gene analysis*

Patient USS-HH4 was found to be a compound heterozygote for ADAMTS13 gene mutations: p.Q449X (c.1345 C > T, exon 12) was inherited from her father and p.Q1374Sfs (c.4119del, exon 29), which resulted in premature stop codon at amino-acid residue 1395, was inherited from her mother. Her parents were heterozygous

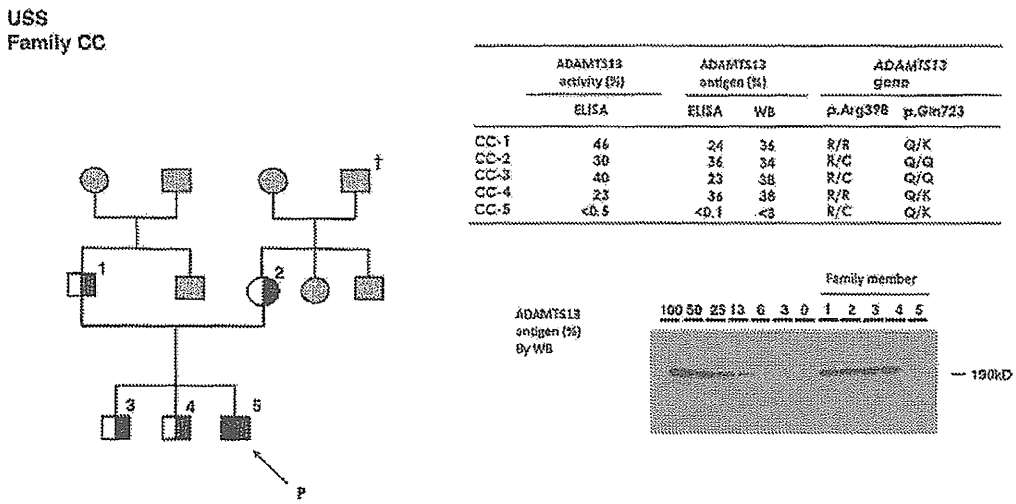


**Fig. 2** Pedigree and ADAMTS13 analysis of USS-CC5 and his family. The propositus (denoted as P) USS-CC5 is the third of three offspring of non-consanguineous parents. His parents and two brothers are asymptomatic and in good health. *Squares* and *circles* indicate males and females, respectively and *shaded symbols* show individuals who were not examined. The *cross* denotes a deceased individual. *Half-black symbols* show asymptomatic carriers. ADAMTS13 activities were determined using activity ELISAs and ADAMTS13 levels were measured using antigen ELISAs and Western blotting. Results are shown as percentages of normal values. Identified mutations in ADAMTS13 are depicted as *one-letter amino-acid abbreviations* (*right upper panel*). Western blot analyses of plasma ADAMTS13 antigen in the patient's family members are shown in the *right lower panel*

carriers of one of the two mutations. No single nucleotide polymorphisms causing missense mutations were identified in this patient or her family members.

Plasma levels of ADAMTS13 antigen based on antigen ELISAs were less than 0.1 % of normal values in the patient, whereas her father, mother and older brother showed antigen levels that were 48, 45 and 54 % of normal values, respectively. Further, plasma levels of ADAMTS13 antigen on Western blots were shown to be <3 % of normal values in the patient, and 47, 45 and 54 of normal values in her father, mother and older brother, respectively. Thus, the p.Q449X/p.Q1374Sfs mutations may have resulted in proteins that were not secreted into plasma (Fig. 4).

### VWF multimer analysis of the patients with USS during remission

Despite the common features in the two USS cases, including a lack of plasma ADAMTS13 activity and severe jaundice as newborns, the subsequent clinical courses of disease markedly differed. To examine potential proteolytic mechanisms other than ADAMTS13, we performed VWF multimer analysis using plasma samples obtained when the patients were in remission. VWF multimer bands from the patients' plasma samples were each represented by a single symmetrical band, which differed from the triplet structure of bands observed with normal plasma (Fig. 5). Moreover, a predominance of high-molecular weight VWF multimers was noted in the plasma samples from both patients, suggesting that their plasma VWF multimers had not been subjected to any alternative proteolytic modifications.

## Discussion

Analysis of the natural history of USS in 43 Japanese patients found that 42 % (18/43) had an episode of severe jaundice as newborns that required exchange blood transfusion, suggesting that jaundice is the earliest clinical sign of bouts of TTP in patients with USS [4]. Although the underlying mechanism has not been fully elucidated, hypoxia can induce the release of UL-VWFMs from vascular endothelial cells by upregulating the production of such inflammatory cytokines as interleukin-6, interleukin-8 and tumor necrotizing factor α [18, 19]. Moreover, newborns can be subjected to hypoxic conditions during prolonged labor, which may induce the release of UL-VWFMs from vascular endothelial cells and cause TTP bouts.

Newborns with USS often show severe jaundice with a negative Coombs test that requires exchange blood transfusion as well as repeated childhood episodes of thrombocytopenia that resolve in response to FFP infusions. Thus, patients with newborn-onset USS have been categorized as having the early-onset phenotype, and are treated throughout their lives with occasional or periodic plasma infusions

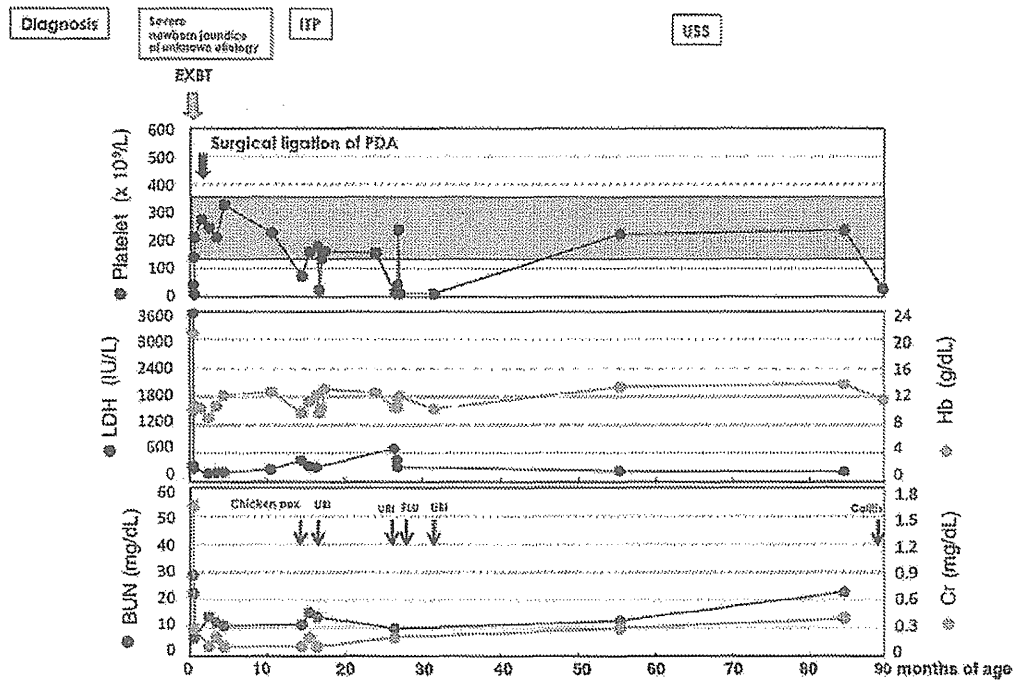


**Fig. 3** Laboratory data and the clinical course of USS in patient HH4. In 2003, the proposita was born and transferred to Nihonkai General Hospital because she developed severe jaundice, cyanosis, and thrombocytopenia. After admission, she received exchange blood transfusions and platelet transfusions. At 14 months of age, she received a misdiagnosis of hemophagocytic syndrome associated with a viral infection. Since that time, she developed several episodes of petechiae with fever due to upper respiratory infection and influenza A. After she turned 3 years old, the incidence of petechiae decreased together with less frequent febrile episodes. In 2008, she received a diagnosis of USS based on severely deficient ADAMTS13 activity (<0.5 % of normal values) and no detected ADAMTS13 inhibitor. Interestingly, because her clinical signs and symptoms were mild, she did not receive FFP infusions. She is now 8 years old and has not developed major complications, such as renal insufficiency or neurologic abnormalities. *BUN* blood urea nitrogen, *Cr* creatinine, *Hb* hemoglobin, *PDA* patent ductus arteriosus, *EXBT* exchange blood transfusion, *ITP* idiopathic thrombocytopenic purpura, *USS* Upshaw–Schulman syndrome, *FLU* influenza A infection, *URI* upper respiratory infection

either prophylactically or in response to bouts of TTP [20]. On the other hand, patients categorized as having the late-onset phenotype develop the first episode of TTP after childhood. Patients with USS, however, occasionally show isolated mild thrombocytopenia during childhood, and, therefore, are overlooked or received a misdiagnosis of idiopathic thrombocytopenic purpura. These results indicate that USS with the late-onset phenotype may result from several different pathogenic processes. Generally, however, bouts of TTP in patients with USS are thought to be induced by various triggers, including pregnancy, upregulated cytokine expression during severe infections and excessive alcohol intake, among others [4].

Camilleri et al. [21] reported that a p.R1060W missense mutation in the ADAMTS13 gene was associated with USS with an ethnically specific late-onset phenotype; a Caucasian patient who was homozygous for the mutation showed plasma ADAMTS13 activity levels that were <5 % of normal values. Recently, we reported that a Japanese patient with USS was homozygous for a p.C1024R missense mutation, resulting in an Asian-specific late-onset phenotype. The patient received a correct diagnosis of USS at 77 years old and was shown to have moderately reduced plasma ADAMTS13 activity levels (2.4–3.4 % of normal values) using a sensitive chromogenic assay [13]. In vitro expression studies using HeLa cells transfected with plasmid encoding the p.C1024R mutant revealed that mutant protein was secreted into culture medium but at a significantly lower level than the wild-type protein. Further, the activity of the secreted p.C1024R mutant protein was three times that of the wild-type protein, indicating that p.C1024R was a gain-of-function mutation [22]. These data suggested that the plasma levels of ADAMTS13 activity in this patient prevented TTP during his childhood unless a strong stimulus-induced UL-VWFM release. Even in normal individuals, however, plasma levels of ADAMTS13 gradually decrease with age, in contrast to increasing plasma VWF levels [23]. Thus, in patients with USS, an

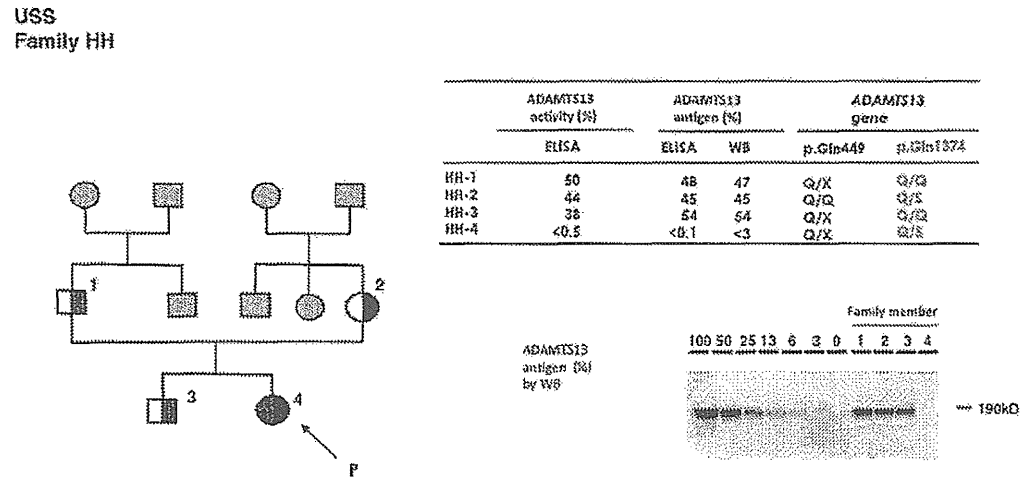


**Fig. 4** Pedigree and ADAMTS13 analysis of USS-HH4 and her family. The proposita (denoted as P) USS-HH4 is the second of two offspring of non-consanguineous parents. Her parents and brother are asymptomatic carriers. ADAMTS13 activities were determined using activity ELISAs and ADAMTS13 antigen levels were measured using antigen ELISAs and Western blotting. Results are shown as percentages of normal values. Identified mutations in ADAMTS13 are depicted as *one-letter amino-acid abbreviations* (*right upper panel*). Western blot analyses of plasma ADAMTS13 antigen in the patient's family members are shown in the *right lower panel*

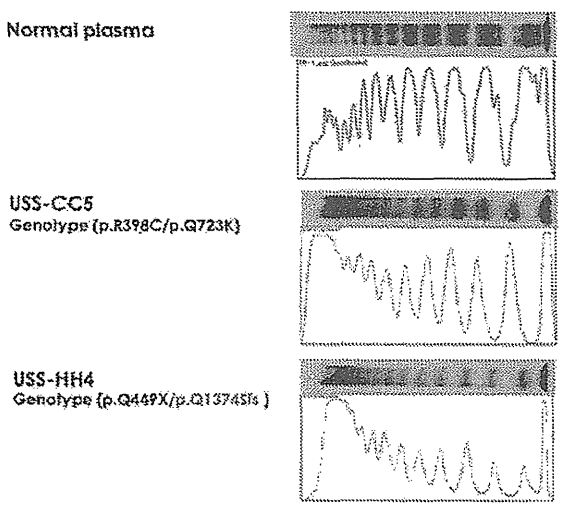


**Fig. 5** Plasma VWF multimers from two patients with USS during remission phases. We analyzed VWF multimers in plasma samples obtained when the patients were in remission. Compared with the triplet bands observed in normal plasma (*upper panel*), VWF multimers in the patients' plasma samples consisted of single symmetrical bands (*middle panel* USS-CC5, *bottom panel* USS-HH4). Further, the patients' plasma samples showed high percentages of high-molecular-weight VWF multimers

imbalance in increased substrate levels (highly multimeric VWF) and the reduced ADAMTS13 enzymatic activity generates prothrombotic conditions, leading to more frequent TTP. Thus, mild or moderate deficiency of ADAMTS13 activity in patients with USS may contribute to conditions that allow the late-onset phenotype to develop.

Here, we have described two patients with USS (USS-CC5 and USS-HH4) who both had severe jaundice as newborns, requiring exchange blood transfusions. The subsequent clinical courses of disease in these patients, however, differed; USS-CC5 experienced chronic thrombocytopenia unless he was treated with prophylactic FFP infusions, whereas USS-HH4 developed transient thrombocytopenia only when she had an infection. USS-HH4 is now 8 years of age and has never been treated with FFP infusions. Notably, severe neonatal jaundice due to feto-maternal ABO incompatibility in USS-CC5—uncovered via an indirect positive Coombs test—masked a correct diagnosis of USS. ADAMTS13 gene analyses revealed that USS-CC5 and USS-HH4 were compound heterozygotes of p.R398C/p.Q723K and p.Q449X/p.Q1374Sfs, respectively. Among these mutations, p.Q449X was found in USS patients [16], but p.R398C, p.Q723K, and p.Q1374Sfs have not been previously reported in USS patient. Of these, p.Q723K alone was found as a rare nonsynonymous mutation in 128 normal individuals [6]. Both patients in this study showed plasma ADAMTS13 activity levels that were less than 0.5 % of normal, and ADAMTS13 antigen levels that were less than 0.1 % of normal. Therefore, it was suspected that p.R398C, p.Q723K, and p.Q1374Sfs were not secreted in plasma. Thus, the pathogenesis of the milder clinical presentation of USS-HH4 probably did not reflect the same mechanisms that contributed to the late-onset phenotype observed in the patient who was homozygous for the p.C1024R missense mutation. To determine whether UL-VWFMs were modulated by proteases other than ADAMTS13, we performed VWF multimer analysis

using patient plasma samples obtained during remission; each VWF multimer band in plasma from the two patients was represented by a single symmetrical band, rather than the triplet structure observed in normal plasma, indicating that VWF multimers from the patients had not been subjected to alternative proteolytic modifications.

In patient USS-HH4, the mechanism regulating the interactions between platelets and hyperactive UL-VWFMs without induction of overt TTP is presently unknown. Because we did not observe alternative proteolytic modifications of VWFMs in the patients with USS, we are now interested in potential fluid-phase regulatory mechanisms during high shear stress-induced platelet aggregation (H-SIPA). H-SIPA is dependent on VWFM size, and is inhibited by compounds that disrupt interactions in the VWF–platelet GPIb axis and subsequent platelet activation. Platelet activation during H-SIPA is mediated by endogenous ADP released from platelet δ-granules in microenvironments; therefore, ADP scavengers block H-SIPA without modifying VWFM structures. Indeed, we previously reported that human placental or vascular endothelial cell ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) efficiently inhibited H-SIPA [24]. Further, we also indicated the presence of soluble E-NTPDase in plasma, which is cleaved from the cell surface or generated by alternative splicing [25]. Thus, studies focusing on potential relationships between E-NTDase and H-SIPA would be of great interest, and may help to elucidate the pathogenesis of TTP in patients with USS.

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**Conflict of interest** Y. Fujimura is a member of clinical advisory boards for Baxter BioScience.

## References

1. Schulman I, Pierce M, Lukens A, Currimbhoy Z. Studies on thrombopoiesis. I. A factor in normal human plasma required for platelet production; chronic thrombocytopenia due to its deficiency. Blood. 1960;16:943–57.
2. Upshaw JD Jr. Congenital deficiency of a factor in normal plasma that reverses microangiopathic hemolysis and thrombocytopenia. N Engl J Med. 1978;298:1350–2.
3. Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, Yang AY, Siemieniak DR, Stark KR, Gruppo R, Sarode R, Shurin SB, Chandrasekaran V, Stabler SP, Sabio H, Bouhassira EE, Upshaw JD Jr, Ginsburg D, Tsai HM. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature. 2001;413:488–94.
4. Fujimura Y, Matsumoto M, Isonishi A, Yagi H, Kokame K, Soejima K, Murata M, Miyata T. Natural history of Upshaw–Schulman syndrome based on ADAMTS13 gene analysis in Japan. J Throm Haemostat. 2011;9(Suppl 1):283–301.
5. Lotta LA, Garagiola I, Palla R, Cairo A, Peyvandi F. ADAMTS13 mutations and polymorphisms in congenital thrombotic thrombocytopenic purpura. Hum Mutat. 2010;31:11–9.
6. Kokame K, Kokubo Y, Miyata T. Polymorphisms and mutations of ADAMTS13 in the Japanese population and estimation of the number of patients with Upshaw–Schulman syndrome. J Throm Haemostat. 2011;9:1654–6.
7. Fujimura Y, Matsumoto M, Yagi H, Yoshioka A, Matsui T, Titani K. Von Willebrand factor-cleaving protease and Upshaw–Schulman syndrome. Int J Hematol. 2002;75:25–34.
8. Furlan M, Lämmle B. Aetiology and pathogenesis of thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome: the role of von Willebrand factor-cleaving protease. Best Pract Res Clin Haematol. 2001;14:437–54.
9. Bartlett A, Dormandy KM, Hawkey CM, Stableforth P, Voller A. Factor-VIII-related antigen: measurement by enzyme immunoassay. Br Med J. 1976;1:994–6.
10. Matsumoto M, Kawaguchi S, Ishizashi H, Yagi H, Iida J, Sakaki T, Fujimura Y. Platelets treated with ticlopidine are less reactive to unusually large von Willebrand factor multimers than are those treated with aspirin under high shear stress. Pathophysiol Haemost Thromb. 2005;34:35–40.
11. Ruggeri ZM, Zimmerman TS. Variant von Willebrand's disease: characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. J Clin Invest. 1980;65:1318–25.
12. Budde U, Schneppenheim R, Plendl H, Dent J, Ruggeri ZM, Zimmerman TS. Luminographic detection of von Willebrand factor multimers in agarose gels and on nitrocellulose membranes. Thromb Haemost. 1990;63:312–5.
13. Kato S, Matsumoto M, Matsuyama T, Isonishi A, Hiura H, Fujimura Y. Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity. Transfusion. 2006;46:1444–52.
14. Yagi H, Matsumoto M, Fujimura Y. Brain nerve symptoms due to thrombotic microangiopathy. Nihon Naika Gakkai Zasshi. 2007;96:353–62.
15. Ishizashi H, Yagi H, Matsumoto M, Soejima K, Nakagaki T, Fujimura Y. Quantitative Western blot analysis of plasma ADAMTS13 antigen in patients with Upshaw–Schulman syndrome. Thromb Res. 2007;120:381–6.
16. Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, Tamai H, Konno M, Kamide K, Kawano Y, Miyata T, Fujimura Y. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. Proc Natl Acad Sci USA. 2002;99:11902–7.
17. Matsumoto M, Kokame K, Soejima K, Miura M, Hayashi S, Fujii Y, Iwai A, Ito E, Tsuji Y, Takeda-Shitaka M, Iwadate M, Umeyama H, Yagi H, Ishizashi H, Banno F, Nakagaki T, Miyata T, Fujimura Y. Molecular characterization of ADAMTS13 gene mutations in Japanese patients with Upshaw–Schulman syndrome. Blood. 2004;103:1305–10.
18. Pinsky DJ, Naka Y, Liao H, Oz MC, Wagner DD, Mayadas TN, Johnson RC, Hynes RO, Heath M, Lawson CA, Stern DM. Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies. A mechanism for rapid neutrophil recruitment after cardiac preservation. J Clin Invest. 1996;97:493–500.
19. Wilkie ME, Stevens CR, Cunningham J, Blake D. Hypoxia-induced von Willebrand factor release is blocked by verapamil. Miner Electrolyte Metab. 1992;18:141–4.
20. Kinoshita S, Yoshioka A, Park YD, Ishizashi H, Konno M, Funato M, Matsui T, Titani K, Yagi H, Matsumoto M, Fujimura Y. Upshaw–Schulman syndrome revisited: a concept of congenital

thrombotic thrombocytopenic purpura. Int J Hematol. 2001;74: 101–8.
21. Camilleri RS, Cohen H, Mackie IJ, Scully M, Starke RD, Crawley JT, Lane DA, Machin SJ. Prevalence of the ADAMTS-13 missense mutation R1060W in late onset adult thrombotic thrombocytopenic purpura. J Throm Haemostat. 2008;6: 331–8.
22. Taguchi F, Yagi H, Matsumoto M, Sadamura S, Isonishi A, Soejima K, Fujimura Y. The homozygous p.C1024R—ADAMTS13 gene mutation links to a late-onset phenotype of Upshaw–Schulman syndrome in Japan. Thromb Haemost. 2012;107: 1003–5.
23. Kokame K, Sakata T, Kokubo Y, Miyata T. von Willebrand factor-to-ADAMTS13 ratio increases with age in a Japanese population. J Throm Haemostat. 2011;9:1426–8.
24. Makita K, Shimoyama T, Sakurai Y, Yagi H, Matsumoto M, Narita N, Sakamoto Y, Saito S, Ikeda Y, Suzuki M, Titani K, Fujimura Y. Placental ecto-ATP diphosphohydrolase: its structural feature distinct from CD39, localization and inhibition on shear-induced platelet aggregation. Int J Hematol. 1998;68:297–310.
25. Matsumoto M, Sakurai Y, Kokubo T, Yagi H, Makita K, Matsui T, Titani K, Fujimura Y, Narita N. The cDNA cloning of human placental ecto-ATP diphosphohydrolases I and II. FEBS Lett. 1999;453:335–40.