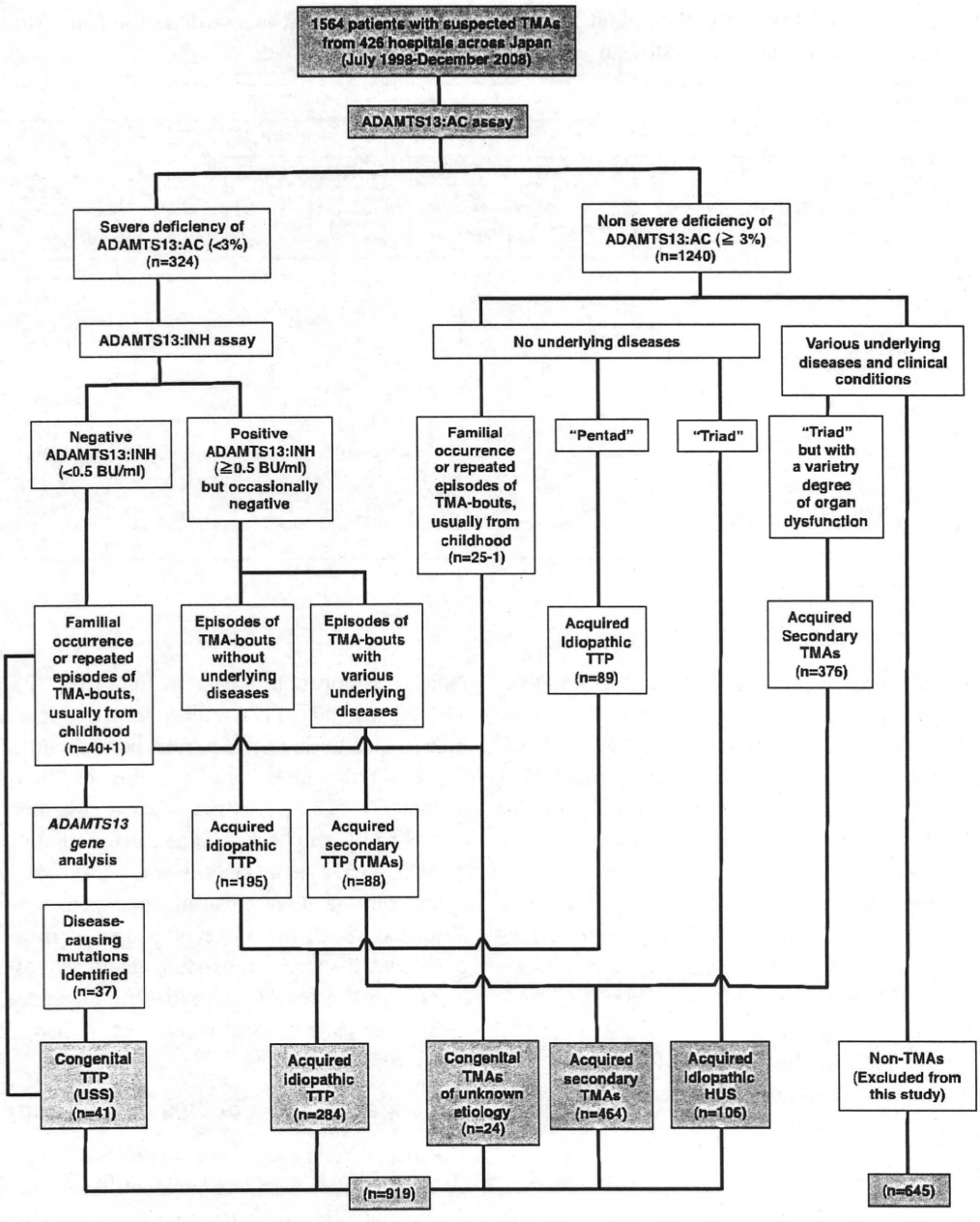

Figure 1. Flow chart of categorization of patients with suspected thrombotic microangiopathies (TMAs) based on ADAMTS13 analysis. Of 1,564 patients with suspected TMAs, 324 had severe deficiency of ADAMTS13 activity and 1,240 did not. In the former category, 40 patients were categorized as USS and 284 as acquired TTP. In the latter category, 24 patients were categorized as congenital TMAs of the unknown etiology, 570 as acquired TMAs, and one patient as USS with moderately reduced plasma ADAMTS13:AC (3.4%), to whom frequent plasma infusions had been made to prevent further aggravation of cerebral infarction. The remaining 645 patients did not have TMAs and were therefore excluded from this study.

cal signs. Second, the patients were grouped as HUS or TTP based on the 'triad' or 'pentad' of clinical signs. This protocol appeared to be important, because our registry includes patients with hereditary deficiency of ADAMTS13: AC or congenital TTP (Upshaw-Schulman syndrome, USS), which generally have less severe clinical signs (often isolated thrombocytopenia) than acquired TTP.

Results and Discussion

A flow chart of patient categorization based on ADAMTS 13 analysis is shown in Fig. 1. Of the 1,564 patients referred to our laboratory, 324 (minor population) had severe deficiency of ADAMTS13: AC and 1,240 (major population) did not. In the population with severe ADAMTS13: AC de-

Table 1. Plasma Levels of ADAMTS13: AC and ADAMTS13: INH in 919 Patients with Thrombotic Microangiopathies (TMAs) Registered at Nara Medical University during July 1988- December 2008

	Congenital TMAs		Acquired TMAs											Total
			Idiopathic		Secondary									
					Drug-Induced									
	Upshaw-Schulman syndrome (USS) (n=41)	Unknown etiology (n=24)	Thrombotic thrombocytopenic purpura (TTP) (n=284)	Hemolytic-uremic syndrome (HUS) (n=106)	Ticlopidine (n=22)/ Clopidogrel (n=1)	Mitomycin C (n=10)	Pegylated-interferon (n=1) / Sildenafil (n=1)	Conective tissue diseases and their allied diseases (CTDs/ADs) (n=221)	Malignancies (n=61)	Hematopoietic stem cell transplant-ation (HSCT) (n=54)	Pregnancy (n=15)	E. coli O157: H7 infection (n=32)	Others (Liver cirrhosis, etc) (n=46)	(n=919)
ADAMTS13:AC (%)	(n=41)	(n=24)	(n=284)	(n=106)	(n=22/n=1)	(n=10)	(n=1/n=1)	(n=221)	(n=61)	(n=54)	(n=15)	(n=32)	(n=46)	(n=919)
<3	40	0	195	0	18	0	2	46	5	0	4	0	13	324
3 ~ <25	1	4	72	20	2	2	0	66	23	18	4	5	16	233
25 ~ <50	0	9	14	48	1	5	0	66	22	24	4	17	6	216
≥50	0	11	3	38	1	3	0	43	11	12	3	10	11	146
ADAMTS13:INH (U/ml)	(n=41)	(n=23)	(n=282)	(n=43)	(n=22/n=1)	(n=7)	(n=1/n=1)	(n=187)	(n=26)	(n=15)	(n=8)	(n=17)	(n=23)	(n=697)
≥2	0	0	120	0	15	0	0	28	5	0	3	0	9	180
0.5 ~ <2	0	0	128	2	6	0	2	80	8	4	2	1	8	242
<0.5	41	23	33	41	2	7	0	79	13	11	3	16	6	275

( ) Sample number determined

ficiency, 40 patients were categorized as USS and 284 as acquired TTP, and no patients with DIC or septic DIC were included. In the population without severe ADAMTS13: AC deficiency, 24 patients were categorized as congenital TMAs of unknown etiology, 570 as acquired TMAs, and only one patient (GG in Table 2) as USS with moderately reduced plasma ADAMTS13: AC (3.4%), to whom frequent plasma infusions had been made to prevent further aggravation of cerebral infarction. Thus, a diagnosis of USS in this patient GG was made after identifying the disease-causing mutations (C1024R/C1024R) in exon 24 by *ADAMTS13* gene analysis. These data will be published elsewhere in detail. The remaining 645 patients did not have TMAs, and were therefore excluded from this study; this group included 64 patients with DIC or septic DIC.

Congenital TMAs

Patients with repeated TMA episodes usually starting in early childhood with or without familial occurrence are usually considered as congenital TMAs; these patients are largely separated into the following two categories, on the basis of plasma levels of ADAMTS13: AC and ADAMTS 13: INH.

1. Upshaw-Schulman syndrome (USS)

USS is alternatively termed congenital TTP and is characterized by severe deficiency of ADAMTS13: AC due to genetic mutations (27). Forty-one patients (25 females and 16 males) belonging to 36 different families, were placed in this category (Table 2). All of these patients were negative for ADAMTS13: INH. USS is inherited in an autosomal recessive fashion, and therefore, the parents of patients are asymptomatic carriers with significantly reduced plasma levels of ADAMTS13: AC. The female-to-male ratio in the USS patient population is theoretically one-to-one, but our results indicate an apparent female predominance (25 to 16). Of the 41 patients, 17 (41%) had a history of exchange blood transfusions during the newborn period, and 32 (78%) had a history of thrombocytopenia during childhood. For the remaining 9 (22%), it was unclear whether their platelet counts had been checked during that period.

ADAMTS13 gene analysis was performed for 38 USS patients, and the disease-causing mutations were identified in 37 of the 38. Of the 37 genotyped patients, 8 were homozygotes and 29 were compound heterozygotes [one *de novo* mutation (28)] for *ADAMTS13* gene mutations. Of the 8 homozygous patients, the parents of 6 had consanguineous marriages.

2. Congenital TMAs of unknown etiology

Patients in this category were characterized by repeated TMA episodes with predominant renal involvement from early childhood, and often with familial occurrence. Twenty-four patients belonging to 12 families were identified, but the etiology of TMAs in these patients remained completely unclear.

In this regard, it is well known that gene mutations in complement regulatory cofactors (factor H, factor I, factor B, and CD46 or membrane cofactor protein) cause excessive complement activation by impairing C3b inactivation, resulting in severe hemolysis, which triggers TMA episodes. Therefore, these patients are commonly termed 'congenital atypical HUS' (19). It is possible that among the patients of this category in this study, some disease might be related to gene mutations of complement regulatory cofactors, but at the time such analysis had not been done in Japan. As a first step toward such analysis, we determined the plasma levels of factor H antigen by immunoassay in our patients, and did not observe reduced levels in any patients (data not shown).

Table 2. Registration of 41 Japanese Patient with Upshaw-Schulman Syndrome (USS)

No	Patient	Year of birth	Sex	Exchange blood transfusion during newborn period	Thrombocytopenia during childhood	Plasma ADAMTS13:AC (%)	*ADAMTS13 gene* mutations
1	A	1989	M	+	+	<0.5	C-Hetero
2	B	1986	F	+	+	<0.5	Homo
3	C	1972	M	-	+	<0.5	Homo
4	D	1978	F	+	+	<0.5	C-Hetero
5	E	1985	M	+	+	<0.5	C-Hetero
6	F	1983	M	+	+	0.6	C-Hetero
7	G	1987	F	+	+	<0.5	C-Hetero
8	H	1951	M	-	-	0.6	C-Hetero
9	I	1972	M	-	+	<0.5	C-Hetero
10	J-3	1977	F	-	+	<0.5	C-Hetero
11	J-4	1979	M	-	+	<0.5	C-Hetero
12	K-3	1976	F	-	+	<0.5	C-Hetero
13	K-4	1978	F	+	+	<0.5	C-Hetero
14	L-2	1967	F	-	-	<0.5	C-Hetero
15	L-3	1972	F	-	+	<0.5	C-Hetero
16	M-3	1969	F	-	-	<0.5	C-Hetero
17	M-4	1971	F	-	-	<0.5	C-Hetero
18	N	1986	F	+	+	<0.5	C-Hetero
19	O-4	1958	F	-	-	<0.5	C-Hetero
20	P	1971	M	-	+	<0.5	C-Hetero
21	Q (1)	1983	M	+	+	<0.5	C-Hetero
22	Q (2)	1988	M	+	+	<0.5	C-Hetero
23	R-5	1982	F	-	+	<0.5	C-Hetero
24	S	1982	F	-	+	0.9	※
25	T	1981	F	-	+	<0.5	C-Hetero
26	U	1980	F	+	+	<0.5	Homo
27	V	1983	F	+	+	<0.5	C-Hetero
28	W-4	1990	F	-	+	<0.5	C-Hetero
29	X-5	1963	F	-	-	<0.5	※
30	Y	1960	F	-	+	<0.5	C-Hetero
31	Z-3	1971	F	-	+	<0.5	Homo
32	AA	1987	F	-	-	<0.5	※
33	BB	1947	M	-	-	<0.5	Homo
34	CC-5	2004	M	+	+	<0.5	C-Hetero
35	DD	2007	F	-	+	<0.5	C-Hetero
36	EE	2003	M	+	+	<0.5	Homo
37	FF	1981	F	+	+	<0.5	Homo
38	GG	1931	M	-	-	3.4	Homo
39	HH	2004	F	+	+	<0.5	C-Hetero
40	II	1977	F	+	+	<0.5	※
41	JJ	1977	M	-	+	<0.5	C-Hetero

C-Hetero: Compound heterozygotes, Homo: Homozygotes, ※: Not determined.

Acquired TMAs

Patients with acquired TMAs are characterized by the following: 1) usually no familial occurrence, 2) presence or absence of underlying diseases or medications associated with TMAs, and 3) common sudden onset of TMA episodes during adulthood. Patients with acquired TMAs are grouped as primary (idiopathic) or secondary, and then further separated into categories as follows, based on the results of ADAMTS 13: AC and ADAMTS13: INH assays.

1. Idiopathic TMAs

The patients in this group lack apparent underlying diseases or medications related to TMA episodes. Idiopathic TMAs can be further categorized into TTP and HUS subgroups. Idiopathic TTP (n=284) included two patient populations: 1) patients (n=195) with severe deficiency of ADAMTS13: AC, commonly positive for ADAMTS13: INH, and 2) patients (n=89) with clinical 'pentad' signs, regardless of plasma ADAMTS13: AC levels. Distribution of plasma ADAMTS13: AC is shown in Table 1. Detailed analysis of the clinical and laboratory features of these patients will be published elsewhere.

In contrast, idiopathic HUS (n=106) consisted of one patient population with clinical 'triad' signs, without severe deficiency of ADAMTS13: AC. Two patients of this category exhibited low levels of ADAMTS13: INH (0.5-<2 BU/mL).

2. Secondary TMAs

Secondary TMAs develop in the setting of various clinical conditions, such as infection, medication, and various underlying diseases. For instance, acquired TMAs are often associated with connective tissue diseases, and also treatment using several specific drugs. In these patients, clinical signs are often highly variable, so diagnostic differentiation of TTP or HUS appears to be insignificant.

(1) Drug-induced TMAs

A significant number of drugs have been associated with TMAs, including anti-platelet thienopyridine derivative drugs, antineoplastic drugs such as mitomycin C, and quinine (29). We have no experience with quinine-associated TMAs, but observed two suspected drug-associated TMAs:

one with sildenafil (Viagra) and the other with pegylated-interferon. Thus, drug-induced TMAs will be discussed in the following 3 subgroups.

a) Thienopyridine derivative-induced TMAs

Ticlopidine (TC) and clopidogrel (CL) are two typical thienopyridine derivatives (30). We identified 22 patients with TC-induced TMAs and one with CL-induced TMA. Nineteen of the 22 patients with TC-TMAs (86%) had severe ADAMTS13: AC deficiency and were positive for ADAMTS13: INH. The mechanism by which TC induces TMAs is still unclear, but it is speculated that TC becomes active in circulation and binds to ADAMTS13, forming a hapten-carrier complex. Antibodies formed against such a complex may be specific for the hapten, the combination hapten-carrier site, or the carrier alone, in a similar fashion to alpha-methyldopa, which may cause the development of anti-red cell antibodies. In approximately 90% of patients with TC-induced TMAs, the onset of TMA episodes occurred within 40 days of treatment (30). The frequency of TC-induced TMAs is estimated to be one per 1,600 to 5,000 patients. In contrast, only one female patient with CL-induced TMA, who developed TMA episodes 4 days after treatment, has been reported in Japan (31). This patient had slightly reduced plasma ADAMTS13: AC (34%), and was negative for ADAMTS13: INH. The pathogenesis of CL-induced TMAs is unclear, but recent studies suggest that ADAMTS13 is released from the liver into circulation, binds to endothelial cell surfaces, and efficiently cleaves UL-VWFMs. Thus, if endothelial cell injuries are present, ADAMTS13 cannot effectively cleave UL-VWFMs; this may lead to TMA episodes. In this regard, Zakarija et al (32) recently addressed two mechanistic pathways in TMAs related to thienopyridine derivatives.

b) Mitomycin C-induced TMAs

Ten patients with mitomycin C (MMC)-induced TMAs were identified. None had severe deficiency of ADAMTS13: AC, and all were negative for ADAMTS13: INH. Previous reports (33) suggest that MMC-induced TMAs develop with a frequency of 4-15% of the patients treated with this drug. The pathophysiology of MMC-TMAs is not well understood, but it is assumed that MMC may cause vascular endothelial cell injuries.

c) TMAs associated with other drugs

We observed two other TMA patients with severe deficiency of ADAMTS13: AC and positive ADAMTS13: INH. Both of these patients were assumed to have drug-associated TMA. One patient was a 62-year-old male with chronic hepatitis C. This patient developed TMA a month after long-term treatment with pegylated-interferon; the detailed clinical course of this patient was previously reported (34). The other patient with possible drug-induced TMA was a 65-year-old male who had taken sildenafil. The patient had taken sildenafil once several months prior to development of TMA, and then he had taken the drug twice within the 2 weeks prior to TMA. Two days after his third intake of sildenafil, the patient developed a low-grade fever, hemolytic anemia (hemoglobin 10.3 g/dL and reticulocyte 3.9%), thrombocytopenia (11,000/μL), and hematuria. ADAMTS13 analysis identified severe deficiency of ADAMTS13: AC (<3%) and ADAMTS13: INH positivity (1.5 Bethesda U/mL). The patient was treated by oral administration of the anti-platelet drug dipyridamole without plasma exchange. Since then, he has recovered, and his ADAMTS13: AC returned to normal range 3 months later.

(2) Connective tissue diseases and their allied diseases (CTD/AD)-associated TMAs

A close relationship between systemic lupus erythematosus (SLE) and TTP was first described in 1939 (35). It is now known that TMAs are frequently associated with CTDs with a frequency of 1-6% of the patient population (36). We have recently reported that severe deficiency of ADAMTS 13: AC and positive ADAMTS13: INH was predominantly detected in patients with rheumatoid arthritis (RA)- and SLE-associated TMAs, via the analysis of 127 patients with CTD-associated TMAs, whose samples were collected between 1998-2006 (37).

In this study, we included other miscellaneous autoimmune diseases, such as antiphospholipid syndrome (APS), as listed in Table 3, in the analysis. Thus, we examined 221 patients with CTD/AD-associated TMAs (Tables 1, 3), of whom 46 (21%) had severe deficiency of ADAMTS13: AC with positive ADAMTS13: INH, while the remaining 175 (79%) had mild-to-moderate deficiency. We presume that the high prevalence of TMA episodes in patients with CTD/AD is closely related to high plasma levels of VWF over the low levels of ADAMTS13: AC (37). Anatomical changes of the microvasculature, namely narrowed vessel cavities due to the proliferation of vascular endothelial cells, result in altered circulation hemodynamics and contribute to the formation of platelet thrombi at sites of vascular injury.

(3) Malignancy-associated TMAs

Sixty-one patients were classified into this category, which largely consisted of 2 groups: one group of patients with hematological malignancies (n=30) and the other group with malignant solid tumors (n=31) (Table 2).

Of the hematological malignancies, lymphoma was the most frequently seen (n=16), and four of the 16 patients had severe deficiency of ADAMTS13: AC with positive ADAMTS13: INH. The clinical course of one patient with intravascular lymphoma (IVL)-associated TMA was previously reported (38). In this case, the aggravation of TMA was dependent on the treatment efficacy of chemotherapy during the early stage of disease progression, but in the later stage was dependent on rituximab after several relapses during a 4-year observation period (39).

Of 31 patients with malignant solid tumor-associated TMAs, stomach cancer (n=10) was most commonly seen,

Table 3. Details of Underlying Diseases or Clinical Conditions of Patients with Acquired Secondary Thrombotic Microangiopathies (TMAs)

Connective tissue diseases and their allied diseases (CTDs/ADs) (n=221)

Systemic lupus erythematosus (SLE)*	92
Systemic sclerosis (SSc)	51
Polymyositis (PM)/Dermatomyositis (DM)	14
Rheumatoid arthritis (RA)**	10
Mixed connective tissue disease (MCTD)	9
Sjogren syndrome	9
Periarteritis nodosa (PN)	7
ANCA***- related nephritis	7
Antiphospholipid syndrome (APS)	4
Anti-GBM**** antibody positive nephropathy	4
Overlap syndrome	3
Vasculitis	3
Wegner syndrome	2
Pemphigoid	2
Goodpasture syndrome	1
Adult Still's disease	1
Amyloidosis	1
Sarcoidosis	1

SLE* (+APS, n=6; +Sjögren, n=4; +RA, n=1)
RA** (+Amyloidosis, n=1)
ANCA***(anti-neutrophil cytoplasmic antibody)
GBM**** (glomerular basement membrane)

Malignancies (n=61)

Hematopoietic malignancies	n=30
Lymphoma*	16
Myelodysplastic syndrome (MDS)	7
Multiple myeloma	2
Acute myeloid leukemia (AML)	2
Acute lymphocytic leukemia (ALL)	2
Chronic myeloid leukemia (CML)	1

Solid tumor	n=31
Stomach cancer	10
Pancreas cancer	3
Lung cancer	2
Ovarian tumor	2
Neuroblastoma	2
Colon cancer	1
Oesophageous cancer	1
Breast cancer	1
Vater's papilla cancer	1
Pharyngeal cancer	1
Uterus cancer	1
Rectum cancer	1
Medulloblastoma	1
Bile duct cancer	1
Bladder cancer	1
Ewing's sarcoma	1
Histiosarcoma	1

Lymphoma*(Non-Hodgkin's lymphoma, n=7; Intravascular lymphoma, n=6; Burkitt lymphoma, n=3)

Hematopoietic stem cell transplantation (HSCT) (n=54)

Bone marrow transplantation (BMT)*	22
Peripheral blood stem cell transpnatation (PBSCT)**	22
Cord blood tranplantation (CBT)***	10

BMT*(unrelated, n=9; related, n=2; unclear, n=11)
PBSCT** (autologous, n=9; allogenic, n=13)
CBT*** (unrelated, n=10)

Others (n=46)

Liver disease	n=16
Liver transplantation*	7
Liver cirrhosis**	3
Hepatitis C	1
Alcoholic hepatitis	1
Veno-occlusive disease (VOD)	1
Liver insufficiency	1
Fulminant hepatitis	1
Autoimmune hepatitis	1

Infections	n=10
Pneumonia	3
Influenza	2
Human immunodeficiency virus (HIV)	1
Pyothorax	1
Liver abcess	1
Sepsis	1
Meningitis	1

Miscellaneous	n=20
Malignant hypertension	5
Diabetes mellitus	2
Renal transplantation	2
Schönlein-Henoch purpura	1
Duchane type muscluar dystrophy	1
Amyotrophic lateral sclerosis (ALS)	1
Interstitial peumanoia	1
Eoshisophilic syndrome	1
Hyperthyroidism	1
Anorexia nervosa	1
Myasthenia gravis	1
Perforation of duodenal ylcer	1
Encephalitis	1
Ulcerative colitis	1

Liver transplantation* (Hepatitis B, n=1; Biliary atresia , n=1; Primary biliary chirrhosis, n=1; Liver cirrhosis, n=1; unclear, n=3)
Liver cirrhosis** (Hepatitis C, n=1; Alcoholic cirrhosis, n=1, unclear, n=1)

but a variety of organs were involved as listed in Table 3. One patient with Vater's papilla cancer showed severe deficiency of ADAMTS13: AC with the presence of ADAMTS 13: INH.

(4) Hematopoietic stem cell transplantation (HSCT)-associated TMAs

Fifty-four patients with TMAs were classified in this category. Of these, 22 were associated with bone marrow transplantation, 22 with peripheral blood SCT, and 10 with cord blood SCT (Table 3). The pathogenesis of TMAs in this category is highly complicated by pre-conditioning regimens of chemotherapies and body irradiation, as well as post-transplantation complications, such as bacterial or viral infections and graft-versus-host disease (GVHD). It was remarkable that none of the patients in this category had severe deficiency of ADAMTS13: AC, and all were negative for ADAMTS13: INH, as previously reported by others (40).

(5) Pregnancy-associated TMAs

TMA episodes are sometimes precipitated by pregnancy and postpartum, and require a rapid differential diagnosis from other thrombocytopenic status, such as ITP, pregnancy toxemia, eclampsia, and HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome. The category of pregnancy-associated TMAs was present in two clinical settings: USS (congenital TTP) and acquired TTP. We have recently reported that pregnancy induced isolated thrombocytopenia or TTP in all female patients with USS examined, and their babies were stillborn or premature when the appropriate plasma therapy was not performed (41).

In the present study, we were able to identify 15 patients with acquired TMAs associated with pregnancy or postpartum. Of these, eight patients developed TMA episodes at 10-40 weeks of gestation, six patients developed TMA episodes soon after delivery, and one patient developed TMA episodes 3 months postpartum. Four of the 15 patients (27%) had severe deficiency of ADAMTS13: AC with positive ADAMTS13: INH (Table 1). These results suggest that the pathogenesis of acquired pregnancy-associated TMAs may be multifactorial, a different setting from USS.

(6) Escherichia coli O157 H7-associated TMAs:

Shigatoxin (1 and 2), composed of one A-subunit of 33 kDa and five B-subunits of 7 kDa each, is produced by the *E. coli* O157: H7 strain. Shigatoxin binds to a receptor, termed globotriaosyl ceramide, which is richly expressed in glomerular endothelial cells. After binding, shigatoxin is internalized and it induces endothelial cell apoptosis; this releases significant levels of UL-VWFMs into the circulation, resulting in platelet thrombi within microvasculatures. Hence, *E. coli* O157: H7-associated TMAs appear to be induced independent of plasma levels of ADAMTS13: AC. Thirty-two patients with TMAs were in this category, and in fact none of them had severe deficiency of ADAMTS13: AC. However, 22 patients had slightly reduced ADAMTS13: AC (Table 1). The reason underlying this is unclear, but we postulate either that ADAMTS13 is partially consumed to cleave the increased plasma VWF or that shigatoxin directly targets ADAMTS13-producing cells.

(7) TMAs associated with other causes

Forty-six TMA patients, who did not fit the aforementioned categories, were classified in this category (Table 3). Because of high heterogeneity in this category, it was subcategorized into patients with liver diseases (n=16), those with infections (n=10), and miscellaneous causes (n=20).

We have reported that numerous liver diseases are associated with reduced plasma ADAMTS13: AC. Notably, plasma levels of ADAMTS13: AC decline in parallel to the progression of liver cirrhosis (42). More interestingly, several patients with advanced liver cirrhosis had severe deficiency of ADAMTS13: AC with positive ADAMTS13: INH. These patients were assumed to have cryptic clinical signs of TMA; therefore, the term 'subclinical TTP' was introduced. In addition, we have reported on recipients of liver transplants with early allograft dysfunction who showed severe thrombocytopenia accompanied by a marked reduction of ADAMTS13: AC one or two days after transplantation, but without any apparent clinical features of TMAs (43). This observation has been confirmed by two recent reports (44, 45), but the mechanism has not yet been addressed.

Viral or bacterial infections can trigger TMA episodes, but the mechanism has not yet been addressed. Most recently, influenza has been revisited by researchers, due to a close relationship between influenza and TMA originally reported in 1980 (46). It is now known that influenza vaccine may induce TTP or disease relapse (47). We have two patients with influenza A-associated TMAs, and one of them had severe deficiency of ADAMTS13: AC with positive ADAMTS13: INH. Influenza virus or vaccination often worsens underlying diseases or conditions, including diabetes mellitus, pregnancy, and ongoing hemodialysis, resulting in multiorgan failure (MOF). Is it possible that such MOF is caused by microcirculatory disturbances, resembling the pathogenesis of TTP.

Human immunodeficiency virus (HIV) infection is also a known trigger of TMAs (48). In our registry, only one HIV-positive patient with severe deficiency of ADAMTS13: AC with positive ADAMTS13: INH was identified.

Finally, the TMAs that fell into the miscellaneous subcategory are too variable to address in this report. The details of some of these patients will be reported in detail elsewhere by referral physicians.

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A 9-MONTH-OLD INFANT WITH ACQUIRED IDIOPATHIC THROMBOTIC THROMBOCYTOPENIC PURPURA CAUSED BY INHIBITORY IgG-AUTOANTIBODY TO ADAMTS13

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□ *Although acquired idiopathic thrombotic thrombocytopenic purpura (ai-TTP) is rare in children, the authors present the case of a 9-month-old boy with ai-TTP showing severe deficiency of ADAMTS13 activity by its inhibitory IgG-autoantibody (4.8 Bethesda units/mL). Plasma exchange therapy was clinically effective but transient. Deficient activity of ADAMTS13 with the presence of its inhibitor persisted for 7 months after the initial diagnosis. However, other laboratory findings improved gradually with steroid (pulse) therapy. The hitherto insufficiently characterized clinical settings of ai-TTP during early childhood underscore the importance of measuring ADAMTS13 activity and its inhibitors for differential diagnosis in patients with thrombocytopenia of unknown etiology.*

Keywords acquired TTP, ADAMTS13, infancy, von Willebrand factor-cleaving protease

Thrombotic thrombocytopenic purpura (TTP) is prominent in disorders with thrombotic microangiopathy characterized by hemolytic anemia, thrombocytopenia, and organ dysfunctions such as neurological

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abnormalities or renal insufficiency. Recent reports have described that von Willebrand factor (vWF) cleaving protease, designated as ADAMTS13, plays important roles in TTP pathophysiology. The lack of ADAMTS13 activity causes the accumulation of unusually large vWF multimers in the plasma, resulting in the disseminated platelet thrombi characteristic of TTP [1, 2]. Previous reports described that 18–72% of clinically diagnosed TTP patients had severe deficiency of ADAMTS13 activity [3].

Decreased activity of ADAMTS13 in patients with TTP might be associated with either inherited or acquired mechanisms. Hereditary TTP, known as Upshaw–Schulman syndrome (USS), results from ADAMTS13 gene mutations [4, 5]. In contrast, acquired TTP—either idiopathic or secondary to drugs, pregnancy, or diseases such as infections, cancers, and autoimmune diseases—is caused mostly by autoantibody against ADAMTS13 [6, 7].

While approximately one-third of USS cases with an inherent deficiency of ADAMTS13 are diagnosed in adolescence or adulthood after passing early childhood [8], acquired TTP in children, including infant patients, has been reported in the relevant literature. This report describes a young infant with acquired idiopathic TTP caused by IgG autoantibody against ADAMTS13. Clinical findings for this patient suggest that assays of ADAMTS13 activity and its inhibitor are indispensable for differential diagnosis with USS and other thrombocytopenic diseases during childhood.

CASE REPORT

In January 2005, a 9-month-old boy with petechial hemorrhage was referred to the Tokyo Medical and Dental University Hospital for suspected idiopathic thrombocytopenic purpura (ITP). He showed nonimmune hemolytic anemia as well as thrombocytopenia. Subsequent examinations revealed that his plasma ADMATS13 activity by vWF multimer assay [6] was markedly decreased (<3%). Moreover, inhibitor activity was detected in the titer of 4.8 BU/mL. This inhibitor activity resided on the purified IgG (data not shown). Based on these findings, he was diagnosed as having acquired idiopathic TTP. Treatment with plasma exchange (PE) performed at the National Center for Child Health and Development was effective to decrease the inhibitor activity (0.2 BU/mL) and increase the serum ADAMTS13 activity to 62.8%, engendering the improvement of his anemia and thrombocytopenia. However, this effect of 6 courses of PE was transient: after about 1 month, the inhibitor activity rebounded to the higher titer at 10 BU/mL with recurrence of low ADAMTS13 activity (<3%) and hematological abnormalities. No effect of administration of fresh frozen plasma (FFP) was observed, nor any obvious sign of renal insufficiency. After PE treatment, as his platelet count decreased rapidly to the critical level lower than 10×10^9/L, presenting the increased risk of hemorrhage, attending

physicians chose to administer low doses of continuous platelet infusion under close observation and started treatment with oral prednisolone (PSL) (2 mg/kg/day). His platelet counts were constantly higher than 1.0×10^9/L after beginning these treatments.

At admission to our hospital in March 2005, he had no petechiae, hepatomegaly, or neurological abnormality. His mental status was normal. His peripheral blood examination showed severe thrombocytopenia (1.6×10^9/L), mild anemia (Hb 7.9 g/dL), and elevation of reticulocyte counts (16.1%). In fact, RBC fragmentation was found in his peripheral blood smear (0.16%). Biochemical examination revealed mild elevation of lactate dehydrogenase (672 IU/L) and indirect bilirubin (0.88 mg/dL), in addition to decreased haptoglobin: lower than 10 mg/dL. Both blood urea nitrogen (14.2 mg/dL) and serum creatinine (0.19 mg/dL) values were within normal ranges. Results of both direct and indirect Coombs tests were negative. Hemostatic examination showed that PT, APTT, and FDP were within normal ranges, but a slightly elevated value of D-dimer was observed. *Escherichia coli* O157 was not detected in his stool culture. His ADAMTS13 activity remained at an undetectable level (<3%). Furthermore, the inhibitor of ADAMTS13 was detected in serum with the titer of 4.3 BU/mL. Moreover, ADAMTS13 gene analysis in this patient revealed no disease-causing mutations for USS (data not shown).

During his stay at our hospital, he received pulse therapy using methylprednisolone (mPSL) (30 mg/kg/day for 3 days). Although the effect of this therapy was not apparent initially, platelet counts of his peripheral blood increased gradually over the 7 weeks subsequent to pulse therapy. Thereafter, the recovery of ADAMTS13 activity with a concomitant disappearance of its inhibitory activity was observed after approximately 5 and 7 months, respectively, following pulse therapy and initial treatment (Figure 1). Oral PSL was stopped when the recovery of ADAMTS13 activity was detected.

DISCUSSION

We report here an infant case with acquired TTP caused by anti-ADAMTS13 autoantibody. Based on findings of low ADAMTS13 activity with serum inhibitors, our patient was considered to have an acquired form of TTP. Only 2 cases diagnosed as acquired TTP during the first year of life have been reported in the relevant literature [9, 10] (Table 1). Ashida et al. reported a 9-month-old girl with high titer of ADMAMTS13 inhibitor (200 Bethesda units/mL), who was treated successfully with mPSL pulse therapy following PE. Schneppenheim et al. also reported an 11-month-old boy with recurrent thrombocytopenia who responded to corticosteroids. For our patient, however, we were unable to conclude simply that steroid therapy was effective because low ADAMTS13 activity with inhibitors was sustained for a certain time after hematological improvement was achieved.

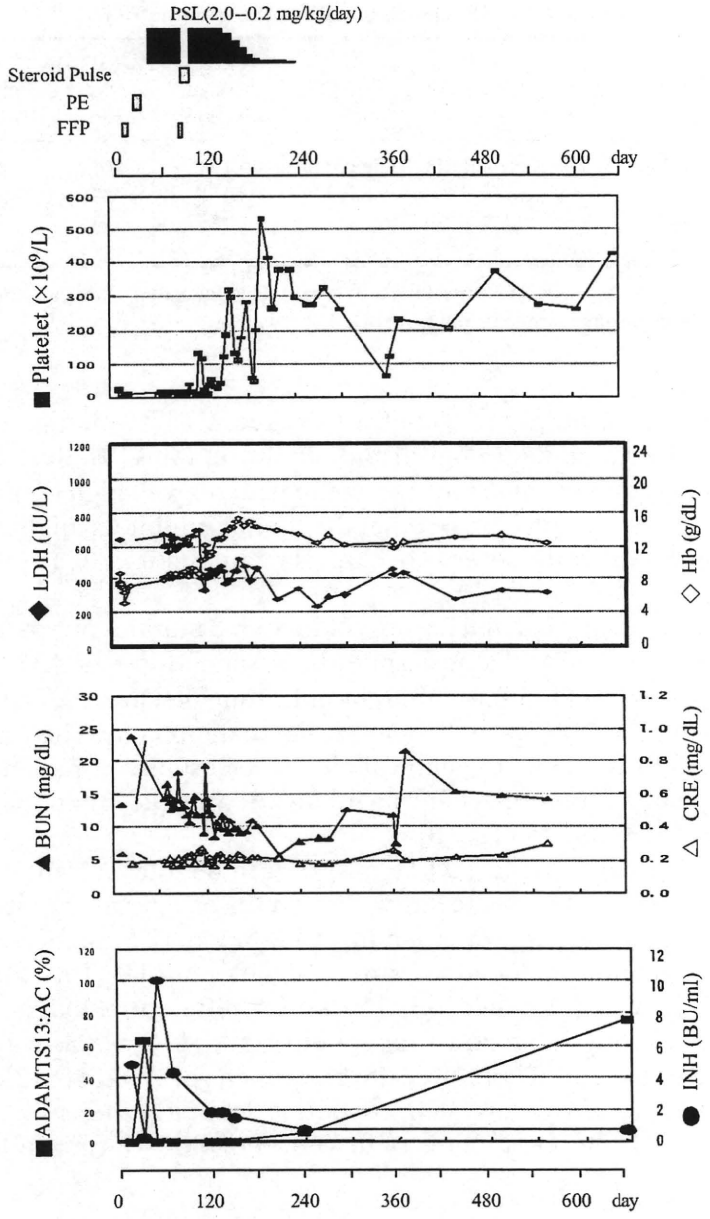

FIGURE 1 Clinical course of the present case of acquired TTP in infancy. ADAMTS13 activity and its autoantibodies were examined using frozen plasma stocked in −80°C with sensitive chromogenic ADAMTS13-act-ELISA method described in a prior study [16]. *Abbreviations*: PSL, prednisolone; FFP, fresh frozen plasma; INH, inhibitors; BU, Bethesda unit.

Neurological abnormalities and renal insufficiency are the hallmarks of acquired TTP in adults. These symptoms are worsened by platelet transfusions through expanding thrombus formation [11]. However, our patient, with a low titer of antibodies, showed no such symptoms at onset or

TABLE 1. Infants with acquired TTP showing anti-ADAMTS13 autoantibody

Patient no.	Sex	Age (months)	Renal impairment	CNS complication	Initial diagnosis	ADAMTS13 activity (%)	Inhibitor (BU/mL)	Ref.
1	F	9	Hematuria	Hemiconvulsion	TTP	<3	200	[9]
2	M	11	(−)	(−)	ITP	<2	(+)	[10]
Present case	M	9	(−)	(−)	ITP	<3	4.8	

Note. M, male; F, female; CNS, central nervous system; ITP, idiopathic thrombocytopenic purpura; TTP, thrombotic thrombocytopenic purpura; BU, Bethesda unit.

worsening in the hospital. In contrast, an infant TTP with high titer of antibodies showed both neurological and renal symptoms [9]. Results show that clinical symptoms become more severe in patients with high titer of inhibitor [12]. Therefore, results suggest that the titer of inhibitors might be a critical factor determining the severity of clinical manifestations of acquired TTP in infancy.

It is noteworthy that, during approximately 3 months, our patient showed hematological improvement despite the sustained low ADAMTS13 activity and the presence of inhibitor, thereby indicating that low ADAMTS13 activity does not necessarily worsen thrombotic microangiopathy. This finding in our patient might resemble the clinical picture of a subset of patients with USS who might be asymptomatic during infancy despite the inherent impairment of ADAMTS13 function [8, 13].

The classical 'pentad' of TTP is known to be fully present in only a minority of patients [3], indicating a limitation of diagnosis based solely on symptoms and routine examinations. Childhood TTP might be diagnosed initially as hemolytic anemia, Evans syndrome, or ITP [10, 14]; the assessment of ADAMTS13 would be of value for differential diagnosis of these diseases. Recently, rapid assays for measuring ADAMTS13 activity have been developed [15, 16]. Therefore, the assessment of ADAMTS13 activity and its inhibitor would be of value as routine laboratory tests for differential diagnosis of thrombocytopenia of unknown etiology during childhood.

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Pivotal role of ADAMTS13 function in liver diseases

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Abstract The liver is a major source of clotting and fibrinolytic proteins, and plays a central role in thromboregulation. Patients with advanced liver diseases tend to bleed because of reduced plasma levels of several clotting factors and thrombocytopenia, but they do also exhibit thrombotic complications. ADAMTS13 is a metalloproteinase, produced exclusively in hepatic stellate cells, and specifically cleaves highly multimeric von Willebrand factor (VWF). VWF plays a pivotal role in hemostasis and thrombosis, and its function is dependent on its multimeric state. Deficiency of ADAMTS13 results in accumulation of unusually large VWF multimers (UL-VWFM) in plasma, in turn induces platelet clumping or thrombi under high shear stress, followed by microcirculatory disturbances. Considering that UL-VWFM, the substrate of ADAMTS13, is produced in transformed vascular endothelial cells at sites of liver injury, decreased ADAMTS13 activity may be involved in not only sinusoidal microcirculatory disturbances, but also subsequent progression of liver injuries, eventually leading to multiorgan failure. This concept can be applied to the development or aggravation of liver diseases, including liver cirrhosis, alcoholic hepatitis, veno-occlusive disease, and adverse events after liver transplantation. These results promise to bring further understanding of the pathophysiology of liver diseases, and offer new insight for development of therapeutic strategies.

Keywords ADAMTS13 · Von Willebrand factor · Liver cirrhosis · Alcoholic hepatitis · Veno-occlusive disease · Liver transplantation · Microcirculatory disturbance · Multiorgan failure

1 Introduction

The liver plays a central role in hemostasis by synthesizing clotting factors, coagulation inhibitors, and fibrinolytic proteins [1]. The hemostatic system is normally in a delicate balance between pro-hemostatic and anti-hemostatic processes [1]. Severe liver diseases are accompanied by multiple changes in the hemostatic system, and the alterations in the system may lead to either a bleeding or thrombosis [1, 2]. Bleeding is clinically evident but hypercoagulability is also an important role in many aspects including poor hepatic blood flow, vasculopathy, and portal and hepatic vein thrombosis, which are closely related to microcirculatory disturbance [2]. Deficiency of anticoagulant proteins and high levels of several procoagulant factors may favor hypercoagulability [2], but the mechanisms underlying this disorder have not been fully elucidated.

ADAMTS13 (*a d*isintegrin-like *a*nd *m*etalloproteinase with *t*hrombo*s*pondin type-1 motifs *13*) is a metalloproteinase that specifically cleaves multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 within its A2 domain [3, 4]. ADAMTS13 deficiency, caused either by mutations in the *ADAMTS13* gene [3–6] or by inhibitory

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autoantibodies against ADAMTS13 [7, 8], results in the accumulation of "unusually large" VWF multimers (UL-VWFM) in plasma; this, in turn, leads to platelet clumping and/or thrombi under high shear stress and subsequent microcirculatory disturbances.

In 2000, we reported that predominantly decreased ADAMTS13 activity (ADAMTS13:AC) in sick children with advanced cirrhotic biliary atresia could be fully restored after living donor liver transplantation, indicating that the liver is the major organ producing ADAMTS13 [9]. In 2001, three other groups indicated that ADAMTS13 mRNA was exclusively expressed in the liver by northern blot analysis [5, 10, 11]. Subsequently, we were able to demonstrate that ADAMTS13 was produced exclusively in hepatic stellate cells (HSCs) using both in situ hybridization and immunohistochemistry [12]. Platelets [13], vascular endothelial cells [14], and kidney podocytes [15] were also shown to be ADAMTS13-producing cells, but the relevance to the pathogenesis of thrombo-regulation in each organ remained unclear.

Since HSCs are the major ADAMTS13-producing cells in human liver [12], we will review the potential functional role of ADAMTS13 in association with the pathogenesis of liver diseases.

2 Hepatic microcirculation and hypercoagulability in liver diseases

Hepatic microcirculation compromises a unique system of capillaries, called sinusoids, which are lined by three different cell types: sinusoidal endothelial cells (SEC), HSC, and Kupffer cells [16]. The SEC modulates microcirculation between hepatocytes and the sinusoidal space through the sinusoidal endothelial fenestration. The SEC has tremendous endocytic capacity, including for VWF and the extracellular matrix, and secretes many vasoactive substances [16]. The HSC is located in the space of Disse adjacent to the SEC, and regulates sinusoidal blood flow by contraction or relaxation induced by vasoactive substances [17]. Kupffer cells are intrasinusoidally located in tissue macrophages, and secrete potent inflammatory mediators during the early phase of liver inflammation [16]. Intimate cell to cell interaction has been found between these sinusoidal cells and hepatocytes [16, 17].

Vascular endothelial cells play a pivotal role in hemostasis and thrombosis [3, 4]. VWF is a marker of endothelial cell activation (damage), and plays an essential role in hemostasis [3, 4]. In the normal state, VWF immunostaining is usually positive in large vessels, but negative in the SEC [18]. On the occurrence of liver injury accompanied by a necroinflammatory process, the SEC becomes positive for VWF, presumably in association with the capillarization of hepatic sinusoids [19]. Subsequently, platelets adhere to subendothelial tissue mediated by UL-VWFM [3, 4]. ADAMTS13 then cleaves UL-VWFM into smaller VWF multimers [3, 4]. This interaction of ADAMTS13 and UL-VWFM is, indeed, the initial step in hemostasis [3, 4]. Recent work has further shown that recombinant ADAMTS13 binds to recombinant CD36 and platelet membrane CD36 in vitro, demonstrating a role for this protein in localizing ADAMTS13 to endothelial cells expressing CD36, where ADAMTS13 regulates the cleavage of VWF [20].

In patients with fulminant hepatic failure and liver cirrhosis, circulating plasma VWF antigen (VWF:AG) levels are extremely high [21–23]. Many fibrin thrombi were found in the hepatic sinusoids in acute liver failure, suggesting a role for intravascular coagulation in the pathogenesis of hepatic necrosis [24]. In cirrhotic liver tissue [25] and even tissue from patients in early stages of alcoholic liver diseases [26], VWF immunostaining shows positive cells predominantly at the scar–parenchyma interface, within the septum, and in the sinusoidal lining. Portal or hepatic vein thrombosis is often observed in advanced cirrhosis [27, 28] and microthrombi formation was found in one or multiple organs in half of autopsied cirrhotics [29]. This hypercoagulable state in liver diseases may be involved in hepatic parenchymal extinction, the acceleration of liver fibrosis, and disease progression.

Considering that ADAMTS13 is synthesized in HSC [12] and its substrate, UL-VWFM, is produced in transformed SEC during liver injury [18], decreased plasma ADAMTS13:AC may involve not only sinusoidal microcirculatory disturbances, but also subsequent progression of liver diseases, eventually leading to multiorgan failure. Based on these findings, it is of particular interest to evaluate plasma ADAMTS13:AC in liver disease patients.

3 ADAMTS13 assays

The classic VWF multimer assay used to be the gold standard method for evaluating plasma ADAMTS13:AC; however, its major disadvantage was that it took several days to provide results [7]. In this regard, the discovery of a minimum 73 amino acid residue sequence within the VWF-A2 domain (VWF73) by Kokame et al. [30], which was prerequisite for the rapid cleavage by ADAMTS13, provided a breakthrough in developing novel methods to assay ADAMTS13:AC. Indeed, a convenient fluorescence method based on FRET-VWF73 is now widely used as the gold standard second generation method [31]. However, the sensitivity of FRET-VWF73 remains approximately 3% of the normal control, and the presence of hemoglobin, bilirubin, and/or chylomicron in samples significantly

influences the results [32]. To solve these problems, a unique method for determining ADAMTS13:AC, termed ADAMTS13-act-ELISA, was developed in our laboratory as a third generation method [33]. This assay was established after production of a novel murine monoclonal antibody to ADAMTS13, termed N-10, which specifically recognizes the Y1605 residue of the VWF-A2 domain, generated by ADAMTS13 cleavage [33]. The lower limit of this assay is 0.5% of the normal control. Developing an automated more rapid assay for ADAMTS13:AC and its usage in hospitals is urged to prevent unnecessary or harmful infusions of platelet concentrates to patients with masked thrombocytopenia, such as "subclinical TTP".

4 The physiological significance of ADAMTS13 in liver diseases

4.1 Liver cirrhosis

Sinusoidal microcirculatory disturbance in liver cirrhosis occurs when the normal hepatic structure is disrupted by fibrin deposition [19] or by impaired balance between the action of vasoconstrictors and vasodilators in hepatic vascular circulation [16]. Studies have shown that cirrhotic liver exhibits a hyperresponse to vasoconstrictors, including catecholamine, endothelin, and leukotrienes D_4 [16]. Now it is well-accepted that thrombocytopenia gradually progresses as functional liver capacity decreases (Fig. 1a). Previously, thrombocytopenia in liver cirrhosis has been speculated to be associated with hypersplenism [34] and decreased synthesis of thrombopoietin in the affected liver [35]. Our recent studies, however, have provided evidence considering that UL-VWFM accumulated in plasmas with far advanced cirrhotic patients enhances high shear-stress-induced platelet aggregation, resulting in thrombocytopenia [36].

Mannucci et al. [37] originally reported a significant reduction of plasma ADAMTS13:AC in advanced cirrhotics. Recently, we showed that ADAMTS13:AC decreased with increasing severity of cirrhosis [36] (Fig. 1b). The values determined by act-ELISA correlated well with those of the classical VWFM assay, and also closely correlated with ADAMTS13 antigen determined by the antigen-ELISA. These results confirmed that both ADAMTS13 activity and antigen decreased with increasing cirrhosis severity [36] (Fig. 1b, c). Our results are consistent with findings described by Feys et al. [38]. In sharp contrast, Lisman et al. [39] showed that both ADAMTS13 activity and antigen levels were highly variable; however, they did not distinguish between patients with varying degrees of cirrhosis. It is unclear why Lisman et al. reached the conclusions different from ours. One possible explanation relates to two distinct clinical settings: a majority of our

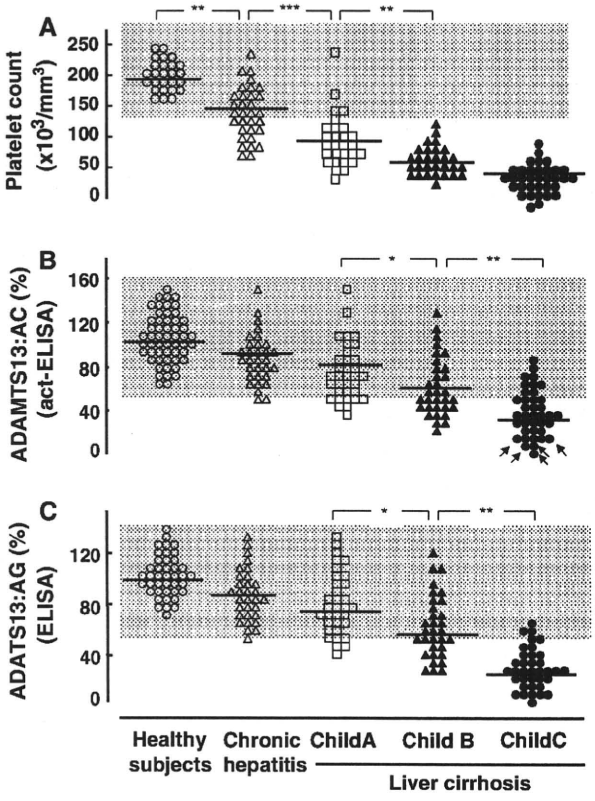

Fig. 1 Platelet counts and plasma levels of ADAMTS13:AC and ADAMTS13:AG in patients with chronic liver diseases. Platelet count decreased with the severity of chronic liver diseases, but no difference was found between Child B and C (**a**). Plasma ADAMTS13:AC determined by the ELISA progressively decreased with worsening cirrhosis (**b**). Severe deficiency in ADAMTS13:AC (<3%) was seen in five liver cirrhosis patients with Child C by the VWFM assay, but by the act-ELISA they ranged from <0.5 to 15.9% of the normal control (**b**, shown by *arrows*). The ADAMTS13:AG levels determined by ELISA also decreased with increasing cirrhosis severity (**c**), which highly correlated with ADAMTS13:AC measured by the act-ELISA ($r = 0.715$, $p < 0.001$). *Open circles* normal controls, *open triangles* chronic hepatitis, *open squares* cirrhosis with Child A, *closed triangles* cirrhosis with Child B, *closed circles* cirrhosis with Child C. *Shaded area* shows normal range. *ADAMTS13:AC* ADAMTS13 activity, *ADAMTS13:AG* ADAMTS13 antigen. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ significantly different between the two groups (partially modified from [36])

patients developed cirrhosis secondary to HCV infection, whereas in the study of Lisman et al. a half of the patients suffered from alcohol abuse-related cirrhosis. Further, the techniques used to determine ADAMTS13:AC differed between our study and theirs. It is assumed that the collagen-binding assay they used can be highly influenced by the increased amount of VWF:Ag in tested cirrhotic plasmas [38], because the substrate in this assay is intact multimeric VWF. In this regard, our act-ELISA is performed using VWF73-based fusion protein, termed GST-VWF73-His, which is readily cleaved by ADAMTS13

without any protein denaturant, and therefore the increased amount of VWF:Ag in tested plasmas does not interfere the assays [36].

Obviously, plasma levels of VWF:Ag substantially increase as liver diseases progress (Fig. 2a) [36], as previously indicated [22, 23]. This is presumably attributed to sinusoidal and/or extrahepatic endothelial damage induced by endotoxin and cytokines [22, 23, 40, 41]. The VWF:RCo was higher (Fig. 2b) [36], but the ratio of VWF:RCo/VWF:Ag was lower in cirrhotic patients than in healthy subjects, suggesting that increased VWF:Ag appears less functional in cirrhosis patients [39]. Nevertheless, our study has clearly shown that the ratio of VWF:RCo/ADAMTS13:AC progressively increases with the worsening of chronic liver diseases (Fig. 2c), more strengthening an enhanced thrombogenesis with the progresses of liver dysfunction and thrombocytopenia [36]. As a part of reflection in our scenario, the decreased platelet counts paralleled to the plasma levels of ADAMTS13:AC [36].

Regarding VWF multimers, the higher molecular weight multimer showed greater degradation than in healthy controls, thus maintaining normal enzyme-to-substrate (ADAMTS13/UL-VWFMs) ratio to maintain blood fluidity [39]. Our recent study showed that there were three different VWFM patterns in cirrhotic patients with lower ADAMTS13:AC (<50% of controls): normal-VWFM was detected in 53%, degraded-VWFM in 31%, and UL-VWFM in 16% (Fig. 3) [36]. UL-VWFM-positive patients showed the lowest ADAMTS13:AC, and the highest values of serum creatinine, blood urea nitrogen, and blood ammonia. In addition, cirrhotic patients with UL- and normal-VWFM had higher levels of VWF:RCo and Child-Pugh score, and lower values of cholinesterase and hemoglobin than those with degraded-VWFM [36]. The pattern, therefore, appears to shift from degraded- to normal-VWFM, and finally to UL-VWFM as functional liver capacity and renal function deteriorate, indicating that advanced cirrhosis may be a predisposing state toward platelet microthrombi formation, even in the absence of clinically overt thrombotic events [36]. In fact, portal or hepatic vein thrombosis is often observed in advanced liver cirrhosis patients routinely screened with Doppler ultrasound [27] and in cirrhotic liver tissue removed at transplantation [28] and at autopsy [29], consistent with our hypothesis.

The mechanism responsible for the decrease in ADAMTS13:AC in advanced cirrhotics may include enhanced consumption due to the degradation of large quantities of VWF:AG [37], inflammatory cytokines [42, 43], and/or ADAMTS13 plasma inhibitor [7, 8]. It is controversial whether ADAMTS13 deficiency is caused by decreased production in the liver; Kume et al. [44] reported that HSC apoptosis plays an essential role in decreased

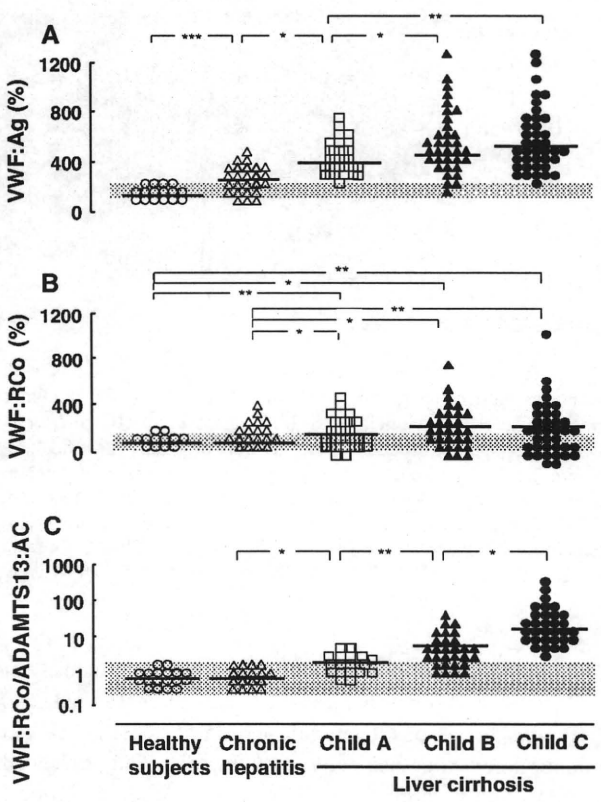

Fig. 2 Plasma levels of VWF:Ag, VWF:RCo, and VWF:RCo/ADAMTS13:AC ratio in patients with chronic liver disease. The VWF:Ag increased with the progression of chronic liver diseases, but the difference between Child B and C did not reach statistical significance (**a**). The VWF:RCo is higher in liver cirrhosis patients than in patients with chronic hepatitis and healthy subjects, but it did not differ among subgroups within liver cirrhosis (**b**). The VWF:RCo relative to ADAMTS13:AC progressively increased with worsening chronic liver disease (**c**). *VWF:Ag* von Willebrand factor antigen, *VWF:RCo* von Willebrand factor ristocetin cofactor activity, *ADAMTS13:AC* ADAMTS13 activity. *Shaded area* shows normal range. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ significantly different between the two groups (partially modified from [36])

ADAMTS13:AC using dimethylnitrosamine-treated rats, but not carbon tetrachloride (CCl_4)-treated animals, whereas Niiya et al. [45] found up-regulation of ADAMTS13 antigen and proteolytic activity in liver tissue using rats with CCl_4-induced liver fibrosis. We observed the inhibitor of ADAMTS13 in 83% of patients with severe to moderate ADAMTS13 deficiency, but its inhibitory activity was in a marginal zone between 0.5 and 1.0 BU/ml in most cases except a TTP patient (2.0 BU/ml) and a patient with severe ADAMTS13 deficiency (3.0 BU/ml) [36]. Interestingly, IgG-type autoantibodies specific to purified plasma derived-ADAMTS13 were detected by western blotting only in five end-stage cirrhotics with severe ADAMTS13 deficiency (<3%) corresponding to TTP [36]. One patient showed an apparent TTP [46], while the other

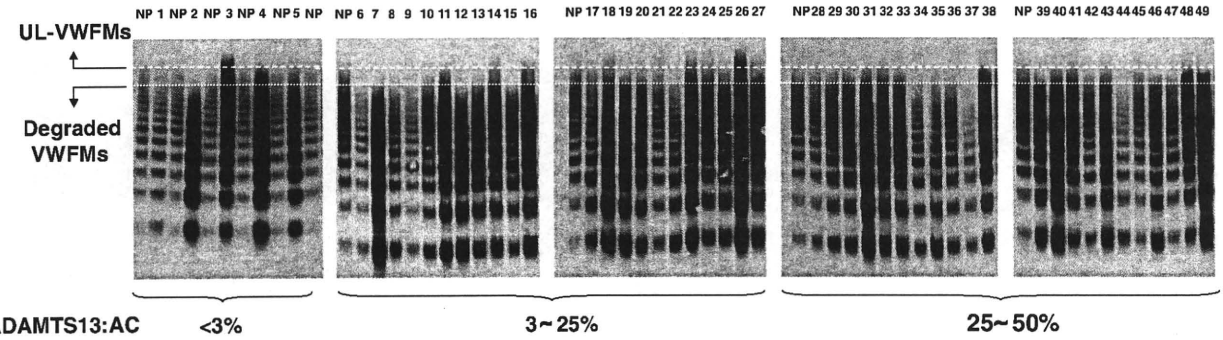

Fig. 3 Plasma VWF multimer in 49 liver cirrhosis patients with severe to mild deficiency of ADAMTS13:AC. The VWF multimer was analyzed by a vertical SDS–1.0% agarose gel electrophoresis system. Five patients (patients no. 1–5) were originally identified as a severe deficiency of plasma ADAMTS13:AC by the von Willebrand factor multimer (VWFM) assay. Twenty-two patients (patients no. 6–27) showed a moderate deficiency (3–25% of the control), and remaining 22 patients (no. 28–49) mild deficiency (25–50% of the control) of plasma ADAMTS13:AC by both methods of VWFM assay and the act-ELISA, without discordant results. There were three different patterns including degraded-, normal-, and UL-VWFM. Out of these 49 patients, 26 (53.1%) showed normal VWFMs, 15 (30.6%) degraded-VWFMs, and the remaining eight (patients no. 3, 4, 11, 14, 16, 18, 23, and 26) (16.3%) UL-VWFMs. *ADAMTS13:AC* ADAMTS13 activity, *VWFM* von Willebrand factor multimer, *UL-VWFM* unusually large von Willebrand factor multimer, *NP* normal control plasma (partially modified from [36])

four cirrhotics did not show apparent clinical features of TTP, but had complications of hepatorenal syndrome (HRS), spontaneous bacterial peritonitis (SBP), marked inflammation together with cytokinemia, and advanced hepatocellular carcinoma (HCC) [36]. Various clinical conditions, including infection, malignancies, and certain drugs, can lead to acquired TTP [47]. In advanced cirrhotics, endotoxemia is frequently detected [23], and SBP sometimes occurs [48]. HCC is highly complicated as the cirrhotic stage progresses [49], suggesting a high-risk state of platelet microthrombi formation. Some end-stage cirrhotics who have extremely low ADAMTS13:AC as well as its IgG inhibitor might be under conditions similar to TTP, or might reflect "subclinical TTP" [36].

With respect to the autoantibodies in patients with HCV-associated liver diseases, there is a general consensus that the overall prevalence of serum non-organ-specific autoantibodies is significantly higher in patients with HCV (about one-third of all cases) than in both healthy subjects and patients with HBV [50–52], but not alcoholic liver injury. That might be additional reason why ADAMTS13:AC significantly decreased in our most patients with HCV-related cirrhosis, but its activity seemed to be highly variable in most patients with alcohol abuse-related cirrhosis as shown by Lisman et al. [39]. Indeed, of our five end-stage LC patients with IgG-type autoantibodies, two were related to HCV, and each one to HBV, PBC and cryptogenic, but none of patients with alcohol abuse-related cirrhosis were found. Further studies will be necessary to clarify whether inhibitors other than the IgG inhibitor might be involved in cirrhotics with lower ADAMTS13:AC.

4.2 Alcoholic hepatitis (AH)

In alcoholic liver diseases, sinusoidal microcirculatory disturbance is thought to play an important pathogenic role [53, 54]. This includes narrowing of the sinusoidal space due to ballooned hepatocytes and perisinusoidal fibrosis, imbalances between endothelin and nitric oxide, and contraction of HSC [53, 54]. AH is a potentially life-threatening complication of alcohol abuse. The severe form of AH, severe alcoholic hepatitis (SAH), is characterized by multiorgan failure with manifestations of acute hepatic failure [55, 56]. In the pathogenesis of SAH, endotoxemia due to hepatic reticuloendothelial dysfunction and increased intestinal permeability may trigger enhanced proinflammatory cytokine production, which potentially causes systemic inflammatory response syndrome together with microcirculatory disturbances, and subsequent multiorgan failure [55, 56].

In our study, plasma ADAMTS13:AC was markedly decreased in the non-survivors of SAH with multiorgan failure; in contrast, mild to moderate decrease was observed in survivors of SAH and those with AH [57]. The VWF:AG was remarkably high in the non-survivors of SAH [58]. At the recovery stage, ADAMTS13:AC returned to the normal range, and the VWF:AG decreased in the survivors, whereas in a non-survivor with SAH, ADAMTS13:AC remained extremely low, and the VWF:AG was still high [57, 58]. UL-VWFM was detected in four of five SAH patients and in five of nine AH patients [58]. The findings of enhanced UL-VWFM production and deficient ADAMTS13:AC may, in part, contribute not only to the development of multiorgan failure but also to the

progression of liver injury through microcirculatory disturbances [57, 58].

Potential mechanism for decreased ADAMTS13:AC may include cytokinemia [42, 43, 59], endotoxemia [59, 60], the inhibitor of ADAMTS13 [7, 8, 59], and the consumption of the protease [37]. Recent investigations demonstrated that IL-6 inhibited the action of ADAMTS13 under flow conditions, and both IL-8 and TNF-α stimulated the release of UL-VWFM in human umbilical vein endothelial cells in vitro [42]. It remains to be clarified whether the IL-6 directly would hamper the cleavage of UL-VWFM or IL-6 would down-regulate gene expression of ADAMTS13 with modifying the promoter activity. IFN-γ, IL-4, and TNF-α also inhibit ADAMTS13 synthesis and activity in rat primary HSC [43]. In addition, inflammation-associated ADAMTS13 deficiency promotes formation of UL-VWFM [61], and intravenous infusion of endotoxin to healthy volunteers caused a decrease in plasma ADAMTS13:AC together with the appearance of UL-VWFM [60]. From these results as well as our own, marked endotoxemia may be closely related to decreased ADAMTS13:AC and the appearance of UL-VWFM through enhanced cytokinemia in AH patients [59]. It will be necessary to clarify what types of inhibitor may be involved in the association with inflammatory cytokines and endotoxin.

4.3 Hepatic veno-occlusive disease (VOD)

Hepatic VOD is a life-threatening complication of patients undergoing allogenic stem cell transplantation (SCT), and occurs at frequencies of 1–54% [62, 63]. Clinically, hepatic VOD is characterized by hyperbilirubinemia, painful hepatomegaly, and fluid retention [63]. Histologically, VOD features sinusoidal fibrosis, necrosis of pericentral hepatocytes, and consequent narrowing of central veins [62, 63]. In these patients, the SEC is the primary site of toxic injury caused by chemotherapy and/or radiation in the setting of SCT, and this initial insult may ultimately lead to the circulatory compromise of centrilobular hepatocytes [62, 63].

Our recent study demonstrated that plasma ADAMTS13:AC is reduced in hepatic VOD patients after SCT (12–32% of normal) compared to non-VOD patients (57–78% of normal), even before any conditioning regimen and throughout SCT, and that the activity might thus be a predictor for the development of hepatic VOD [64]. A multicenter, prospective, randomized controlled study revealed that prophylactic fresh frozen plasma (FFP) infusion as a source of ADAMTS13 may be instrumental in preventing the development of hepatic VOD after SCT [65]. In two typical cases with hepatic VOD, plasma levels of VWF:AG progressively increased and ADAMTS13:AC gradually decreased from preconditioning or the early period after the SCT to the later period at the occurrence of hepatic VOD [65].

Interestingly, in VOD patients, VWFM corresponding to high and intermediate molecular weight, which is usually seen in normal plasma, were lacking at preconditioning or the early period after SCT, and thereafter gradually appeared [65]. Furthermore, in the group without prophylactic FFP infusion, high and/or intermediate molecular weight VWFM was also lacking in the early stage and even in the later stage after SCT. In contrast, in the group with FFP infusion, no apparent changes in VWFM patterns were found throughout SCT [65]. It remains unclear why such a phenomenon occurred, but one possible explanation may be the SEC injury caused by intensive chemotherapy and/or total body irradiation in the setting of SCT. Indeed, chemotherapy before SCT is a regimen with a high incidence of hepatic VOD, and total body irradiation causes radiation-induced liver disease [62, 63]. The amount of VWF released from injured SEC may be increased at first, but may thereafter decrease because the endothelial cells are extensively damaged [65]. After SCT, as damaged endothelial cells gradually regenerate, the release of VWF may increase, resulting in the appearance of high and intermediate VWFM. Under these circumstances, plasma ADAMTS13 may be consumed to degrade the large amounts of VWF. The imbalance caused by decreased ADAMTS13:AC versus increased production of VWF:AG before and during the early stage after SCT would contribute to a microcirculatory disturbance that could ultimately lead to VOD, especially in zone 3 of the hepatic lobule where hepatocytes are susceptible to damage induced by hypoxia [65]. The supplementation of ADAMTS13 by prophylactic FFP infusion may suppress the increase in VWF:AG that is extensively released from damaged SEC.

4.4 Liver transplantation

One of the serious complications in solid organ transplantation is the occurrence of sporadic thrombotic microangiopathies (TMAs) at an estimated frequency of 0.5–3.0% [66–68]. For instance, various degrees of thrombocytopenia are commonly observed after liver transplantation, especially during the first postoperative week, and some clinical studies have demonstrated that thrombocytopenia was significantly associated with poor prognosis [69]. The imbalance between endothelin and nitric oxide produced by the SEC may lead to active vasoconstriction, narrowing of the sinusoidal lumen, and subsequent sinusoidal microcirculatory disturbance [70]. During the past decade, the measurement of plasma ADAMTS13:AC was utilized as a differential diagnostic tool for TMAs [68], but its relevance to organ transplantation itself was not well evaluated.

In this regard, we first reported in 2006 that a significant reduction of ADAMTS13:AC with a concomitant appearance of UL-VWFM was consistently observed in patient plasma soon after liver transplantation [71]. Consecutive analysis of ADAMTS13:AC indicated that these changes reflected liver graft dysfunction, including ischemia–reperfusion injury and acute rejection. The ADAMTS13:AC in these patients often decreased to less than 10% of normal controls, concurrent with severe thrombocytopenia. These clinical and laboratory features appeared to be similar to TMAs, and more specifically to TTP, which is typically defined by severe deficiency of plasma ADAMTS13 with or without neutralizing autoantibodies to this enzyme. However, different from TTP, the liver transplant recipients in our study had no additional clinical signs of TTP, such as neurological manifestation, fever, or renal dysfunction. Thus, the organ dysfunction appeared to be restricted to the liver graft. From these observations, we suggested that a decrease of plasma ADAMTS13:AC coupled with the appearance of UL-VWFM in liver transplant recipients was caused by the mechanism of "local TTP" within the liver graft [71]. It is assumed that the primary target is vascular endothelial cells within the liver graft in both ischemia–reperfusion injury and acute rejection after liver transplantation [72–74]. Indeed, depositions of activated platelets on the sinusoidal endothelium with a concomitant increase of VWF expression have been found in the liver immediately after reperfusion or cold preservation [73, 74]. In addition, the up-regulated VWF expression has been observed in liver allografts during acute rejection [74]. Thus, newly released UL-VWFM from vascular endothelial cells [71], together with consumption of ADAMTS13, induces platelet aggregation or thrombi formation at the hepatic sinusoid, and results in microcirculatory disturbance. This hypothesis might address why organ dysfunction restricts in the graft liver in liver transplantation-associated 'subclinical' TMA, distinct from systemic organ involvements found in "classical TTP".

Recently, two groups of investigators from Japan [75] and the Netherlands [76] reported interesting results as compared with ours. The report by Kobayashi et al. [75] appeared to be in good agreement with ours, because by examining a large number of liver transplant patients ($n = 81$) they provided solid data showing decreased platelet counts and plasma ADAMTS13:AC levels in the early stage of transplantation. Further, they were able to show increased plasma levels of VWF with the appearance of UL-VWFMs, as a reflection of the reduced plasma ADAMTS13:AC. On the other hand, Pereboom et al. [76] reported that a reduction of ADAMTS13:AC occurred within 1 day after liver transplantation, and was followed by an increased plasma level of fully functional VWF; however, they did not address platelet count in their patients ($n = 20$). One of their patients with severe deficiency of ADAMTS13 indeed had thrombotic complications after transplantation, but the patient did not have UL-VWFMs in the plasma. As a partial explanation for this reason, the authors suggested that plasmin activity was increased in these patients by demonstrating increased plasma levels of tissue plasminogen activator. But, if this hypothesis is true, these patients should have severe bleeding symptoms rather than thrombotic complications, or the investigators might be able to demonstrate the presence of VWF fragments specifically generated by plasmin cleavage in patient plasmas [77]. If not, it will be necessary that the presence of UL-VWFMs is carefully re-examined.

Through our experience, we would like to emphasize here that it is extremely important to monitor plasma ADAMTS13:AC in the treatment of thrombocytopenia associated with allograft dysfunction after liver transplantation. This is because the infusions of platelet concentrate under an imbalance of decreased ADAMTS13:AC to enhanced UL-VWFM production might further exacerbate the formation of platelet aggregates mediated by uncleaved UL-VWFM, leading to graft failure via the "local TTP" mechanism [71]. To date, FFP is a unique source of ADAMTS13 replacement therapy, and may improve both liver dysfunction and thrombocytopenia in liver transplant patients. From this point of view, we are particularly interested in the start of clinical trials on recombinant ADAMTS13 preparations.

5 Conclusion and future perspectives

The introduction of ADAMTS13 to the field of hepatology not only enabled us to confirm the diagnosis of TTP early, but also provided novel insight into the pathophysiology of liver diseases. Some diseases were shown to be TTP itself, but others did not show any apparent clinical features of TTP, even in the presence of extremely decreased ADAMTS13:AC and increased UL-VWFM corresponding to TTP. Such TTP-like states, but without disseminated intravascular coagulation, might be "subclinical TTP" as seen in advanced liver cirrhotics [36] and SAH patients [57, 58], or "local TTP" as shown in patients with hepatic VOD after SCT [64, 65] and patients with adverse events after living donor liver transplantation [71]. One would essentially be unable to detect such TTP-like phenomena without the determination of ADAMTS13:AC, because the interaction of ADAMTS13 and UL-VWFM is the initial step in hemostasis, and their abnormalities do occur in the absence of apparent imbalance in other hemostatic factors and/or irrespective of the presence or absence of abnormal