Table 1 (Continued).

No.	Patient	Year of birth	Gender	ADAMTS13 Activity (%)	ADAMTS13 Inhibitor (BU mL^{-1})	ADAMTS13 Titer of IgG-type binding antibody (Year of examination)	*ADAMTS13* gene mutations: Father's origin DCM	Father's origin Missence SNP	Mother's origin DCM	Mother's origin Missence SNP	Reference numbers and remarks
38	GG2	1931	M	2.4–3.4	< 0.5	< 25 × (2008)	p.C1024R		p.C1024R		†
39	HH4	2003	F	< 0.5	< 0.5	< 25 × (2008)	p.Q449X		c.4119delG		†
40	II3	1977	F	< 0.5	< 0.5	50 × (1998), 50 × (2009)	*		*		†
41	JJ3	1980	M	< 0.5	< 0.5	< 25 × (2009)	c.1885delT		p.C908Y		Parent's origin is unknown†
42	KK3	1976	F	< 0.5	< 0.5	< 25 × (2011)	*		*		†
43	LL4	1981	F	< 0.5–1.8	< 0.5–1.4	> 400 × (2002), 200 × (2011)	p.C438S		p.G909R	p.T339R, p.Q448E, p.P618A	†

DCM, disease causing mutation; SNP, single nucleotide polymorphism; USS, Upshaw–Schulman syndrome. *Undetermined. †Unpublished.

Brief clinical data

USS-B3 (*ADAMTS13* genotype: **p.Q449X/p.Q449X**) is an only child who was born in Hokkaido to non-consanguineous parents. Her history prior to reaching 5 years of age was previously described [11,26,38]. Since childhood, she has received prophylactic FFP infusions. As a consequence, she was infected with hepatitis C and has received interferon therapy on two different occasions. In both instances, accelerated thrombocytopenia was observed despite the regular prophylactic FFP infusions. Furthermore, during her early childhood, she received DDAVP (1-desamino-8-D-arginine vasopressin) infusion once that immediately aggravated her clinical signs, including haematuria and thrombocytopenia. Currently, her renal function is normal and her liver function is well preserved (communication with Dr Mutsuko Konno). Her parents initially stated that they had a non-consanguineous marriage. However, a subsequent ancestral analysis revealed that two great-grandparents of USS-B3 on the paternal and maternal sides migrated from the same area (a small fisherman's village) of Iwate to Hokkaido at the end of the 19th century when Hokkaido was an undeveloped island, and the pioneers settled from the Japanese mainland (Honshu). This fisherman's village is located in the northern part of Honshu (Tohoku) facing the Pacific ocean, an area severely damaged several times by earthquake and tsunami – most recently in March 11, 2011. In the old days, this small village was isolated from neighbours, and was surrounded by mountains, suggesting that there were many consanguineous marriages within this village.

Family USS-C

Patient

One male (USS-C3) born in 1972.

Brief clinical data

USS-C3 (*ADAMTS13* genotype: **c.414+1G>A/c.414+1G>A**) is the last of four children to consanguineous parents (first cousins). Notably, the patient's elder brother (third sibling) died of melena soon after birth. The history of this patient was previously described [39,40]. At 8 years of age, USS-C3 was clinically diagnosed with USS. Since then, he has received prophylactic FFP (160 mL) infusions every 2–4 weeks. However, his renal function due to chronic nephritis gradually deteriorated, and in 1995 he required continuous ambulatory peritoneal dialysis (CAPD). Because of repeated peritonitis associated with CAPD, his therapy for renal insufficiency was changed to haemodialysis (three times per week) in 1999. However, his cardiac function decreased, and he eventually died of chronic heart failure in 2010 at 38 years of age.

Family USS-D

Patient

One female (USS-D4) born in 1978.

Brief clinical data

USS-D4 (*ADAMTS13* genotype: **p.I673F/c.414+1G>A**) was born as the second of 2 children to non-consanguineous parents. Her history was previously described [40,41].

Family USS-E

Patient

One male (USS-E4) born in 1985.

Brief clinical data

USS-E4 (*ADAMTS13* genotype: **p.I673F/p.C908Y**) was born as the second of three children to non-consanguineous parents. His history was previously described [40]. The third sibling had Down's syndrome and died of an unknown cause soon after birth.

Family USS-F

Patient

One male (USS-F3) born in 1993.

Brief clinical data

USS-F3 (*ADAMTS13* genotype: **p.R193W/c.1244+2T>G**) was born as the first of three children to non-consanguineous parents. His history was previously described [40]. He currently receives 120 mL FFP infusions when he develops an occasional haemolytic crisis.

Family USS-G

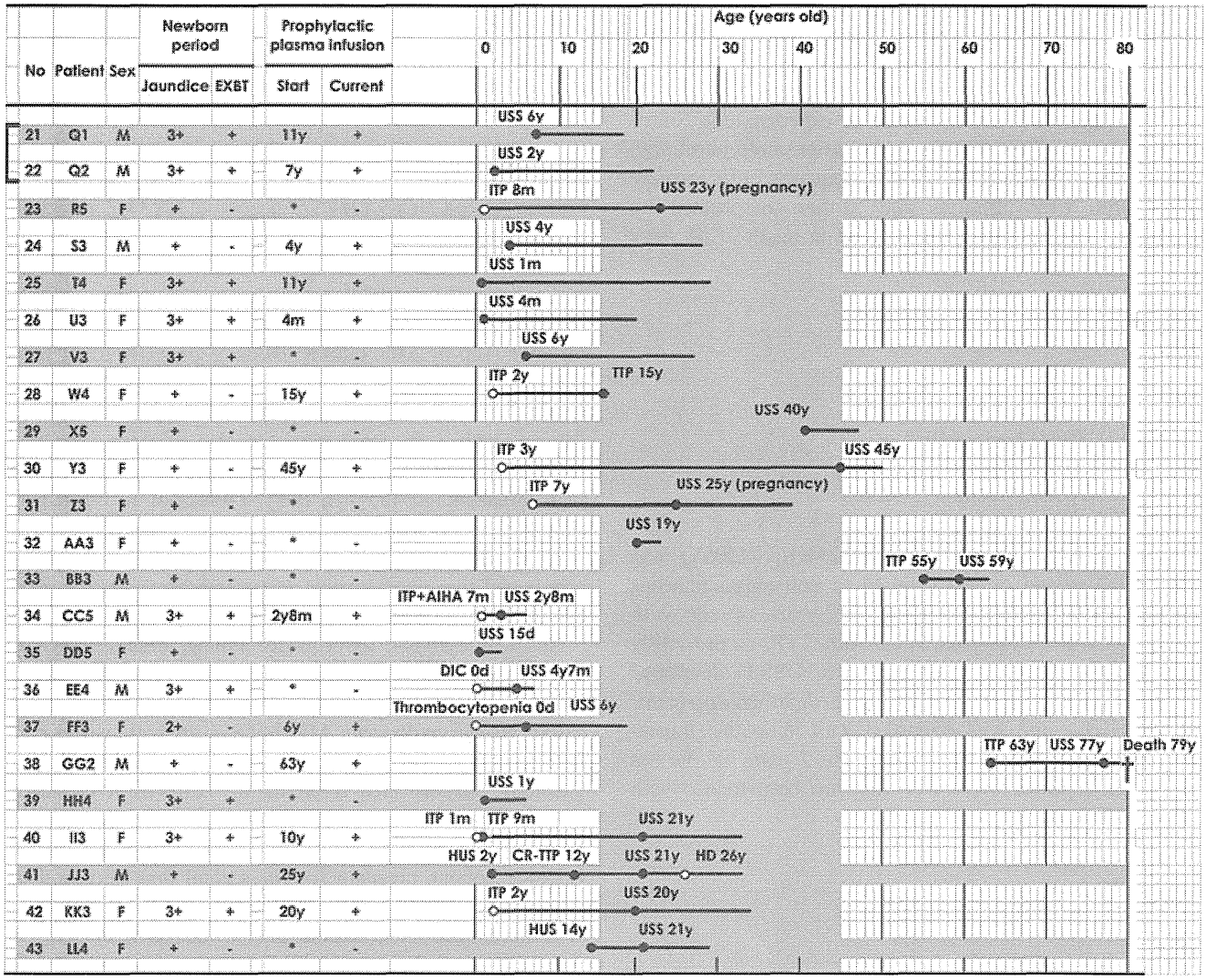
Patient

One female (USS-G3) born in 1987.

Table 2 Clinical course of 43 Japanese patients with USS

No	Patient	Sex	Newborn period: Jaundice	Newborn period: EXBT	Prophylactic plasma infusion: Start	Prophylactic plasma infusion: Current	Age (years old)
1	A4	M	3+	+	4m	+	USS 4m
2	B3	F	3+	+	11m	+	USS 2m
3	C3	M	2+	-	8y	+	USS 8y; CAPD 23y HD 27y; Death 38y †
4	D4	F	3+	+	4y	+	USS 4y
5	E4	M	3+	+	*	-	USS 5y
6	F3	M	3+	+	*	-	ITP 10m; USS 2.5y
7	G3	F	3+	+	-	-	Evans synd 5y; USS 14y
8	H3	M	+	-	*	*	Thrombocytopenia; TTP 51y Death 52y †
9	I4	M	2+	-	2y	+	ITP 3m; TTP 2y
10	J3	F	+	-	*	-	TTP 3y
11	J4	M	+	-	*	-	TTP 5y
12	K3	F	+	-	27y	+	ITP 6y; USS 27y (Pregnancy)
13	K4	F	3+	+	25y	+	ITP 4y; USS 25y (Pregnancy)
14	L2	F	+	-	*	-	USS 25 y (Pregnancy)
15	L3	F	+	-	*	-	ITP 3y; USS 25 y (Pregnancy)
16	M3	F	+	-	*	-	USS 33y (Pregnancy)
17	M4	F	+	-	*	-	USS 30y (Pregnancy)
18	N6	F	3+	+	4y	+	USS 4y
19	O4	F	+	-	26y	+	USS 26y (Pregnancy)
20	P3	M	+	-	21y	+	TTP 3y

Table 2 Continued



No	Patient	Sex	Newborn period: Jaundice	Newborn period: EXBT	Prophylactic plasma infusion: Start	Prophylactic plasma infusion: Current
21	Q1	M	3+	+	11y	+
22	Q2	M	3+	+	7y	+
23	R5	F	+	-	*	-
24	S3	M	+	-	4y	+
25	T4	F	3+	+	11y	+
26	U3	F	3+	+	4m	+
27	V3	F	3+	+	*	-
28	W4	F	+	-	15y	+
29	X5	F	+	-	*	-
30	Y3	F	+	-	45y	+
31	Z3	F	+	-	*	-
32	AA3	F	+	-	*	-
33	BB3	M	+	-	*	-
34	CC5	M	3+	+	2y8m	+
35	DD5	F	+	-	*	-
36	EE4	M	3+	+	*	-
37	FF3	F	2+	-	6y	+
38	GG2	M	+	-	63y	+
39	HH4	F	3+	+	*	-
40	II3	F	3+	+	10y	+
41	JJ3	M	+	-	25y	+
42	KK3	F	3+	+	20y	+
43	LL4	F	+	-	*	-

EXBT, exchange blood transfusion; TTP, thrombotic thrombocytopenic purpura; HUS, hemolytic uremic syndrome; ITP, idiopatic thrombocytopenic purpura; DIC, disseminated intravasclar coagulation; AIHA, autoimmune hemolytic anemia; CAPD, continuous ambulatory peritoneal dialysis; HD, hemodialysis; USS, Upshaw–Schulman syndrome. *Undertermined.

Brief clinical data

USS-G3 (*ADAMTS13* genotype: **p.R1123C/c.686+1G>A**) was born as the third of four children to non-consanguineous parents. Her history before she reached 14 years of age was previously described [40]. Of note, her elder sister (second child) had severe jaundice soon after birth and received an exchange blood transfusion. She died of intracranial bleeding after a trivial traffic accident when she was 8 years old. USS-G3 received prophylactic FFP infusions after she was diagnosed with USS, but the infusion intervals gradually increased. Under these circumstances, she became pregnant at 21 and 22 years of age. During the first pregnancy, she had a spontaneous abortion at 5 weeks of gestation. During the second pregnancy, she delivered a premature baby girl (1446 g BW) at 35 weeks of gestation after a caesarean section under extensive prophylactic FFP infusions (normal Japanese female baby at 35 weeks of gestation has a BW of median 2173 g). Interestingly, many of the placental small vessels were occupied with hyaline thrombosis (communication with Dr Michiko Kajiwara, details will be published elsewhere by the physicians in charge).

Family USS-H

Patient

One male (USS-H3) born in 1951.

Brief clinical data

USS-H3 (*ADAMTS13* genotype: **p.A250V/c.330+1G>A**) was born to non-consanguineous parents, but the details were unclear [42]. At 51 years of age, he visited a nearby

hospital because of a convulsive seizure after haemorrhoidectomy where he was diagnosed with TTP. He had an episode of childhood thrombocytopenia, but there is no additional information. After 51 years of age, he had two episodes of overt TTP, and both were efficiently treated with FFP infusions. In July 2002, he experienced a fourth episode of overt TTP that developed after cholecystectomy, followed by gastrointestinal bleeding that was unsuccessfully treated with FFP infusions and further complicated by renal failure, which ultimately resulted in death at 52 years of age.

Family USS-I

Patient

One male (USS-I4) born in 1972.

Brief clinical data

USS-I4 (*ADAMTS13* genotype: **p.H234Q/p.R1206X**) was born as the second of two children to non-consanguineous parents. His elder brother died at 2 years of age with clinical signs that were compatible with TTP, as previously described [43]. At the age of 3 months, USS-I4 developed thrombocytopenia after receiving the diphtheria/pertussis/tetanus vaccine and was diagnosed with idiopathic thrombocytopenic purpura (ITP). Since he was 2 years old, he has experienced repeated overt TTP that has been treated with plasma infusions.

Family USS-J

Patients

One female (USS-J3) born in 1977 and one male (USS-J4) born in 1979.

Brief clinical data

USS-J3 (*ADAMTS13* genotype: **p.R312C/c.3198delCT**) and -J4 (*ADAMTS13* genotype: **p.R312C/c.3198delCT**) are the first and second of three children to non-consanguineous parents, respectively. For these two patients, severe jaundice was not noted during the newborn period. At 3 years of age, USS-J3 developed a cold followed by purpura with thrombocytopenia and was diagnosed with disseminated intravascular coagulation (DIC). Since then, she has experienced repeated episodes of thrombocytopenia and haemolytic anaemia, and was diagnosed with CR-TTP at 6 years of age. USS-J4 had an episode of purpura and thrombocytopenia when he was 5 years old. In 2000, both patients were shown to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. These two patients were not given prophylactic FFP infusions.

Family USS-K

Patients

Two females (USS-K3 born in 1976 and USS-K4 born in 1978).

Brief clinical data

Patients USS-K3 (*ADAMTS13* genotype: **p.Y304C**/p.T339R-p.Q448E-**p.G525D**-p.P618A) and -K4 (*ADAMTS13* genotype: **p.Y304C**/p.T339R- p.Q448E-**p.G525D**-p.P618A) were the first and second of two children of non-consanguineous parents, respectively. The history of these two patients was previously reported [12]. In 2003, USS-K3 became pregnant at 27 years old and developed overt TTP at 25 weeks of gestation. She experienced intrauterine foetal death followed by a caesarean delivery with a hysterectomy. On this occasion, she was diagnosed with USS. Since then, she has received prophylactic FFP infusions (80–120 mL) every 4 weeks in an out-patient clinic with a good clinical course. However, at the end of 2010, she had H1N1 influenza A virus infection that remarkably aggravated thrombocytopenia and was hospitalised for treatment (communication with Dr Junji Tomiyama).

In 2003, USS-K4, the younger sibling, became pregnant 2 months after her elder sister. She developed mild thrombocytopenia without significant clinical signs at 22 weeks of gestation. She underwent ADAMTS13 analysis, which confirmed a diagnosis of USS. While being treated with FFP infusions, she delivered a premature baby by a caesarean section [12]. Since then, she has received FFP infusions of 80 mL every 3 weeks. In 2008, 5 years after her first pregnancy, USS-K4 became pregnant for the second time and received more frequent FFP infusions (160 mL biweekly). At 29 weeks of gestation, her platelet count suddenly and severely dropped. Thus, at 30 weeks of gestation, a caesarean section was performed, and she delivered a baby (1522g BW) with congenital heart failure due to a ventricular septum defect (details will be published elsewhere by the physicians in charge).

Family USS-L

Patients

Two females (USS-L2 born in 1967 and USS-L3 born in 1972).

Brief clinical data

Both patients USS-L2 (*ADAMTS13* genotype: p.618A-**p.Q1302X/p.R125fsX6**-p.T339R-p.Q448E) and -L3 (*ADAMTS13* genotype: p.618A-**p.Q1302X/p.R125fsX6**-p.T339R-p.Q448E) were born as the second and fifth of five children to non-consanguineous parents. The history of these two patients was previously described [12]. At 27 years of age, USS-L2 became pregnant. At 27 weeks of gestation, she had intrauterine foetal death due to a suspected diagnosis of HELLP (haemolysis, elevated liver-enzymes, low platelets) syndrome. However, she

subsequently had four children who were all premature and uniformly born at approximately 30 weeks of gestation by a caesarean section with oral aspirin. Patient USS-L3, the younger sister of USS-L2, was diagnosed with ITP at 3 years of age. She had two pregnancies at 25 and 27 years of age. However, she lost both babies at 23 and 24 weeks of gestation, respectively, under a suspected diagnosis of 'habitual abortion'.

Family USS-M

Patients

Two females (USS-M3 born in 1969 and USS-M4 born in 1971).

Brief clinical data

Patients USS-M3 (*ADAMTS13* genotype: **p.R193W/p.R349C**) and USS-M4 (*ADAMTS13* genotype: **p.R193W/p.R349C**) were born as the second and third of four children to non-consanguineous parents. The history of USS-M3 was previously described [12]. USS-M3 was primigravida at 33 years of age, and at 20 weeks of gestation she miscarried with overt TTP. The history of her younger sister, USS-M4, was also previously reported [12]. However, recently Kato *et al.* [44] reported a more detailed account of the pregnancy of USS-M4, to which we have to make some corrections. According to that report, USS-M4 became primigravida at 28 years of age. Until 28 weeks of gestation, the pregnancy was uneventful when she suddenly stopped feeling foetal movement, resulting in intrauterine foetal death and a subsequent diagnosis of HELLP syndrome. One year later, at the age of 29, she became pregnant for the second time. She was diagnosed with ITP and treated with prednisolone therapy until 37 weeks of gestation, but with incremental low platelet counts (approximately 23×10^9 L^{-1}). Soon after this, she underwent a caesarean section after receiving concentrated platelet infusions that transiently increased her platelet counts to 96×10^9 L^{-1}. As a result, she delivered a healthy baby. At 32 years of age, she became pregnant for the third time. At 20 weeks of gestation, she developed DIC followed by multi-organ failure, despite extensive treatments, including platelet transfusions. By this time, she had been diagnosed with USS and had undergone ADAMTS13 analysis, along with her elder sister, USS-M3. At the age of 36, USS-M4 became pregnant for a fourth time. With extensive FFP infusions, she continued her pregnancy until 36 weeks of gestation and delivered a healthy baby (2506 g BW) by natural birth with a skin incision [44].

Family USS-N

Patient

One female (USS-N6) born in 1986.

Brief clinical data

Patient USS-N6 (*ADAMTS13* genotype: p.**H234R**-p.P475S/**c.3220delTACC**) was born as the last of four children to non-consanguineous parents. She had a history of severe neonatal jaundice and childhood thrombocytopenia. Her clinical data were previously reported [11,37]. Of note, she developed a thrombotic occlusion of the left carotid artery at 11 years of age that resulted in right hemiparesis. Subsequently, she developed hypertension and proteinuria, but these clinical signs have significantly improved during a long clinical course with prophylactic FFP infusions, although some neurological sequelae have persisted (communication with Dr Seiji Kinoshita).

Family USS-O

Patient

One female (USS-O4) born in 1958.

Brief clinical data

Patient USS-O4 (*ADAMTS13* genotype: **p.I178T/p.Q929X**) was the second of two children to non-consanguineous parents. The history of USS-O4 was previously described [12]. At the age of 26, USS-O4 became pregnant. At 23 weeks of gestation, she developed thrombocytopenia and delivered a premature infant at 25 weeks of gestation who died soon after birth. After delivery, she developed overt TTP that was rescued with plasma exchange. At 31 years of age, she became pregnant for the second time while receiving prophylactic FFP infusions every 1–2 weeks. At 8 weeks of gestation, she developed proteinuria and thrombocytopenia, and therefore received more frequent FFP infusions. At 36 weeks of gestation, she delivered a healthy baby girl.

Family USS-P

Patient

One male (USS-P3) born in 1971.

Brief clinical data

The clinical data for patient USS-P3 (*ADAMTS13* genotype: **p.C908Y/p.C322G-p.T323R-p.F324L**, *de novo* mutation) were previously described [45]. Briefly, USS-P3 was the second of four children to non-consanguineous parents. The first and fourth siblings died of an abortion at 6 and 22 weeks of gestation, respectively, due to unknown causes. At 3 years of age, USS-P3 had clinical signs of overt TTP, which was efficiently treated with FFP infusions. He was repeatedly treated with FFP infusions when overt TTP developed. Thus, after 21 years of age, the prophylactic FFP infusions were continued.

Family USS-Q

Patients

Two males, (USS-Q1) born in 1983 and (USS-Q2) born in 1988.

Brief clinical data

Patients USS-Q1 (*ADAMTS13* genotype: **p.G227R**-p.G1181R/**p.C908Y**) and -Q2 (*ADAMTS13* genotype: **p.G227R**-p.G1181R/**p.C908Y**) were the first and third of three children to non-consanguineous parents. Their detailed clinical data during childhood were reported in 1990 [46].

Family USS-R

Patient

One female (USS-R5) born in 1982.

Brief clinical data

USS-R5 (*ADAMTS13* genotype: **p.R193W**/p.T339R-p.Q448E-**p.A606P**-p.P618A) was the last of three children to non-consanguineous parents. The history of USS-R5 was previously reported [12]. Briefly, at 23 years of age, she became pregnant. At 23 weeks of gestation, she developed mild thrombocytopenia, and at 31 weeks of gestation, she had sudden intrauterine foetal death. After a caesarean section, she developed overt TTP, which was treated with plasma exchange and steroids. On this occasion, she was diagnosed with USS after her ADAMTS13 activity and ADAMTS13 inhibitor status were analysed. This patient did not receive prophylactic FFP infusions.

Family USS-S

Patient

One male (USS-S3) born in 1982.

Brief clinical data

USS-S3 (*ADAMTS13* genotype: undetermined) was born to non-consanguineous parents. Neither his childhood nor family history have been obtained. The patient was clinically diagnosed with USS at a nearby hospital when he was 4 years old. Since then, he has received prophylactic FFP infusions every 1 weeks at the same hospital. In 2002, USS-S3 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. Furthermore, the ADAMTS13 activities for his father and mother were 34.2% and 47.6%, respectively. This family has not been examined for *ADAMTS13* gene mutations.

Family USS-T

Patient

One female (USS-T4) born in 1981.

Brief clinical data

USS-T4 (*ADAMTS13* genotype: **c.3220delTACC/c.3220del-TACC**) was born as the second of two children to non-consanguineous parents. Soon after birth, she developed severe neonatal jaundice and received exchange blood transfusion for three times [47]. One month after birth, she developed haematuria with thrombocytopenia, which led to a clinical diagnosis of USS. She received DDAVP infusion once at the age of 4, by which her platelet count promptly dropped and her clinical signs were aggravated, in accord with a transient disappearance of larger VWFMs from plasma [47]. Thus, she has received prophylactic FFP infusions every 2 weeks since 1992.In 1998, USS-T4 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. She had a homozygous *ADAMTS13* gene mutation of **c.3220del TACC/c.3220delTACC** (exon 24).

Family USS-U

Patient

One female (USS-U3) born in 1990.

Brief clinical data

USS-U3 (*ADAMTS13* genotype: **c.2259delA/c.2259delA**) was born as the second of two children to consanguineous parents (second cousins). Soon after birth, she developed severe neonatal jaundice that required an exchange transfusion. She was clinically diagnosed with USS at 4 months of age. In 1998, USS-U3 was confirmed to have a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. She was homozygous for an *ADAMTS13* gene mutation of **c.2259delA/c.2259delA** (exon 19). This patient has continued prophylactic FFP infusions.

Family USS-V

Patient

One female (USS-V3) born in 1983.

Brief clinical data

USS-V3 (*ADAMTS13* genotype: **p.W1081X/p.R193W**) was born as the second of two children to non-consanguineous parents. Soon after birth, she developed severe neonatal jaundice that required an exchange blood transfusion. She was clinically diagnosed with USS at 4 years of age. In 1998,

USS-V3 was confirmed to have a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. She had a compound heterozygous *ADAMTS13* gene mutation with **p.W1081X** (exon 24) from her father and **p.R193W** (exon 6) from her mother. The patient has been administered FFP infusions on demand.

Family USS-W

Patient

One female (USS-W4) born in 1990.

Brief clinical data

USS-W4 (*ADAMTS13* genotype: p.Q448E-**p.G550R**/p.P475S) was born as the second of two children to non-consanguineous parents. She did not have episodes of severe jaundice as a newborn. At 2 years of age, she developed pneumonia followed by thrombocytopenia. Since then, she has had repeated episodes of thrombocytopenia and haemolytic anaemia that have coincided with various infections, resulting in a diagnosis of Evans syndrome. In 2005, USS-W4 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. *ADAMTS13* gene analysis in USS-W4 suggested that she was a compound heterozygote with a **p.G550R** (exon 14) mutation from her father and an unidentified DCM from her mother. This patient has received prophylactic FFP infusions every 2 weeks.

Family USS-X

Patient

One female (USS-X5) born in 1963.

Brief clinical data

USS-X5 (*ADAMTS13* genotype: p.G1181R/p.P475S) was the last of four children to non-consanguineous parents. She did not have severe neonatal jaundice or childhood thrombocytopenia. She had two pregnancies at the ages of 24 and 26 years that yielded two children. During her first pregnancy, she had pregnancy-induced hypertension, but the details are unknown. At 32 years of age, she developed nephrotic syndrome, followed by repeated haemolytic anaemia and thrombocytopenia of an unknown cause. None of the laboratory markers were indicative of connective tissue disease. She underwent a splenectomy at the age of 36. In 2004, she had a relapse of nephrotic syndrome with haemolytic anaemia and thrombocytopenia that was treated with high-dose steroid therapy with limited success. At this time, her plasma ADAMTS13 activity levels and ADAMTS13 inhibitor status were examined, and she was determined to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. The same results were obtained 6 months later with a different plasma specimen. An *ADAMTS13* gene analysis in USS-X5 identified no DCMs, but revealed two SNPs of p.P475S from her mother and p.G1181R from her father. In 2007, she developed systemic lupus erythematosus (SLE) and was moved to a different hospital, after which we were unable to follow her clinical and laboratory data. From these results, USS-X5 could be considered to be a possible USS.

Family USS-Y

Patient

One female (USS-Y3) born in 1960.

Brief clinical data

USS-Y3 (*ADAMTS13* genotype: **p.G385E/p.R1206X**) was the last of three children to non-consanguineous parents. It is unclear whether this patient had a history of severe neonatal jaundice. However, during childhood she had an episode of thrombocytopenia and was diagnosed with ITP. She has a history of fresh whole blood transfusions, although the details are unclear. Since then, she had no remarkable changes. However, at 45 years of age, she suddenly developed thrombocytopenia and haemolytic anaemia, leading to a diagnosis of Evans syndrome. On this occasion, her physician noted many schistocytes on her blood film, and USS-Y3 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. An *ADAMTS13* gene analysis determined that she was a compound heterozygote with **p.G385E** (exon 10) from her father and **p.R1206X** (exon 26) from her mother.

Family USS-Z

Patient

One female (USS-Z3) born in 1971.

Brief clinical data

USS-Z3 (*ADAMTS13* genotype: **p.R193W/p.R193W**) was the last of three children to consanguineous parents (second cousins). Her clinical data were previously described [12]. Briefly, she became pregnant for the first time at 25 years of age, and at 12 weeks of gestation, she developed thrombocytopenia and was diagnosed with pregnancy-associated ITP. At 32 weeks of gestation, she had a live birth by caesarean section, and then developed overt TTP, which was treated with daily plasma exchange. This patient was referred to our laboratory in 1998, and USS-Z3 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. This patient did not receive prophylactic FFP infusions, and she had more than five TTP episodes between 1998 and 2005. Each episode was treated with 320 mL plasma infusions. She has been receiving prophylactic FFP infusions every 2 weeks.

Family USS-AA

Patient

One female (USS-AA3) born in 1987.

Brief clinical data

USS-AA3 (*ADAMTS13* genotype: not performed) was the first of two children born to non-consanguineous parents. She had neither an apparent history of severe neonatal jaundice nor thrombocytopenia during childhood. At 19 years of age, she suddenly developed petechiae, and her laboratory data indicated severe thrombocytopenia and haemolytic anaemia. Thus, her ADAMTS13 activity was examined and revealed a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. Plasma exchange therapy was performed, and her platelet counts normalised. One month later, her ADAMTS13 activity and ADAMTS13 inhibitor status were re-tested and yielded the same results. In addition, her family members had the following ADAMTS13 activities: father (32%), mother (53%), and younger sister (46%). An *ADAMTS13* gene analysis was not performed in this family because permission was not obtained. In 2009, we determined that USS-AA3 had a normal platelet count (201×10^9 L^{-1}), but her ADAMTS13 activity was still very low (< 0.5% of normal) with no ADAMTS13 inhibitors. Since this point, we have been unable to obtain more up-dated information on this patient.

Family USS-BB

Patient

One male (USS-BB3) born in 1947.

Brief clinical data

USS-BB3 (*ADAMTS13* genotype: **p.R193W/p.R193W**) was the first of three children to consanguineous parents (first cousins). His younger sister died of 'purpura of unknown cause' at 23 years of age. It is unclear whether USS-BB3 experienced episodes of severe jaundice as a newborn or childhood thrombocytopenia. He was married and had three children. At 55 years of age, he developed overt TTP, which was successfully treated with plasma exchange. When he was 59 years old, he developed haematuria and was admitted to a nearby hospital, where an ADAMTS13 analysis showed that he had a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. An *ADAMTS13* gene analysis indicated that he was a homozygote with **p.R193W** (exon 6) (communication with Dr Toshi Imai, details will be reported by the physicians in charge).

Family USS-CC

Patient

One male (USS-CC5) born in 2004.

Brief clinical data

USS-CC5 (*ADAMTS13* genotype: **p.Q723K/p.R398C**) was the last of three children to non-consanguineous parents. Soon after birth, he developed Coombs-negative haemolytic anaemia and was treated with an exchange blood transfusion. At 7 months of age, he became infected with influenza A virus that aggravated his thrombocytopenia and haemolytic anaemia. At 32 months of age, he suddenly developed a transient disturbance in his ability to walk and converse. On this occasion, an ADAMTS13 analysis revealed that USS-CC5 had a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. An *ADAMTS13* gene analysis indicated that he was a compound heterozygote with **p.Q723K** (exon 18) from his father and **p.R398C** (exon 10) from his mother. Since he was diagnosed with USS, he has received prophylactic FFP infusions every 2 weeks.

Family USS-DD

Patient

One female (USS-DD5) born in 2007.

Brief clinical data

USS-DD5 (*ADAMTS13* genotype: **p.R268P/p.Y304C**) was born as the last of three children to non-consanguineous parents. One day after birth, the patient developed haematuria, petechiae, moderate jaundice, and thrombocytopenia, suggesting immune thrombocytopenia. A platelet transfusion was performed that subsequently aggravated her jaundice, which was ameliorated with albumin infusions and phototherapy from three directions. Therefore, an exchange blood transfusion was not performed. Her platelet counts were maintained around $60–100 \times 10^9$ L^{-1}, and at 15 days of age the physician infused FFP at a dose of 10 mL kg^{-1} due to suspected USS. This treatment markedly increased her platelet counts (written information from Dr Hitoshi Miyabayashi). One month after birth, ADAMTS13 analysis showed that the patient had a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. An *ADAMTS13* gene analysis determined that USS-DD5 was a compound heterozygote with **p.R268P** (exon 7) from her father and **p.Y304C** (exon 8) from her mother. The patient did not receive prophylactic FFP infusions.

Family USS-EE

Patient

One male (USS-EE4) born in 2003.

Brief clinical data

USS-EE4 (*ADAMTS13* genotype: **c.2259delA/c.2259delA**) was born as the second child of bi-ovular twins by a caesarean delivery at 37 weeks of gestation to consanguineous parents (second cousins). Soon after birth, USS-EE4 received an exchange blood transfusion under a diagnosis of DIC. However, the other twin did not have these complications. Since then, USS-EE4 has continued to experience mild thrombocytopenia. At 18 months of age, his platelet count dropped to 11×10^9 L^{-1}, and schistocytes appeared on a blood film when the patient had a rotavirus infection. The patient subsequently experienced repeated episodes of thrombocytopenia and haemolytic anaemia associated with a variety of infectious diseases. At the age of 4 years and 7 months, the patient was admitted to a nearby hospital because of exacerbated asthmatoid bronchitis together with severe thrombocytopenia (4×10^9 L^{-1}). After being diagnosed with ITP, the patient was administered high-dose immunoglobulin therapy with steroid therapy, but there was no clinical improvement (written information from Dr Masahiro Migita). ADAMTS13 analysis showed severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. *ADAMTS13* gene analysis in USS-EE4 identified a homozygous mutation of **c.2259delA** (exon 19). This patient did not receive prophylactic FFP infusions.

Family USS-FF

Patient

One female (USS-FF3) born in 1991.

Brief clinical data

USS-FF3 (*ADAMTS13* genotype: **p.Q449X/p.Q449X**) was born as the first of two children to non-consanguineous parents [48]. As a newborn, the patient had moderate jaundice that required phototherapy, but no exchange blood transfusion was required. She also had a history of chronic thrombocytopenia as a newborn, but did not receive specific treatment. At 6 years of age, she developed severe thrombocytopenia and haemolytic anaemia, and ADAMTS13 analysis revealed a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. *ADAMTS13* gene analysis was performed at the laboratory of Dr David Ginsburg, where a homozygous mutation of **p.Q449X** (exon 6) was identified ([40] and written communication with Dr Yoji Sasahara). Since the USS diagnosis was confirmed, the patient has received FFP infusions (5 mL kg^{-1}) every 2 weeks.

Family USS-GG

Patient

One male (USS-GG2) born in 1931.

Brief clinical data

USS-GG2 (*ADAMTS13* genotype: **p.C1024R/p.C1024R**) was born as the fifth of seven children to consanguineous parents (first cousins). The ancestors of this family can be traced back to Kochi on Shikoku Island. The first two siblings died of an unknown aetiology during childhood. Interestingly, USS-GG2 suddenly developed overt TTP with neurological signs at 63 years of age and was admitted to a nearby hospital. Before this, he had never had an episode of anaemia or thrombocytopenia. He was treated with plasma infusions because plasma exchange was not readily available at that hospital. The next day, his neurological signs dramatically improved. He subsequently has experienced repeated episodes of overt TTP, resulting in a clinical diagnosis of CR-TTP, which was treated with biweekly prophylactic FFP infusions (320–480 mL per each). However, at 77 years of age, he had cerebellar bleeding. Thus, he received an ADAMTS13 analysis that showed a significant reduction in ADAMTS13 activity (2.4–3.4% of normal on three different occasions) but no ADAMTS13 inhibitors. An *ADAMTS13* gene analysis revealed that he was a **p.C1024R/p.C1024R** (exon 24) homozygote, confirming the USS diagnosis. Under prophylactic FFP infusions, he was alive until 79 years old, but he suddenly died of stroke in 2011 at the age of 79 (communication with Dr Fumihiro Taguchi, details will be published elsewhere by the physician in charge).

Family USS-HH

Patient

One female (USS-HH4) born in 2003.

Brief clinical data

USS-HH4 (*ADAMTS13* genotype: **p.Q449X/c.4119delG**) was born as the second of two children to non-consanguineous parents. Soon after birth, she developed Coombs-negative haemolytic anaemia that was treated with an exchange blood transfusion. In 2005, she had three episodes of thrombocytopenia and haemolytic anaemia that occurred concomitantly with fever or the chicken pox. Therefore, her ADAMTS13 activity was assayed, and she was determined to have a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. *ADAMTS13* gene analysis revealed that she was a compound heterozygote with **p.Q449X** (exon 12) from her father and **c.4119delG** (exon 29) from her mother. Although she had a history of severe neonatal jaundice followed by an exchange blood transfusion, she subsequently has only had mild clinical signs and has not received prophylactic FFP infusions. She receives FFP infusions only when her platelet count severely drops.

Family USS-II

Patient

One female (USS-II3) born in 1977.

Brief clinical data

USS-II3 (*ADAMTS13* genotype: not performed) was born by a caesarean section as the fourth and final pregnancy of her mother at 40 weeks of gestation. Her parents were non-consanguineous. Her mother had previously had two abortions (5 and 3 months of gestation) and a stillbirth (9 months of gestation) before USS-II3 was born. On the second day after birth, USS-II3 was treated with an exchange blood transfusion because of severe jaundice and thrombocytopenia. One month later, the patient was discharged but the thrombocytopenia continued, suggesting ITP. Since then, she has received whole blood transfusions when her platelet counts have dropped to 10×10^3 L^{-1}. At 9 months of age, the patient was clinically diagnosed with TTP. She was administered FFP infusion when severe thrombocytopenia developed. At 10 years of age, she underwent a splenectomy but there was no clinical improvement. The prophylactic FFP infusions have continued. At 21 years of age, she was diagnosed with USS after it was determined that she had a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. She currently receives prophylactic FFP infusions (120 mL) every week.

Family USS-JJ

Patient

One male (USS-JJ3) born in 1980.

Brief clinical data

USS-JJ3 (*ADAMTS13* genotype: **c.1885delT/p.C908Y**) was born as the last of four children to non-consanguineous parents. He had no history of exchange blood transfusions as a newborn. At 2 years of age, he suddenly complained of abdominal pain and developed haemolytic anaemia, haematuria, and thrombocytopenia. On this occasion, he was diagnosed with acute renal insufficiency due to diarrhoea-negative atypical HUS at a nearby hospital. Under this diagnosis, he received conservative therapy, including heparin, anti-platelet drugs, and red blood cell transfusion, but no platelet or FFP infusions. Over the next 14 years, he occasionally experienced overt HUS. At 12 years of age, his physician noticed that the FFP infusions were highly effective and improved his clinical manifestations, suggesting a clinical diagnosis of CR-TTP. Since 1996, he has received FFP infusions (160–240 mL per each) when his platelet counts have dropped below 100×10^9 L^{-1}, and has been administered FFP infusions of greater volumes (320–480 mL) during instances of overt TTP. In 1998, he was diagnosed with USS after an ADAMTS13 analysis revealed a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. Furthermore, he was a heavy drinker, which increased the frequency of overt TTP. Under these unhealthy conditions, the prophylactic FFP infusions were sometimes interrupted. Thus, when he was 25 years old, he experienced a cerebral infarction and the prophylactic FFP infusions were re-started. Nevertheless, 1 year later, he had severe renal insufficiency that required haemodialysis. Thus, he currently receives maintenance dialysis therapy and prophylactic FFP infusions of 240 mL per week. In 2009, an ADAMTS13 activity analysis revealed a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. *ADAMTS13* gene analysis revealed that he was a compound heterozygote with **c.1885delT** (exon 16) and **p.C908Y** (exon 21), but that was not performed to his parents.

Family USS-KK

Patient

One female (KK3) born in 1976.

Brief clinical data

USS-KK3 (*ADAMTS13* genotype: not performed) was born as the second of three children to non-consanguineous parents. She had no history of exchange blood transfusion during the newborn period. At the age of 2, she developed thrombocytopenia and was diagnosed of ITP. She received a steroid therapy for thrombocytopenia at the age of 17 but without improvement, and then received splenectomy. As a university student at the age of 20, she developed thrombocytopenia and haemolytic anaemia after heavily drinking alcohol, and on this occasion she was clinically diagnosed of TTP at Shinshu university hospital in Nagano. A diagnosis of CR-TTP was made by Dr Miha Furlan at University of Bern in 1998, after ADAMTS13 analysis, which showed a severe deficiency of the activity but without its inhibitors (these results were re-confirmed in March 2011 using chromogenic act-ELISA). Her mother and two siblings had a slightly decreased ADAMTS13 activity (25–50%) (communication with Drs Fumihiro Ishida and Hikaru Kobayashi). Now, the patient receives the prophylactic FFP infusions (240 mL per each) every 3 weeks. *ADAMTS13* gene analysis has not been performed.

Family USS-LL

Patient

One female (LL4) born in 1981.

Brief clinical data

USS-LL4 (*ADAMTS13* genotype: **p.C438S**/p.T339R-p.Q448E-p.P618A-**p.G909R**) was born as the last of two children to non-consanguineous parents. She had no history of exchange blood transfusion during the newborn period. At the age of 14, she was diagnosed of HUS of unknown aetiology,

and received haemodialysis. During 1996–2001, she repeated overt TTP when she had various infectious diseases, and in each occasion she was treated with FFP infusions. In 2002, she was diagnosed of USS after analysing ADAMTS13, showing a severe deficiency of the activity but without its inhibitors in our laboratory. Since then, however, a low-titer ADAMTS13 inhibitor (< 1.4 BU mL^{-1}) was detected on a few occasions, but its clinical significance was not well evaluated. Her parents and elder sister are asymptomatic and have a slightly decreased ADAMTS13 activity (27–57%). The *ADAMTS13* gene analysis in this patient revealed a compound heterozygote of **p.C438S** (exon 12) from her father and **p.G909R** (exon 21) from her mother. She had been treated with FFP infusions on demand. Most recently, she has become pregnant, and her inhibitor titers have remained below 0.5 BU mL^{-1}. Thus, the prophylactic FFP infusions (10 mL kg BW^{-1}) have been started biweekly, and so far no increase of ADAMTS13 inhibitor titer has been observed (communication with Dr Yoshiyuki Ogawa).

Characterisation and allelic numbers of ADAMTS13 gene mutations in Japanese patients with USS

Of our 43 USS-patients, 39 received an *ADAMTS13* gene analysis while it was not performed in four patients (USS-S3, AA3, II3 and KK3). Nine of these 39 USS-patients were homozygous for *ADAMTS13* gene mutations, and 29 were the compound heterozygotes, including one patient (USS-W4) with **p.G550R** mutation on one allele while DCM on the other allele was unidentified. In the remaining patient (USS-X5), two SNPs (p.P475S/p.G1181R) but no DCMs were identified on each allele. Of these 39 USS-patients, five were siblings that each belonged to different families. Thus, the 65 [2 × (39 − 5) − 3] allelic numbers of DCMs in these patients are summarised in Table 3. Interestingly, these mutations are quite different from those reported in the US and Western countries [3,49–66], except for **p.R268P**. However, the **p.R349C** mutation was previously reported in a Chinese USS patient in Hong Kong [67], and **c.330 + 1G > A** was identified in a Korean patient [68]. Thus, it is likely that specific *ADAMTS13* gene mutations are more common among certain ethnicities. In this regard, the mutation of **p.R268P** is quite unique, as the same mutation was reported by Veyradier *et al.* [55] in France, but in a Haitian patient.

The *ADAMTS13* gene mutation with the highest frequency in Japan was **p.R193W** (n = 8), followed by the remaining alleles in order of descending frequency: **p.Q449X** (n = 5), **p.C908Y** (n = 4), **c.2259delA** (n = 4), etc. The **p.Q449X** mutation was localised to the northern part (Tohoku) of Honshu, **c.2259delA** to Kyushu, **p.C908Y** to western Japan, and **p.R193W** to a relatively wide area across Japan but more frequently in western Japan, suggesting some geographical specificity in these mutations (Fig. 1).

Plasma levels of ADAMTS13 activity, ADAMTS13 inhibitor, and IgG-type anti-ADAMTS13 binding antibody in USS-patients

Most of our USS-patients had the plasma levels of ADAMTS13 activity with a < 0.5% of the normal (Table 1), but USS-GG2 alone had the ADAMTS13 activity of 2.4–3.4% of the normal, measured in three different occasions, as described above. Further, seven USS-patients (USS-F3, J3, K3, H3, Q1, S3, and LL4) had a trace amount of ADAMTS13 activity (0.6–1.8% of the normal) on some occasions, of whom four patients (USS-J3, K3, Q1, and LL4) had the ADAMTS13 activity below 0.5% of the normal in different occasions. The reason for this slight variation of plasma ADAMTS13 activity in our patients is presently unknown.

As for the ADAMTS13 inhibitors, all of our USS-patients had plasma levels of < 0.5 BU mL^{-1}, with one exception (USS-LL4), who showed the inhibitor titers ranging from < 0.5–1.4 BU mL^{-1}.

In regard to the IgG-type anti-ADAMTS13 binding antibody, 36 of 43 USS-patients did not have it (shown as the titer of 25 or < 25 × in Table 1). However, seven patients (USS-K3, K4, X5, AA3, EE4, II3, and LL4) had the antibody titers ranged from 50 to 400 × on some occasions. Clinical significance of the IgG-type anti-ADAMTS13 binding antibody is also unclear at moment, but notably six of these seven patients are female.

Table 3 Summary of 65 allelic numbers of ADAMTS13 disease-causing gene mutation out of 69 mutations in 35 Japanese patients with USS (five siblings)

≥ 2 Allelic numbers (n = 11)	Allelic numbers	One allelic number (n = 28)	
p.R193W	8	p.I178T	p.A606P
p.Q449X	5	p.G227R	p.Q723K
p.C908Y	4	p.H234R	p.G909R
c.2259del A	4	p.H234Q	p.Q929X
c.414 + 1G > A	3	p.A250V	p.W1081X
c.3220delTACC	3	p.R312C	p.R1123C
p.R268P	2	p.C322G/p.T323R/ p.F324L	p.Q1302X
p.Y304C	2	p.R349C	c.372insGT
p.I673F	2	p.G385E	c.1885delT
p.C1024R	2	p.R398C	c.3198delCT
p.R1206X	2	p.C438S	c.4119delG
		p.C508Y	c.330 + 1G > A
		p.G525D	c.686 + 1G > A
		p.G550R	c.1244 + 2T > G

Fig. 1. Geographical distribution of 43 Japanese patients with USS and their *ADAMTS13* gene mutations. Among 43 USS patients, an *ADAMTS13* gene analysis was performed in 39 patients. Nine of the 39 USS patients had homozygous *ADAMTS13* gene mutations, and 29 were the compound heterozygotes, including one patient (USS-W4: patient no 28) with disease-causing mutation (DCM) on one allele while the other was unidentified. In the remaining one patient (USS-X5: patient no 29), two single nucleotide polymorphisms (SNPs), p.P475S and p.G1181R, but not DCMs were identified on each allele. The p.Q449X mutation localised to the northern part (Tohoku) of Honshu, c.2259delA to Kyushu, p.C908Y to western Japan, and p.R193W to a relatively wide area across Japan. Circled numbers indicate the patients shown in Tables 1 and 2.

Discussion

Since ADAMTS13 was originally discovered, one major question has been why USS-patients who consistently lack ADAMTS13 activity do not always experience acute symptoms of overt TTP. Furthermore, symptoms often become evident only when the patients have infections or become pregnant [12,69]. In both instances, vascular endothelial cell injury may be involved, and these cases have been indirectly associated with elevated plasma levels of cytokines or soluble thrombomodulin [70]. Consistent with these observations, studies on two different groups of *ADAMTS13* gene knockout mice revealed that UL-VWFMs were detectable in the blood, although the mice did not exhibit acute symptoms [71,72]. Considering these results, investigators have assumed that a deficiency in ADAMTS13 activity is prothrombotic, but alone is insufficient to provoke acute symptoms. Thus, second hits or triggers must exist. Related to this hypothesis, it has been said that there are two clinical features of USS, termed the 'early-onset' and 'late-onset' phenotypes. To partially address this question, we have extensively analysed the natural histories and *ADAMTS13* genotypes of 43 Japanese patients with USS.

This study has two advantages. One advantage is that Japan basically has four small islands, Hokkaido, Honshu, Shikoku, and Kyushu that make tracing the ancestral roots of a targeted USS family favourable. This is because USS patients tend to live near their parents or healthy relatives to receive medical support when they develop overt signs of TTP. In fact, before ADAMTS13 was discovered in 2001, nine patients were clinically diagnosed with USS or congenital CR-TTP in Japan, and none of these patients have moved to other areas or countries. The other advantage of this study can be attributed to the development of two convenient ADAMTS13 activity assays in our country, FRETS-VWF73 [29] and the chromogenic ADAMTS13-ac-ELISA [28]. Both assays are now used worldwide, and in 1998 Nara Medical University started voluntarily using the VWFM assay to meet the requests of clients across Japan. In 2005, the act-ELISA shortened the time required to diagnose TTP, and more importantly facilitated the identification of new USS-patients in Japan.

Although severe neonatal jaundice that requires exchange blood transfusion has been a hallmark of USS, this clinical sign was only present in 18 of 43 (42%) patients in this study. Because of this just four (/18) physicians correctly diagnosed their patient with USS before the patient reached 6 months of age, whereas 10 (/18) physicians required 6 years to reach a diagnosis of USS. On the other hand, among 25 USS patients without severe newborn jaundice, two (/25) were correctly diagnosed within

6 months of age, and six (/25) were diagnosed within 6 years. As a whole, 25 of 43 (58%) USS patients were correctly diagnosed before they reached 15 years of age, including 12 females and 13 males, indicating that there is no gender disparity in diagnosing USS during childhood. These 25 patients would be unanimously considered to have the 'early-onset phenotype'. However, the remaining 18 USS patients were diagnosed after 15 years of age. This raises the question of whether these patients were the true 'late-onset phenotype' or not. One particularly interesting result was that 15 (/18) patients were diagnosed between 15 and 45 years of age, and interestingly they were all female. Furthermore, among these 15 female patients, nine were diagnosed in association with pregnancy. The remaining three patients (USS-H3, -BB3, and -GG2) were diagnosed after 45 years of age, and they were all male, which sharply contrasts the previous scenario. Thus, the natural history of these three male patients appeared to be an excellent means to analyse the pathogenesis of the 'late-onset phenotype'. Among these patients, USS-H3 with a p.A250V/c.330 + 1G > A genotype had an episode of thrombocytopenia, but there are few clinical details and the patient died of renal failure in 2002 [42], Thus, no further results on USS-H3 are available.

However, two other males, USS-BB3 and USS-GG2, had received annual health examinations during adulthood, and there were no apparent abnormalities until sudden and overt TTP developed at 55 and 63 years of age, respectively. This may indicate that the clinical signs of TTP were very mild during their childhood and adulthood, and any symptoms might have been attributed to isolated mild thrombocytopenia. Interestingly, these two elderly men carried two different homozygous *ADAMTS13* gene mutations, p.R193W/p.R193W and p.C1024R/p.C1024R, respectively. We previously reported that the p.R193W protein was present in the plasma of patient USS-Z3 [12,73]. In this study we also determined that the p.C1024R protein was present in the plasma of patient USS-GG2 (data not shown). Furthermore, *in vitro* expression studies using HeLa cells that were transfected with either of these two mutant gene plasmids showed that each protein was consistently secreted into the culture medium but had much reduced activity compared to the wild-type protein ([39] and unpublished data). Consistent with these observations, the ADAMTS13 activity of patient USS-GG2 was mildly reduced (2.4–3.4% of the normal) on three different occasions. As for the homozygous p.R193W/p.R193W mutation, we identified another female patient (USS-Z3) who was correctly diagnosed with USS at 27 years of age as a result of pregnancy-associated TTP at 25 years of age. Her past history was well recorded, and indicated that she had mild jaundice as a newborn and thus did not receive an exchange blood transfusion. However, she was diagnosed with ITP with isolated thrombocytopenia at 7 years of age. Taken together, these results indicate that the phenotype of the homozygous p.R193W/p.R193W mutation is mild. Therefore, patients carrying this mutation would presumably have mild thrombocytopenia during childhood, as shown in USS-Z3, unless they are exposed to strong stimuli such as a cytokine storm during influenza virus infection. However, after adolescence the gender disparity apparently determines the fate of these USS-patients. Pregnancy undoubtedly is a strong inducer of overt TTP in female USS-patients, although the pathogenesis is not fully elucidated. However, it is now well established that plasma VWF levels remarkably increase as gestation progresses, along with the appearance of UL-VWFMs, which are accompanied by reduced ADAMTS13 activity due to consumption, even in normal pregnant women [74,75]. Thus, in pregnant USS women, an enormous excess of the substrate (larger VWFM) relative to the ADAMTS13 enzyme is the most plausible pathogenic mechanism.

As a consequence, our studies here have re-confirmed that pregnancy, influenza infection, and DDAVP administration can be the strong triggers inducing overt TTP in USS-patients. Besides, now it is indicated that the aging, interferon therapy, and heavily drinking alcohol could be additional modifiers aggravating clinical signs of USS-patients.

Given that the p.R193W mutation is a frequent DCM for USS in Japan, male patients carrying this mutation might not exhibit clinical signs of thrombosis at a younger age. However, as they age, multi-factorial endogenous and exogenous causes mentioned above would facilitate thrombotic events, leading to brain infarctions and chronic renal failure as a result of microcirculation disturbances. We speculate that thrombotic events in the brain or kidney, which still have an unknown pathogenesis, might result from *ADAMTS13* gene abnormalities. Our examination of the natural history in this large cohort of USS-patients with *ADAMTS13* mutations may shed light on these important diseases. Thus, here we emphasise again an importance of the assay for ADAMTS13 activity as a routine test to make and/or exclude a diagnosis of USS, when physicians meet the patients with thrombocytopenia of unknown aetiology, not only in childhood but also in adulthood.

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Disclosure of Conflict of Interests

YF is a clinical advisory board for Baxter Bioscience.

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Reduced larger von Willebrand factor multimers at dawn in OSA plasmas reflect severity of apnoeic episodes

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ABSTRACT: **Plasma von Willebrand factor (VWF), produced in and released from vascular endothelial cells by various stimuli including hypoxia, induces platelet aggregation under high shear stress and plays dual pivotal roles in haemostasis and thrombosis within arterioles, which are regulated by the size of vWF multimers (VWFMs).**

Patients with obstructive sleep apnoea (OSA) have increased risk of thrombotic cardiovascular events, but the pathogenesis is unclear. We examined the relationship between VWF and OSA by measuring VWF antigen (VWF:Ag), VWFMs, VWF collagen binding activity (VWF:CB) and a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13. A total of 58 OSA patients were enrolled. Blood samples were collected before sleep, after sleep, and after one night of nasal continuous positive airway pressure therapy.

Based on VWFM analysis, OSA patients were classified into three groups; consistently normal VWFMs (group 1, n=29), increased high molecular weight (HMW)-VWFMs at 06:00 h (group 2, n=18), and decreased or absent HMW-VWFMs at 06:00 h (group 3, n=11). Patients in group 3 had significantly worse apnoea/hypopnoea index; VWF:CB followed a similar pattern. We observed a significant decrease in platelet count between 21:00 h and 06:00 h in OSA patients, potentially associated with reduced larger VWFMs together with decreased VWF:Ag levels. Severe OSA may contribute to an arterial pro-thrombotic state.

KEYWORDS: **ADAMTS13, obstructive sleep apnoea, von Willebrand factor**

Obstructive sleep apnoea (OSA) is characterised by the collapse of the upper airway and associated intermittent hypoxia during sleep [1]. OSA is associated with excessive daytime sleepiness and cardiovascular disease. Patients with OSA often suffer from obesity, hypertension, hyperlipidaemia, and impaired glucose tolerance, and OSA is an independent risk factor for cardiovascular diseases [2–4]. Consistent with this, cardiovascular risk returned to baseline in OSA patients treated with nasal continuous positive airway pressure (CPAP), whereas those with severe untreated OSA maintained a high risk [5]. Recently, some association of OSA with venous thromboembolism in regard to pulmonary embolism has been implicated [6, 7]. However, the mechanism of OSA-associated thrombosis might be multifactorial, and in fact has not been evaluated on a basis of arterial thrombosis, which is generated under high shear stress in microvasculatures, where von Willebrand factor (VWF) plays a critical role as a molecular glue that facilitates platelet aggregation or thrombi.

VWF is a macromolecular plasma protein, which is exclusively produced in and released from vascular endothelial cells, and exerts pivotal effects on both haemostasis and thrombosis. VWF assembles into unusually large VWF multimers (UL-VWFMs) consisting of identical 250 kDa subunits, before its release into the circulation. Under normal circumstances, UL-VWFMs are rapidly cleaved by a specific plasma protease, ADAMTS13 (a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13), under the high shear stress generated in the microvasculature; consequently, VWF circulates in the plasma as a heterogeneous family of multimers ranging in size from 500 to 15,000 kDa. UL-VWFMs play an essential role in primary haemostasis by binding platelets to denuded vascular endothelial tissue. However, in the absence of ADAMTS13 activity (ADAMTS13:AC) due to gene mutation or acquired autoantibodies, UL-VWFMs remain uncleaved and generate platelet hyperaggregation. Uncleaved UL-VWFMs lead to the formation of vast platelet thrombi, known as thrombotic

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TABLE 1 Characteristics of patients with obstructive sleep apnoea (OSA) and sleep controls

	OSA	Sleep controls	p-value
Sex n (M/F)	58 (55/3)	25 (22/3)	NS
Blood type			NS
A	18	12	
B	8	2	
O	26	8	
AB	6	3	
Age yrs	44.7±9.9	38.3±7.1	<0.01
BMI kg·m^{-2}	28.2±3.7	27.7±3.0	NS
AHI	50.5±22.2	4.5±2.8	<0.01
ODI3%	41.6±19.9	7.8±5.1	<0.01
Lowest S_{p,O_2} %	76.0±10.0	88.8±5.0	<0.01
Systolic blood pressure mmHg	129±16	122±28	NS
Diastolic blood pressure mmHg	82±12	81±10	NS
vWF:Ag levels % at 06:00 h	103.1±61.4	143.5±63.8	<0.01
ADAMTS13:AC levels % at 06:00 h	56.8±22.6	61.7±20.6	NS

Data are presented as mean±SD, unless otherwise stated. M: males; F: females; BMI: body mass index; AHI: apnoea/hypopnoea index; ODI3%: oxygen desaturation index ⩾3%; S_{p,O_2}: arterial oxygen saturation measured by pulse oximetry; vWF:Ag: von Willebrand factor antigen; ADAMTS13:AC: a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity; NS: not significant.

thrombocytopenic purpura, a life-threatening generalised disease [8–11].

It is now well established that high plasma levels of VWF antigen (VWF:Ag) are linked with an increased risk for ischaemic heart disease and ischaemic stroke [12–14]. Furthermore, the relative risks of stroke and acute myocardial infarction are higher in individuals with lower ADAMTS13:AC [14, 15]. Furthermore, hypoxia leads to increased VWF release from cultured vascular endothelial cells, both directly, by up regulating VWF expression, and indirectly *via* autocrine and paracrine signalling downstream of hypoxia-induced inflammatory cytokines including interleukin (IL)-6, IL-8, and tumour necrosis factor-α [16, 17]. Despite these important reports of hypoxia-induced VWF secretion, no subsequent studies have addressed the relationship between VWF and the severity of OSA [18, 19]. In particular, no studies have been performed on plasma samples obtained in chronological order relevant to the sleep cycle.

In this study, we sequentially analysed plasma VWF:Ag levels, VWFM patterns, and ADAMTS13:AC in OSA patients not only before and after sleep, but also before and after CPAP treatment. We found that the reduced larger VWFMs together with decreased VWF:Ag levels in the plasma of OSA patients taken at dawn correlate with the clinical severity of apnoeic episodes.

PATIENTS, MATERIALS AND METHODS

Patients

Between February 2004 and April 2011, 284 patients received full standard diagnostic polysomnography (PSG) at Nara Medical University Hospital (Nara, Japan). Among them, 86 patients were diagnosed with normal or mild OSA (apnoea/

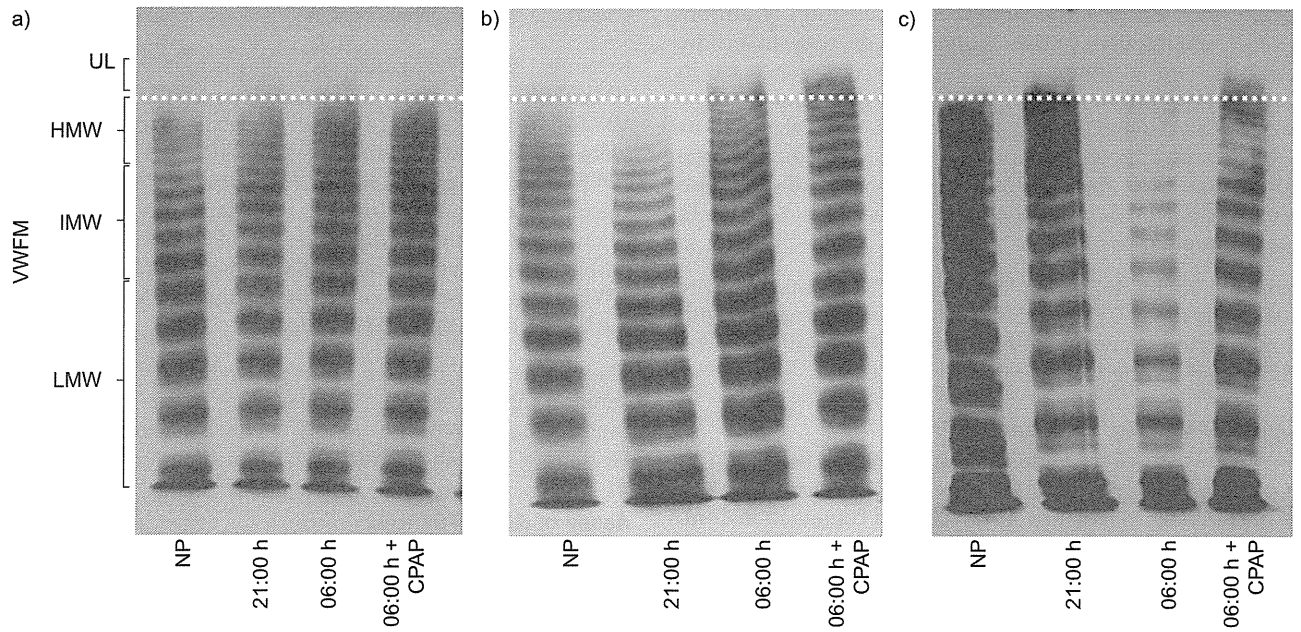

FIGURE 1. Patterns of von Willebrand factor multimers (VWFMs) corresponding to three patient groups. Obstructive sleep apnoea (OSA) patients were categorised into three groups based on the results of VWFM analysis, using sequential samples. Representative results from each group are shown. a) Group 1, patients (n=29) showed a consistently normal pattern of VWFMs. b) Group 2, patients (n=18) had increased, unusually large (UL)- and high molecular weight (HMW)-VWFMs at 06:00 h compared to 21:00 h. c) Group 3,patients (n=11) had decreased UL- and HMW-VWFM at 06:00 h compared to 21:00 h.

hypopnoea index (AHI) <15), and 198 patients were diagnosed with moderate or severe OSA (AHI ⩾15) and received nasal CPAP therapy. Within the latter group, 140 patients with the following underlying diseases were excluded: stroke, coronary artery disease, asthma, chronic obstructive pulmonary disease, arthritis, autoimmune disease, rhinitis, and malignant diseases. The 58 remaining OSA patients were enrolled in this study; detailed clinical information for these 58 patients is shown in table S1. Written informed consent was obtained from all patients, and the study was approved by the Human Subjects Ethics Committee of Nara Medical University (No. 04-012). 25 healthy volunteers (88% male), as shown in table 1, that had undergone PSG studies without OSA were also enrolled and used as the sleep controls.

Blood sampling

Plasma samples were collected from OSA patients at three time points throughout the day; 21:00 h before PSG, at 06:00 h after the PSG without CPAP, and at 06:00 h after CPAP treatment. For the sleep control subjects, plasma samples were collected at 06:00 h. Blood was collected in plastic tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) containing a tenth volume of 3.8% trisodium citrate an anticoagulant, and platelet-poor plasma was prepared by centrifugation at 3,000 × g for 15 min at 4°C. Aliquots were stored at -80°C prior to use. To obtain platelet counts, blood was collected into plastic whole blood tubes with spray-coated EDTA (Becton, Dickinson and Co.) tubes containing EDTA as an anticoagulant and analysed with a Coulter counter (Beckman Coulter, Tokyo, Japan).

Sleep study

PSG was performed using a computerised polysomnography system (Alice 4; Respironics, Pittsburgh, PA, USA). Data acquisition began at 21:00 h and continued until 06:00 h the following day. Apnoea was defined as a cessation of airflow for ⩾10 s, and hypopnoea was defined as a decrease in airflow at least 50% for a minimum of 10 s or a clear decrease in airflow (⩾20%) followed by either oxygen desaturation ⩾3% or signs of physiological arousal. The AHI was calculated as the number of apnoea/hypopnoea events per hour of total sleeping time. We also calculated the oxygen desaturation index ⩾3% (ODI3%), defined as the number of ⩾3% dips in oxygen saturation per hour of sleep.

During the night, following diagnostic PSG, patients were treated with nasal CPAP (REMstar Auto; Respironics), with PSG monitoring. Apnoeic episodes were substantially reduced or eliminated during treatment with nasal CPAP.

Analyses of VWF:Ag, VWF, and VWF:CB

Plasma VWF:Ag levels were measured by sandwich ELISA using a rabbit anti-human VWF polyclonal antiserum (DAKO, Glostrup, Denmark) [20]. The VWF:Ag level contained in 1 mL of pooled normal human plasma was defined as 100%; VWF:Ag levels in the 20 healthy controls were 102±33% (mean±SD) [21].

VWFMs were analysed by sodium dodecyl sulphate-1.2% agarose gel electrophoresis followed by Western blotting with luminographic detection [22, 23]. The blots were scanned and subjected to densitometric analysis using ImageJ (National Institutes of Health (NIH), Bethesda, MD, USA). Multimers were classified as low molecular weight (LMW-VWFMs; corresponding to bands 1-5 in VWFM analysis), intermediate molecular weight (IMW-VWFMs; bands 6-10), and high molecular weight (HMW-VWFMs; bands ⩾11) [24]. High molecular weight bands that were not detected in normal plasma (NP) were defined as UL-VWFMs. The levels of LMW-, IMW- and HMW-VWFMs were calculated using NIH ImageJ. For quantitative analyses, we calculated the ratios of the densities of VWFMs, LMW, IMW, and HMW relative to total VWFM density. Further, multimeric VWF:Ag levels were calculated by multiplying VWF:Ag level by the LMW, IMW, and HMW ratios.

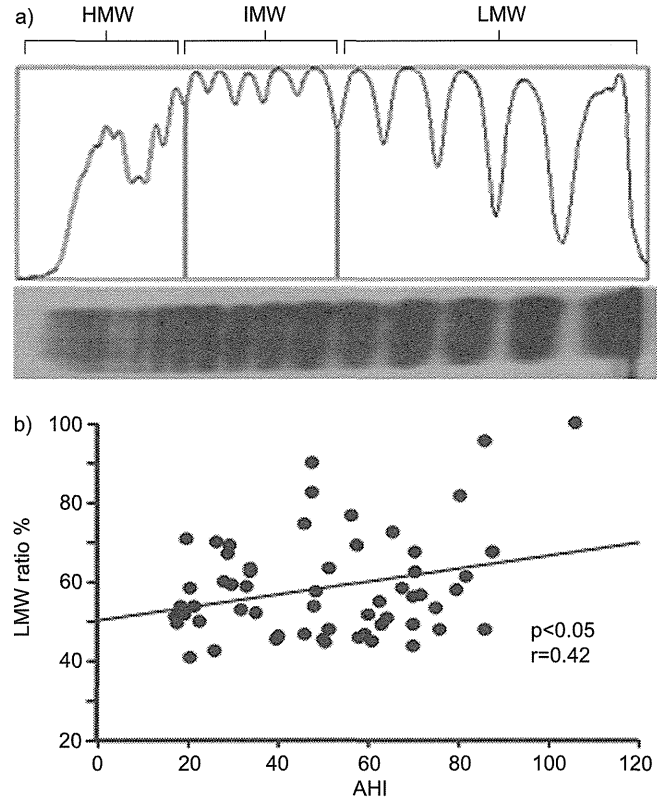

FIGURE 2. Relationship between low molecular weight (LMW) von Willebrand factor multimers (VWFMs) to total VWFMs (LMW ratio) and hypoxia. a) Quantitative analysis of VWFMs was performed by calculating the density of LMW-VWFMs relative to total M density. A representative result of VWF analysis at 06:00 h is shown. b) The LMW ratio of obstructive sleep apnoea patients was significantly correlated to apnoea/hypopnea index (AHI). IMW: intermediate molecular weight.

The plasma VWF collagen binding activity (VWF:CB) was measured using an enzyme immunoassay using a commercially available kit (VWF-CBA ELISA, PROGEN Biotechnik GmbH, Heidelberg, Germany) according to the manufacturer's instructions.

Assay of ADAMTS13:AC

ADAMTS13:AC was determined using a commercially available chromogenic ELISA/ACT (Kainos Co., Tokyo, Japan). The detection limit of this assay was 0.5%; the values obtained from 55 healthy controls were 99.1±21.5% (mean±SD) [25].

Statistical analysis

Laboratory data are expressed as the mean±SD. Comparisons between OSA patients and controls were analysed using the

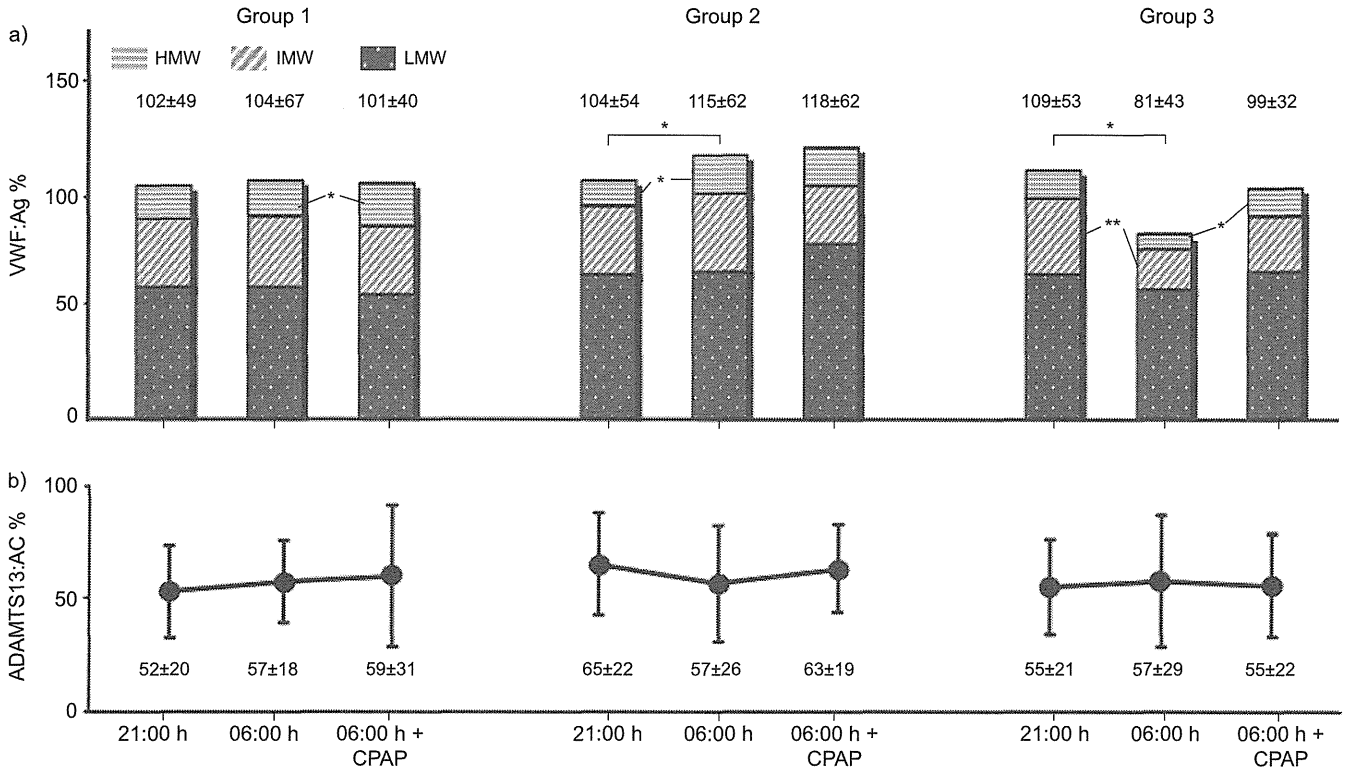

FIGURE 3. Changes in serial von Willebrand factor antigen (VWF:Ag) levels and a disintegrin-like, metalloproteinase, and thrombospiondin type 1 motifs 13 activity (ADAMTS13:AC) in groups 1–3. VWF:Ag levels were divided into high molecular weight (HMW)-, intermediate molecular weight (IMW)-, and low molecular weight (LMW)-VWFM groups by multiplying the VWF:Ag level by the results of the multimeric analyses. Data are presented as mean±SD . Groups were first compared using the Kruskal-Wallis H-test; significantly different groups were then analysed using the Mann-Whitney U-test. *: $p<0.05$; **: $p<0.01$.

Mann-Whitney U-test or Chi-square test. All comparisons among the three groups were tested for statistical significance using the Kruskal-Wallis H-test or Chi-square test, with Yates' correction for 2×3 tables; significant differences between the three groups (overall $p<0.05$) were further analysed using the Mann-Whitney U-test or Chi-square test. All analyses were carried out using StatView (SAS Institute Inc., Cary, NC, USA). A p-value <0.05 was considered significant.

RESULTS

Characteristics of patients with OSA and controls

The demographics and sleep characteristics of patients with OSA and controls are shown in table 1. Patients with OSA were slightly older than the control population but were otherwise similar demographically. 18, seven, and four patients in the OSA group were being treated for hypertension, hyperlipidaemia, and diabetes mellitus, respectively, but no diabetic patients were receiving insulin therapy. Based on the PSG results, the two populations differed significantly with respect to AHI, ODI3%, and lowest S_{p,O_2} %.

Plasma VWF:Ag levels at 06:00 h were significantly lower in patients with OSA compared with the controls, but plasma ADAMTS13:AC at 06:00 h did not differ between these groups. Interestingly, the plasma ADAMTS13:AC at 06:00 h in both OSA patients and sleep controls were lower than those of the above mentioned healthy controls ($p<0.01$).

Chronological changes of plasma VWFM patterns categorise the patients with OSA into three groups

We analysed VWFM patterns in plasmas taken from OSA patients, obtained at 21:00 h and 06:00 h following sleep with or without CPAP. Based on these results, we categorised the patients with OSA into three groups (fig. 1). Patients in group 1 (n=29) had a consistently normal pattern of VWFM, almost indistinguishable from that of the sleep controls (n=6). Patients in group 2 (n=18) exhibited reduced HMW-VWFMs at 21:00 h and persistent UL-VWFMs at 06:00 h, with or without CPAP. Patients in group 3 (n=11) had normal VWFM patterns at 21:00 h, reduced predominantly HMW-VWFMs at 06:00 h without CPAP, and returned to a normal VWFM pattern after CPAP therapy.

The decrease in HMW-VWFMs and concomitant increase in LMW-VWFMs could reflect either enhanced proteolysis by ADAMTS13 or extensive consumption secondary to platelet aggregation. Therefore, we first calculated the ratio of LMW-VWFMs to total VWFMs (LMW ratio) at 06:00 h without CPAP (fig. 2), and subsequently determined the relationship between LMW ratio and AHI. As shown in figure 2, these two parameters are significantly correlated ($p<0.05$), suggesting that the

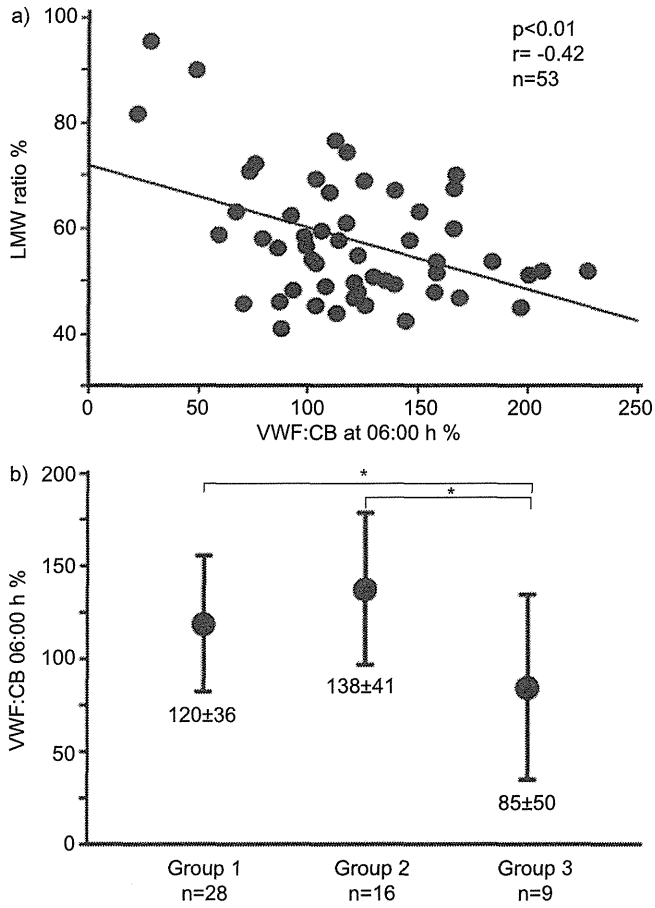

FIGURE 4. Relationship von Willebrand factor (VWF) collagen binding activity (VWF:CB) and ratio of low molecular weight (LMW)-VWF multimers (Ms) to total VWFMs (LMW ratio) and comparison of vWF:CB at 06:00 h in each group. vWF:CB was measured in 53 out of 58 obstructive sleep apnoea (OSA) patients. a. Significant inverse correlation between LMW ratio and VWF:CB at 06:00 h in OSA patients. b) VWF:CB at 06:00 h in group 3 was significantly lower than in groups 1 and 2. Data are presented as mean±SD *: $p<0.05$.

degree of hypoxia during apnoeic events is related to vFWMs processing and/or consumption.

Chronological changes of plasma levels of VWF:Ag, VWFM ratio, and ADAMTS13:AC in three patient groups with OSA

Plasma levels of VWF:Ag at 21:00 h, 06:00 h without CPAP, and 06:00 h with CPAP were determined in all three groups of OSA patients. As shown in figure 3, plasma VWF:Ag levels were almost unchanged in group 1 patients, but significantly increased between 21:00 h and 06:00 h in group 2 patients. Notably, VWF:Ag levels remarkably decreased between 21:00 h and 06:00 h in group 3.

We then determined levels of HMW, IMW, and LMW in all three groups. In group 1, HMW-VWFM showed a slight increase at 06:00 h with CPAP, relative to 06:00 h without CPAP. In group 2, HMW-VWFMs significantly increased at 06:00 h compared to 21:00 h confirming the results of the VWFM analysis used for defining groups 1–3. Consistent with this, in group 3, the IMW-VWFMs at 06:00 h was significantly lower than that at 21:00 h; CPAP treatment reversibly increased the HMW-VWFM at 06:00 h, in accordance with the increase in plasma VWF:Ag level.

In contrast, no change in the plasma ADAMTS13:AC was seen at 21:00 h, 06:00 h, or 06:00 h with CPAP in any of the three groups. These data argue that consumption of the HMW-VWFMs occurred overnight in OSA patients.

Plasma levels of VWF:CB activity

We observed dynamic chronological changes in plasma VWF:Ag levels and VWFM patterns in our subjects, especially in group 3. VWF:CB represents a biological function of VWF, in which HMW-VWFM adheres to collagen with a higher binding affinity than IMW- or LMW-VWFM. In this study, we were able to examine plasma VWF:CB levels in 53 out of 58 OSA patients. As expected, plasma levels of VWF:CB at 06:00 h without CPAP were inversely correlated with the LMW ratio ($p<0.01$), as shown in figure 4. Furthermore, as shown in figure 4, plasma levels of VWF:CB at 06:00 h was significantly lower in group 3 (85±50%) than in either group 1 (120±36%) or group 2 (138±41%). These results argue that structurally and functionally impaired VWFMs were present at 06:00 h in group 3 patients.

Decreased platelet counts at dawn in the untreated patients with OSA

A pair of platelet counts at 21:00 h and 06:00 h without CPAP was determined in 31 of the 58 OSA patients and in six of the 25 sleep controls, all of whom were involved in the later phase of this study. To correct for a possible hydration effect during sleep, we calculated the ratio of platelet count to haematocrit. The ratios in sleep controls did not exhibit significant changes between 21:00 h and 06:00 h (fig. 5), whereas they were lower at 06:00 h in untreated OSA patients ($p<0.01$) (fig. 5). However, none of the patients who received CPAP treatment developed overt clinical signs of thrombotic complications. These results suggest that platelet consumption, to a lesser extent, might occur during sleep without distinct thrombotic symptoms in untreated OSA patients.

Patient characteristics of groups 1, 2, and 3

Table 2 summarises the demographic and measured parameters of OSA patients categorised into groups 1–3. These three groups did not differ demographically, but AHI was significantly higher in group 3 than in groups 1 and 2. ODI3% in group 3 was also significantly higher than in group 1. These results unambiguously indicate that patients in group 3, who exhibit lower levels of large VWFM at 06:00 h represent the highest severity of OSA among the three groups.

Consistent with these results, decreased plasma levels of VWF:Ag in the two different time intervals (06:00 h and 21:00 h) was remarkable in group 3, in comparison to those in groups 1 and 2. Interestingly, the differences of LMW ratio in the two times (06:00 h and 21:00 h) was significantly higher in group 3 than those of groups 1 or 2. These results indicated that decreased VWF:Ag at 06:00 h was caused primarily by the reduction in larger VWFMs. Alternatively, no significant change in ADAMTS13:AC between the two times (06:00 h and 21:00 h) was observed in group 3, whereas such a change was observed in groups 1 and 2, leaving the physiological relevance unaddressed.