FVIII, thus inactivating the enzymes [2]. Therefore, PC-deficient persons have a decreased capacity to control the propagation of thrombin generation by FVa and FVIIIa after the activation of coagulation cascade, according to the circulating functional PC irrespective of the defective production or increased consumption.

Congenital PC deficiency is an autosomal recessive thrombophilia [3]. FVLeiden (G1691A) and FII mutation (G20210A) are the major predisposition to thrombosis in Caucasians, but not in Asian ancestries. Severe PC deficiency is exceptionally rare in all racial backgrounds, despite the frequency of mutation carriers [4–6]. Recent reviews described only eight and 12 survivors with long-term therapy in the United Kingdom [7] and North America [3] respectively. Purpura fulminans (PF) is the major presentation of biallelic *PROC* mutants [7], while it occurs in children with acquired PC deficiency. Infectious PF arises from PC-deficient hypercoagulability incited by the production of lipopolysaccharide or auto-antibodies to PC [8]. However, it remains unclear why PF is an exclusive phenotype in patients with severe PC deficiency. Several reports have uncovered the genetic background of thrombophilic adults [9,10]. Two major studies in Japan demonstrated that 65% of adult patients with deep vein thrombosis (DVT) showed low activity of PS, PC and/or antithrombin (AT), and half of them carried heterozygous mutation of the genes [11,12]. Considering the frequency of each mutant, heterozygous *PROC* mutations may greatly contribute to the development of venous thromboembolisms (VTE) and strokes in Japanese adults. However, there is limited information about PC-deficient children in Japan [13–16].

aPC exerts anticoagulant and cytoprotective effects [17]. The latter actions of anti-inflammation, antiapoptosis and endothelium stabilization are facilitated via the specific receptor expressed on immunocompetent cells [18–20]. aPC may thus be the sole anticoagulant agent that reduced the mortality of sepsis patients [21]. Plasma-derived aPC concentrate (Anact®C, Teijin, Tokyo & Kaketsuken, Kumamoto, Japan) is a PC agent licensed in Japan, and has been used in limited cases with congenital PC deficiency.

In this study, we analysed the clinical features of PC-deficient children based on our experiences with the genetic study, postmarketing study and intensive review of patients in Japan. The diagnosis and use of PC agent at the onset of PC deficiency were discussed.

Materials and methods

Patients and data collection

Clinical data were collected from our genetic study, postmarketing study and literature review. Seven children were diagnosed to have symptomatic PC deficiency as assessed by their plasma PC activity and the genetic study at Kyushu University from 2008 to 2010. This study was certified by the Institutional Review Board of Kyushu University (#232–02). Anact®C is only licensed for the treatment of VTE due to congenital PC deficiency in Japan. Twenty-nine children treated with human plasma-derived aPC concentrate (Anact®C, Teijin, Tokyo & Kaketsuken, Kumamoto, Japan) were collected in the registry between December 2000 and March 2011. The indication for aPC treatment was limited to patients who fulfilled at least one of the five diagnostic criteria for congenital PC deficiency; 1) ≤60% PC activity and a ratio of <0.7 for the PC activity to FVII activity, 2) ≤60% PC activity and a history of thrombosis, 3) ≤60% PC activity and congenital PC-deficient family members, 4) 60~80% PC activity, <0.7 PC to FVII activity or antigen ratio, and a history of thrombosis or congenital PC-deficient family members, or 5) a genetic diagnosis of PC deficiency. We obtained permission to access anonymous data from the postmarketing survey from each attending doctor via the pharmaceutical company. The data covered 66% of all aPC-treated cases. Of the 75 patients who had 99 aPC-treated episodes, 34 patients who did not fulfil the above criteria for congenital PC deficiency were excluded. The data collected included gender, age at each treatment, family history of PC deficiency, previous history of VTE and associated conditions, PC antigen levels and activity, FVII activity, genetic study results, outcomes and complications and the utility, efficacy and adverse events of aPC concentrate treatment. We further reviewed all publications and sentinel sources, including conference presentations in Japan, using Japana Centra Revuo Medicina, PubMed, and Google Scholar for citations published from 1981 to March 2011. The search terms were congenital, inherited, hereditary or heritable PC or PS deficiency, PF and thrombophilia. Finally, we determined that there were 27 PC-deficient patients <20 years of age who could be analysed; seven from our genetic study, and 20 from the aPC agent study and literature review.

Coagulation study

Coagulation tests were performed as described previously [22]. The anticoagulant activities of PC and PS were determined using the Staclot Protein C kit and the Staclot Protein S kit (Diagnostica Stago, Asnieres, France) respectively. A chromogenic substrate was used to assay for AT activity as the heparin-dependent inhibition of bovine thrombin (Chromostrate ATIII kit, Hitachi, Tokyo, Japan). Pooled normal plasma was used as the adult standard. A level within 2SD was regarded to be the reference interval, and a level below 3SD was defined as reduced activity. The PC activity/antigen levels for term and preterm infants

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were assessed by previous reports [23,24]. The severity of PC deficiency was defined based on the PC activity [3]; <0.01 U mL^{-1} (<1%) as 'severe', 0.01–0.2 (1–20%) U mL^{-1} as 'moderately severe', and >0.2 (>20%) U mL^{-1} as 'mild'.

Gene analysis of PC, PS and AT

Genomic DNA was extracted from peripheral blood leucocytes after informed consent was obtained. Direct sequencing of the polymerase chain reaction (PCR) products was performed for the coding regions of PC gene (*PROC* exons 1–9) as described previously [11]. When patients had low PS or AT activity, PS (*PROS1* exons 1–15) and AT (*SERPINC1* exons 1–6) genes were also analysed. The exon and exon–intron boundary regions of each gene, including the promoter region, were amplified by PCR, and the products were then subjected to direct sequencing using an ABI 377 (Perkin Elmer Applied Biosystems, CA, USA).

Statistical analysis

The median of the continuous variables was assessed by the Mann–Whitney *U*-test. The difference in the distribution of countable variables was analysed by chi-square test or Fisher's exact test. *P*-values <0.05 were considered to be significant.

Results

aPC concentrate-treated children in the postmarketing study

The demographics of 29 patients <20 years of age treated with Anact®C are shown in Table 1. The male/female ratio was 11/18. The median age at presentation was 3 months (range: 3 days–16 years). The median PC activity was 21% (range: 0–57%). There were no adult patients who developed PF included in the registry data. Five of nine PF patients developed ICTH concurrently. Six patients suffered from ICTH but not PF. Fourteen others had pulmonary thromboembolism, DVT and hepatic thrombosis with or without venoocclusive disease during the course of malignancies. The clinical profiles did not differ between the PF and ICTH groups, except for a higher incidence of concurrent infections in PF patients ($P = 0.044$). The ICTH patients had no triggers at the presentation. Although 14 others met the aforementioned criteria of 1) higher PC activity, absent family history and associated conditions suggested a low possibility of inherited PC deficiency. The clinical outcomes after aPC therapy did not differ among the three groups of ICTH, PF and other VTE patients.

Symptomatic congenital PC deficiency in Japanese children

A total of 27 PC-deficient patients <20 years of age were finally collected in Japan for this analysis, including a pair of first cousins (Pt2–1, Pt2–2) and a set of twins (Pt9–t1, Pt9–t2). There was no consanguinity. The patients were divided into two groups; 18 inherited (Table 2) and nine non-inherited deficiencies (Table 3) based on the PC activity of their parents. The patients in each group are listed in order of their age at the first presentation of ICTH (*upper*) or other conditions (*lower*). Of the 19 ICTH patients, 10 had PF that did not precede ICTH. Although three had foetal hydrocephalus (inherited Pt1, Pt2–1; non-inherited Pt1), no antenatal diagnosis of PC deficiency was made. Of the eight others (inherited Pts15–18, non-inherited Pts4–7), PF was the first presentation of six patients. One developed acute renal failure (non-inherited Pt6), and the other suffered from a chance

Table 1. Demographics of activated PC concentrate-treated children according to the major lesions.

	PF[a]	ICTH[b]	Other VTE[c]	*P* value a vs. b	*P* value a+b vs. c
Number of Pts, male : female	9, 3 : 6	6, 1 : 5	14, 7 : 7	0.604	0.264
Age*, median (range)	15 days (3–422)	14 days (7–317)	4.4 yrs (58 days–16 yrs)	0.768	<.0001
PC activity, median (range) %	0 (0–31)	18 (0–40)	28 (0–57)	0.195	0.006
Family history positive, %	78	83	0	>.999	<.0001
Associated conditions					
No	4	6	0	0.044	0.0002
Yes infection	5	0	1		
malignancy	0	0	12		
cardiac disease	1	0	1		
Efficacy; yes/no/unknown	5 / 4 / 0	1 / 1 / 3	7 / 4 / 1	>.999	>.999
Clinical utility; yes/no/unknown	3 / 1 / 5	3 / 0 / 3	7 / 0 / 7	>.999	>.999
Survival outcome, (%)	7 (78)	6 (100)	7 (50)	0.486	0.050

*The age at onset of disease was defined as the first time PC activity was determined.
Pts: patients, PF: purpura fulminans, ICTH: intracranial thrombosis and/or haemorrhage, VTE: venous thromboembolism
Other VTE included pulmonary thromboembolism ($n = 1$), DVT with haemothorax ($n = 1$), hepatic thrombosis with or without venoocclusive disease ($n = 12$) during the course of aplastic anaemia, myelodysplastic syndrome, haemophagocytic syndrome, leukaemia, neuroblastoma, or leukodystrophy.

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Table 2. Reports of symptomatic children with inherited protein C deficiency in Japan.

		Age at presentation, days			Patient						
Pt	Neonatal asphyxia	ICTH	PF	Ocular bleed	PC-act (%)	*PROC*	type*	Parents low PC-act	FH†	aPC therapy	Outcome
Intracranial lesions											
1	yes	GA33w	3	no	<10	ex9:1235G>A(V339M)	homo	M F	no	yes	died (6 m)
2–1	yes	GA34w	26	46	<10		c.heter	M F	yes	yes	amputation, PMR
2–2	yes	0	no	12	<10		heter	M	yes	yes	PMR
3	no	0	2	yes	3	ex9:1362delG*(stop420→462)	homo	M F	no	yes	NR
4	no	0	0	NR	<10	ex9:1190G>A(V297M)/ex9:1362delG*	c.heter	M	yes	yes	amputation, PMR
5	no	0	7	yes	<10		heter	M	no	no	PMR,VP-shunt
6	no	1	1	NR	5	ex3:296G>A(E26K)/ex9:1362delG*	c.heter	M F	no	no	favourable, PMR
7	no	2	2	yes	<10		c.heter	M F	no	yes	discharge (2 m)
8	NR	>3	3	NR	1.5		c.heter	M F	NR	yes	PMR
9–t1	no	6	no	6	<10	ex8:887C>T(L223F)/ex9:1360G>C(W380C)	c.heter	M F	twin	yes	PMR
9–t2	no	6	no	NR	<10	ex8:887C>T(L223F)/ex9:1360G>C(W380C)	c.heter	(M F)	twin	yes	PMR
10	no	7	no	NR	19		heter	M	no	yes	NR
11	no	13	16	NR	8	ex4:258delT 18Stop/ex9:905C>T(R229W)	c.heter	M F	no	yes	Ep, PMR
12	NR	16	no	no	17		heter	M	yes	yes	NR
13	NR	10 m	no	no	40		heter	F	yes	yes	NR
14	no	13 m	13 m	no	8	ex4:356G>T(D46Y)	heter	M	yes	yes	severe PMR
Extracranial lesions											
15	no	no	0	NR	<5	ex7:725C>T(R169W)/ex9:1362delG*	c.heter	M F	no	no	normal develop
16	no	no	1	yes	20		heter	M	no	no	no PMR, died
17	no	no	2	yes	24		c.heter	M F	NR	yes	NR
18	yes	no	10	yes	<10		c.heter	M F	yes	yes	NR

*The allele type of the *PROC* mutation was estimated by each parent's PC activity unless the genetic study was performed.

†Family history (FH) includes young thrombosis, habitual abortion, foetal/neonatal hydrocephalus and consanguineous marriages.

Pt: patient, PC: protein C, aPC: activated PC, NR: not recorded, ICTH: intracranial thrombosis, haemorrhage and/or hydrocephalus, PF: purpura fulminans, homo: homozygote, c.hetero; compound heterozygote, PMR: psychomotor retardation, Ep: epilepsy, Pt2–1 and Pt2–2 are first cousins. Pt9–t1 and Pt9–t2 are twins.

Four of 11 genetic studies (Pt4, 9–t1, 9–t2, 11 and 14) were completed at Kyushu University. The base number was referred to Accession No. NM 000312 Version 3, 1790np (mRNA) PRI 27-NOV-2011. Four patients among eight unrelated families carried PC nagoya 1362delG* (stop420→462).

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Table 3. Reports of symptomatic children with non-inherited protein C deficiency in Japan.

		Age at presentation, days			Patient						
Pt	Neonatal asphyxia	ICTH	PF	Ocular bleed	PC-act (%)	*PROC*	type*	Parents low PC-act	FH†	aPC theropy	Outcome
Intracranial lesions											
1	no	GA36w	no	NR	37		unknown	NR	NR	no	PMR
2	no	3	no	NR	24	no mutation	unknown	normal	no	no	PMR
3	no	5	no	NR	34		unknown	NR	no	no	discharge (18d)
Extracranial lesions											
4	NR	no	0	NR	23		unknown	normal	no	yes	died
5	no	no	1	NR	30		unknown	normal	NR	no	NR
6	yes	no	no	no	6	no mutation	unknown	normal	no	yes	renal failure
7	no	no	no	11	28		unknown	normal	no	no	blindness

*The allele type of the *PROC* mutation was estimated by each parent's PC activity unless the genetic study was performed.
†Family history (FH) includes young thrombosis, habitual abortion, foetal/neonatal hydrocephalus and consanguineous marriages.
Pt: patient, PC: protein C, aPC: activated PC, NR: not recorded, ICTH: intracranial thrombosis, haemorrhage and/or hydrocephalus, PF: purpura fulminans, homo: homozygote, c.hetero; compound heterozygote, PMR: psychomotor retardation.
Two genetic studies (Pt2 and Pt6) were completed in Kyushu University.

vitreous haemorrhage (non-inherited Pt7). Postnatal-onset infants showed normal birthweight (median 2848 g) at full-term delivery. Severe or moderately severe PC deficiency was found in 85% of the patients in the inherited group and 15% in the non-inherited group. The genetic study and parents' PC activity indicated that the 20 inherited patients had two homozygous [14], 11 compound heterozygous [13,16] and seven heterozygous PC deficiency [15]. PC nagoya was found in half of the unrelated patients with genetic diagnosis. There were no genotype-specific features of PC-deficient children. Only five patients declared a family history suggestive of inherited thrombophilias. Mothers ($n = 18$) or fathers ($n = 12$) had mild PC deficiency. None of them had experienced thromboembolism before the diagnosis of their children (Table 2).

The aPC agent was given to 18 patients (67%) for the treatment of PF (100 U kg^{-1} bolus, and 600–800 U kg^{-1} per day continuous infusion for 6 days), or other VTE (200–300 U kg^{-1} per day continuously infusion for 6 days). Of the 18 aPC-treated patients, two died of infection, and eight of 10 survivors with recorded outcomes had neurological sequelae.

Discussion

This study documented that the first presentation of Japanese children with PC deficiency was exclusively ICTH and/or PF within 3 weeks after birth. Although each incidence was similar, PF did not precede ICTH in patients having two lesions. ICTH developed prenatally without apparent triggers, while PF occasionally occurred with infection. PC nagoya was prevalent in Japanese children with PC deficiency. The mothers of >75% of infants showed low PC activity, but only 25% infants had a declared thrombophilic family history at presentation. The mortality rate was 8%, and most of the survivors had neurological deficits irrespective of aPC therapy. Screening for PC-deficient mothers may allow for earlier intervention to improve the outcome of affected newborns.

Neonatal PF is the hallmark of heritable PC deficiency. Of more than 30 reported PF cases, one PS-deficient homozygote developed neonatal PF [25], and five patients had a *PROS* mutation in association with three FVLeiden [25–29]. In our review of Japanese patients, there were no PF cases with inherited PS/AT-deficiency. Four PS-deficient Japanese children developed DVT after the age of 3 years [30]. There is a missing link between the rare *PROC* mutants and the exclusive phenotype of PF neonates. The frequency of *PROS* mutant heterozygotes is higher in the general Japanese population (1.12~1.8%) [31–33] than was seen in Caucasians (0.03~0.13%) [34]. The founder effects of PS-tokushima might account for PS-deficiency as the leading cause of genetic thrombophilia in Japanese adults [12,32]. On the other hand, the frequency of *PROC* mutations in Japanese subjects (0.16%) [35] is similar to that in Caucasians (0.2–0.3%) [4,5]. PC nagoya is one of the five major genotypes of PC deficiency in Japan. However, no PC nagoya but, recurrent mutations related to CG>TG and CG>CA transitions, were found in adult Japanese VTE cases [11,12]. The high frequency of PC nagoya in paediatric, but not adult, patients suggested a distinct contribution of this gene to the development of thrombosis between biallelic mutants and heterozygous ones, as the founder effect in Japan.

Perinatal ICTH and neonatal PF were the exclusive phenotypes of paediatric PC deficiency. ICTH occurred earlier than PF as the first presentation. Hydrocephalus was a unique manifestation in PC-deficient foetuses. Fong *et al.* [36] have recently reported sisters with cerebral palsy who experienced periventricular haemorrhagic infarction caused by heterozygous *PROC* mutations. Postnatal magnetic resonance imaging showed that the brain lesions were consistent with bilateral cerebral intramedullary venous thrombosis occurring at under 28 weeks of gestation for the

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older sister and around the time of birth for the younger sister. Mothers with FVLeiden or FII G20210A are at risk of delivering low birthweight infants [37]. On the other hand, in the series of subjects with PC deficiency (Tables 2 and 3), premature birth or intrauterine growth retardation was unremarkable. Postnatal cases had appropriate birthweight at full-term deliveries. This may be corroborated by the fact that homozygous PC-deficient mice are born and die shortly after birth, whereas homozygous PS- or AT-deficient mice result in foetal loss.

PC-deficient heterozygotes were occasionally found in the series of paediatric patients. Of 16 PF children in Turkey (median age: 2 years, range 3.5 months ~12 years) [38], six patients (38%) carried FVLeiden (five heterozygotes and one homozygote), but none had FII G20210A. Of the six PC- and nine PS-deficient patients, only one had inherited thrombophilia of PS-deficiency. All six PC-deficient patients were <2 years of age, and four of them had severe infection. Hypercoagulability of inherited thrombophilia depends on the genetic background of patients [39]. Acute infectious PF occurs across all age groups, and the patients have been rarely assessed by genetic study [15]. Taken together, these data indicate that additional insults, including infection, might contribute to the development of PF in heterozygous PC-deficient infants.

The plasma PC concentration physiologically increases from birth until age 6 months [3]. Vitamin K deficiency predisposes infants to depressed PC activity. Of 11 patients assessed by *PROC* sequencing, two infants (Table 3; Pt2 and Pt6) carried no PC mutation. Selective low PC activity continued in both patients, despite their normal PS/AT/FVII levels and absent protein induced by Vitamin K absence or antagonists-II. Our study demonstrated no polymorphisms in the promoter regions of *PROC* which might have affected the PC levels of patients. The plasma PC and PS levels could be differentially influenced by other factors in early infancy. The presence of borderline PC activity in infancy hardly predicts the PC-deficient heterozygotes. Although the genetic study is essential for the diagnosis of paediatric thrombophilias, severe PC deficiency may be a critical perinatal condition beyond the patient genotype. Although >75% of mothers showed low plasma PC levels, all PC-deficient mothers were healthy up until the delivery. Pt3 in Table 2 underwent successful aPC prophylaxis at surgery, as reported elsewhere [40]. PC replacement may be useful for VTE prophylaxis in pregnancy and Caesarean section. The aPC therapy might be also life saving for Japanese children with PC deficiency; however, most survivors had serious long-lasting sequelae despite treatment. Further studies should be directed towards early maternal screening and optimal PC therapy for newborns at risk of PC deficiency.

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Contributions to authorship

SO was the principal investigator taking primary responsibility for the study. DK organized coagulation and genetic studies. TK, MO, TD, MI, JY and SO treated and enrolled the patients. YK and MU managed and performed the laboratory work for this study. TI, AS, HK and TH helped to collect information of all paediatric/perinatal cases of PC deficiency in Japan. SO wrote the manuscript.

Disclosures

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

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PERINATAL/NEONATAL CASE PRESENTATION

Neonatal asphyxia and renal failure as the presentation of non-inherited protein C deficiency

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Inherited or acquired protein C (PC) deficiency leads to thromboembolic events. Plasma PC activity in infancy is physiologically lower than in adults. We describe a case of neonatal asphyxia and acute renal failure associated with isolated PC deficiency. A full-term male infant was born to a healthy mother by caesarean section because of fetal distress. The small-for-gestational age infant showed 2 and 7 of Apgar scores at 1 and 5 minutes, respectively. Hypercoagulability required repeated infusions of fresh frozen plasma. Coagulation study revealed PC activity, 6%, protein S activity, 61%, and high D-dimer levels, along with normal factor VII activity and absent vitamin K deficiency. Anticoagulant and activated PC therapy improved coagulopathy and nephropathy. Imaging analyses indicated no visceral infarctions. Renal function and PC activity have been slowly normalized until 6 months of age. He had no *PROC* mutation or PC-deficient parents. Selective PC deficiency may occur as an acquired cause of hypercoagulable crisis in the stressed newborn.

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Keywords: protein C deficiency; purpura fulminans; activated protein C therapy

Introduction

Protein C (PC) is a vitamin K-dependent serine protease zymogen produced by the liver. The anticoagulant factor is activated by the thrombin (factor IIa: FIIa)-bound thrombomodulin expressed on the endothelium. Activated PC (aPC) inactivates FVa and FVIIIa in the presence of protein S (PS) as the cofactor.[1] Administration of aPC products inhibits clot formation and augments fibrinolysis by blocking plasminogen activator inhibitor-1. aPC also exerts anti-inflammatory and cytoprotective effects on neutrophils, macrophages, dendritic cells and endothelial cells via specific receptors.[2] Inherited PC deficiency is an autosomal recessive thrombophilia.[1] Homozygotes and compound heterozygotes of *PROC* mutation present with purpura fulminans (PF) during early neonatal period.[3] Heterozygous *PROC*-mutated adults are at risk of deep vein thrombosis, pulmonary thromboembolism and disseminated intravascular coagulopathy, being often triggered by infection, vasculopathy and malignancy. Sepsis and liver dysfunction precipitate PC deficiency via the consumption, impaired synthesis and both. Plasma PC activity is physiologically low until 6 months of age. Neonates are vulnerable to vitamin-K-deficient coagulopathy arising from the immaturity, nutrition and enteral microbiota. Nevertheless, there is little information about neonatal thromboembolism arising from non-familial PC deficiency.

We report a case of neonatal asphyxia and renal failure associated with non-inherited PC deficiency. The etiology and management of hypercoagulable crisis in the newborn was discussed.

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Case

A male newborn was hospitalized because of neonatal asphyxia showing 2 and 7 of Apgar scores at 1 and 5 min, respectively. He was born to a healthy 30-year-old mother, gravida 0 and para 0, at 41 weeks of gestation by urgent caesarean section for non-reassuring fetal status on the labor induction, after uneventful pregnant course. The small-for-gestational age infant weighing 2404 g had normal umbilical cord and placenta. There was no consanguineous marriage or contributory family history. On day 6 after birth, he was transferred to our tertiary neonatal intensive care unit because of anuria and thrombocytopenia.

On admission, the vigorous infant showed 70 min^{-1} of tachypnea, normal pulse rate (136 min^{-1}) and blood pressure (89/67 mm Hg). Body temperature was 37.6 °C. There was no desaturation. He had occipital cephalhematoma and faint purpura (3 × 5 cm) on the right back. Auscultation was unremarkable. No hepatosplenomegaly was found. Complete blood counts and coagulation study 2 days after the transfusion of packed platelets and fresh frozen plasma (FFP) revealed white blood cells

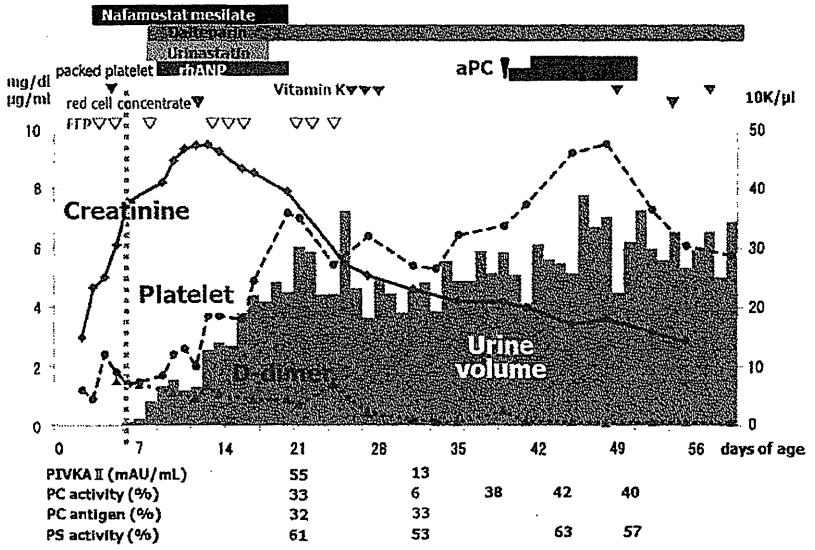

Figure 1 Treatment course of the infant. Intensive therapy was directed to anticoagulant and nephroprotective effects. Repeated infusions of fresh frozen plasma (FFP) increased platelet counts and urine volume. Serum creatinine peaked at 9.47 mg dl^{-1} and slowly decreased. The activated protein C (aPC) therapy further improved platelet counts, D-dimer and creatinine levels. PIVKA-II, protein induced by vitamin K absence or antagonists-II; PS, protein S; rhANP, recombinant human atrial natriuretic peptide.

7.540 × 10^{9} l^{-1}, hemoglobin 9.9 g dl^{-1}, platelets 69 × 10^{9} l^{-1}, fibrinogen 103 mg dl^{-1}, prothrombin time-INR 1.56, activated partial thromboplastin time 48.1 s, fibrinogen degradation products 15.0 μg ml^{-1} and D-dimer 7.9 μg ml^{-1}. Antithrombin activity was 47%, thrombin–antithrombin complex was 7.3 ng ml^{-1} and plasmin α2–antiplasmin complex was 2.5 μg ml^{-1}. C-reactive protein concentration was 0.13 mg dl^{-1}. Serum levels of creatinine (7.54 mg dl^{-1}), urea nitrogen (59 mg dl^{-1}), uric acid (22.5 mg dl^{-1}), aspartate aminotransferase (113 U l^{-1}), alanine aminotransferase (345 U l^{-1}) and creatinine kinase (904 U l^{-1}) were high. Blood gas analysis indicated metabolic acidosis with −7.1 mmol l^{-1} of actual base excess. Dark red urine indicated hemolysis. Echographies revealed intact brain, heart, liver and kidney.

Intensive therapy was started for renal failure and disseminated intravascular coagulopathy (Figure 1). Dalteparin, nafamostat mesilate, urinastatin, antithrombin products and FFP but not packed platelets were infused for the hypercoagulability of unknown cause. Diuretics, catecholamine, recombinant human atrial natriuretic peptide and packed red cells were administered. Urine volume slowly increased. Serum creatinine levels at the peak of 9.47 mg dl^{-1} on day 13 of age decreased gradually. During repeated FFP infusions, low PC activity (33%, reference range (rr): 67–130) was dissociated with subnormal PS activity (61%, rr: 73–121) on day 21 of age. The steady improvement of coagulopathy and renal function after each FFP substitution was suggestive of inherited thrombophilia. The coagulation profile was reassessed after vitamin K therapy and 10 days after the last FFP infusion. PC activity was 6%, PC antigen level was 33% (rr: 65–135) and PS activity was 53%. Protein induced by vitamin K absence or antagonists-II levels (PIVKA-II; 13 mAU ml^{-1}, rr: ⩽40) and FVII activity (75%, rr: 75–140) were normal. These findings indicated the type 2 PC-deficient heterozygote. Administration of human plasma-derived aPC (Anact C; a bolus infusion of 50 U kg^{-1} and a continuous infusion of 50–100 U kg^{-1} for consecutive 7 days, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) led to the drastic increase of platelet counts, undetectable D-dimer and decreasing creatinine levels. Magnetic resonance imaging of the brain and abdomen, scintigraphy of the lung and heart, renogram and funduscopy revealed no infarctions. Direct sequencing of the coding and promoter regions of *PROC* revealed no mutation in the infant.[4] Healthy father and mother had normal PC activity of 135% and 165%, respectively. Thereafter, PC activity of the patient increased to 55% until the age of 3 months, and attained 102% at the age of 10 months. Serum creatinine levels fell to 0.87 mg dl^{-1} until 10 months. The infant shows normal development at the writing of the manuscript.

Discussion

The hypercoagulable neonate presented renal failure, cephalhematoma, purpura and hemoglobinuria. Extremely low PC but not PS activity, the response to aPC therapy and no *PROC* mutation determined the diagnosis of acquired PC deficiency. Despite the low frequency of *PROC* mutation, the thrombotic risk of PC mutant is higher than other congenital thrombophilias.[5,6] Neonatal PF is the hallmark of severe PC deficiency (<0.01 U ml^{-1} or <1% of PC activity). Heterozygous PC-deficient infants are at

risk of PF in the association with additional triggers.[4] Cerebral infarction and/or vitreous bleeding were the other presentations of fetal/neonatal PC deficiency.[7] Kidney is a target organ of thrombosis in neonatal thrombophilias.[8] In this context, selective PC deficiency has a greater impact on the thromboembolic events especially in early infancy than other thrombophilias.

The major concern is the etiology of transient PC deficiency in the newborn. Manco-Johnson *et al*[9] described 11 infants initially seen in the newborn period with undetectable PC activity and/or antigen, which proved on subsequent follow-up, to be acquired. About 5 of the 11 infants manifested thrombotic events, including renal, aortic and cerebral sites. They further evaluated PC, D-dimer and other regulatory proteins in 164 newborn infants. In this report,[10] prevalence of PC $<0.1\ U\ ml^{-1}$ or $<10\%$ of healthy adults ranged from 0 to 37% with the highest rates in twin gestation (either term or preterm) and preterm infants with respiratory distress, and including a rate of 5% in term singleton infants with distress. Although they showed no genetic study, severe to moderately severe PC activity ($<0.1\ U\ ml^{-1}$ or $<10\%$) could occur and often led to thrombosis in the stressed newborn as non-inherited PC deficiency.

At the time of diagnosis, the discrepancy between 6% of PC activity and 33% of PC antigen levels was unexplainable. The lower PC activity than PC antigen levels at first suggested type 2 deficiency, which accounts for 15% of symptomatic deficiency. Genetic PC deficiency arises from a gross deletion in patients with no *PROC* mutation.[11] However, the qualitative and quantitative abnormality of PC molecule associated with *de novo* heterozygous PC deficiency was excluded by 102% of PC activity at the age of 10 months. The blood samples at diagnosis showed undetectable PIVKA-II and normal FVII activity. It may raise the possibility that a certain inhibitor selectively reduced the PC activity but not PS activity, while no such factors have been suggested.[12] Shorter half-life of PC and aPC than PS might contribute to persistently low PC activity. To clarify the cause of neonatal PC deficiency, genome-wide study should be directed toward the genotyping associated with the functional maturation of PC activity.

The other issue is the indication of aPC concentrate. The efficacy of aPC therapy is still controversial in the treatment of sepsis and/or PF in childhood.[3,7] During the disease course, the image analyses disclosed no evidence of thrombosis. However, the early anticoagulant therapy protected irreversible organ damages with impaired circulation. After the start of aPC administration, the increase of platelet counts and disappearance of D-dimer were drastic. Subsequent recovery of renal function might corroborate the additional profibrinolytic and cytoprotective effects of aPC on the ischemic kidney. Although genetic study is indispensable for PC deficiency, the entity of neonatal PC deficiency might emphasize the feasibility of aPC therapy beyond the *PROC* mutation. Thromboprophylaxis should be augmented for PC-deficient patients. Warfarin control is not easy in young children. In this line, regular PC substitution may be limitedly recommended in early infancy. Further study is needed to search the non-inherited causes of neonatal isolated PC deficiency as well as to establish the optimal aPC therapy for PC-deficient infants.

Conflict of interest

The authors declare no conflict of interest.

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Thrombocytosis in asplenia syndrome with congenital heart disease: A previously unrecognized risk factor for thromboembolism

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ABSTRACT

Background: Thrombocytosis and thromboembolic complications occur after splenectomy. However, there is no previous report investigating the presence of thrombocytosis and its association with thromboembolic events in patients having asplenia syndrome with congenital heart disease.
Methods: Enrolled were 161 consecutive patients with functionally single ventricle who underwent cardiac catheterization between 1997 and 2010. They were divided into two groups: patients having asplenia (Group A, n=46) and patients having no asplenia (Group B, n=115). Aspirin therapy was employed in all patients after surgical interventions except for pulmonary artery banding. We retrospectively reviewed the platelet counts at each seven stage of cardiac catheterization (for pre- and postoperative evaluation of the first palliation, Glenn operation, and Fontan operation, and for late evaluation after Fontan operation), incidence of thromboembolic events, and other possible risk factors for thromboembolism.
Results: The median platelet counts in Group A were consistently higher than those in Group B at any of the seven stages of cardiac catheterizations ($p<0.002$). The incidence of thromboembolic complications was also higher in Group A than that in Group B (28% vs. 10%, $p=0.030$). Univariate and multivariate logistic regression analyses showed that a platelet count of more than 550×10^9/L at the first cardiac catheterization was associated with thromboembolic complications (Odds ratio 3.17; $p=0.046$).
Conclusions: Persistent thrombocytosis is present in patients with asplenia syndrome. It may greatly contribute to the development of thromboembolism during the management of congenital heart disease than expected.

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1. Introduction

Post-splenectomy thrombocytosis is reported to be a predisposing factor for thromboembolism. Thromboembolic complications following splenectomy occur in up to 10% of patients. These range from myocardial infarction, portal vein thrombosis, and pulmonary embolism to deep vein thrombosis [1–3]. Such thrombotic events could be observed not only immediately after surgery, but also several months or even years later in the patients in whom thrombocytosis persisted [4].

Congenital asplenia syndrome is a form of heterotaxy that is also known as right atrial isomerism. This syndrome is typically associated with severe heart defects and needs staged cardiac operations from infancy as a functionally single ventricle [5]. Thromboembolic events are often experienced before and after the completion of Fontan operation [6].

There are a few reported cases with secondary thrombocytosis caused by isolated spleen agenesis without cardiac defects, mimicking essential thrombocythemia [7,8]. Thromboembolism is one of the major complications during and after surgical intervention in patients with congenital heart disease. However, there is no previous report investigating the relationship between platelet count and thromboembolic events in patients with asplenia syndrome having congenital heart disease.

The objective of this study is to clarify the clinical significance of thrombocytosis and its impact on thromboembolic events in patients with congenital heart disease and asplenia.

2. Materials and methods

2.1. Patients

A total of 161 consecutive patients with functionally single ventricles who underwent cardiac catheterization at Kyushu University Hospital and Kyushu Koseinenkin

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Table 1
Patient characteristics.

	Group A (n=46)	Group B (n=115)
Anatomy of the congenital heart disease		
SRV	19	13
AVSD	14	6
DORV	7	23
SLV	2	15
CCTGA	2	13
TA	0	19
PAIVS	0	10
HLHS	0	8
MA	0	5
Others	0	3
Age	7 years (6 months–13 years)	7 years (4 months–13 years)
Sex	M/F=22/24	M/F=57/58
First palliation		
Blalock–Taussig shunt	38 (83%)	66 (57%)
Pulmonary artery banding	8 (17%)	41 (36%)
Norwood operation	0 (0%)	8 (7%)
Survival	33 (72%)	107 (93%)

SRV: single right ventricle, AVSD: atrioventricular septal defect, DORV: double outflow right ventricle, SLV: single left ventricle, CCTGA: congenitally corrected transposition of the great arteries, TA: tricuspid atresia, PAIVS: pulmonary atresia with intact ventricular septum, HLHS: hypoplastic left heart syndrome, MA: mitral atresia.

Hospital between 1997 and 2010 were enrolled in this study. No patients had any family history of thrombophilia. The patients were divided into two groups: patients having asplenia syndrome (Group A, n=46) and those having no asplenia syndrome (Group B, n=115). Patient characteristics of each group are shown in Table 1.

All patients underwent staged surgical procedures. Initial palliation included Blalock–Taussig (BT) shunt, pulmonary artery banding or Norwood operation as needed. The second stage operation was a bidirectional Glenn cavopulmonary connection performed on cardiopulmonary bypass. The third stage operation was Fontan operation using expanded polytetrafluorethylene vascular graft as an extracardiac conduit. Fenestration was only performed for two patients with high pulmonary vascular resistance. All patients received cardiac catheterization for pre- and postoperative evaluation of each surgical procedure and for late evaluation after Fontan operation (Fig. 1).

Postoperative and interstage anticoagulation therapy was identically performed as follows: aspirin (3–5 mg/kg/day) after BT shunt, Norwood procedure, and Glenn operation; aspirin and warfarin (targeted prothrombin time-international normalized ratio [PT-INR] 1.5–2.0) for 1 year following Fontan operation; and only aspirin after 1 year following Fontan operation.

The diagnosis of asplenia syndrome was determined based on the presence of Howell–Jolly bodies in the peripheral blood smear and the absence of spleen assessed by computed tomography (n=31) and/or ultrasonography (n=46). The diagnosis of thromboembolic complications was determined using objective methods when they were suspected by clinical manifestation or when they were coincidentally found by the regular examinations. BT shunt malfunction was defined as shunt occlusion or stenosis which needs catheter intervention or surgery, assessed by computed tomography or cardiac catheterization. Cerebral infarction was diagnosed by computed tomography or magnetic resonance imaging. Diagnosis of venous thromboembolism was made by ultrasonography, computed tomography, or venography.

3. Methods

We retrospectively reviewed the medical records of all 161 patients to determine (i) the platelet counts at each seven stage of cardiac catheterization; (ii) the incidence of thromboembolic complications; and (iii) other clinical data that may be possible risk factors for thromboembolic complications in each group.

For the purpose of elucidating the association between platelet counts and thromboembolic events, we investigated the sequential changes of platelet counts, the timing of thromboembolic events, and the correlations among platelet counts at each stage of cardiac catheterizations.

We evaluated the effect of clinical parameters (platelet count, age, birth weight, hemoglobin concentration, activated partial thromboplastin time, ejection fraction of the systemic ventricle, atrioventricular valve regurgitation, presence or absence of BT shunt, size of the BT shunt, and presence or absence of pulmonary vein obstruction) as a risk factor of thromboembolic complications with univariate and multivariate logistic regression analyses.

3.1. Statistical analysis

Continuous variables were analyzed using the Mann–Whitney *U* test and categorical variables were analyzed using the chi-square test. The analysis of Pearson's correlation coefficient was used to evaluate correlations between platelet counts at each other stage of catheterization. Dichotomous variables were created out of continuous variables by using clinically important cutoff points. Univariate and

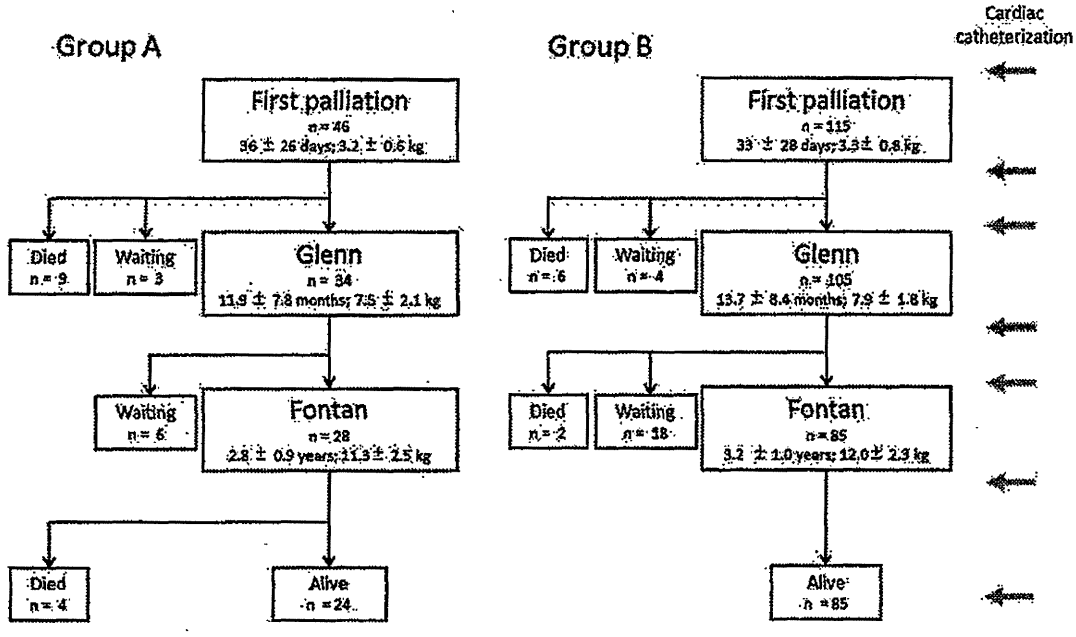

Fig. 1. Number of study patients, mean age and mean weight at each stage.

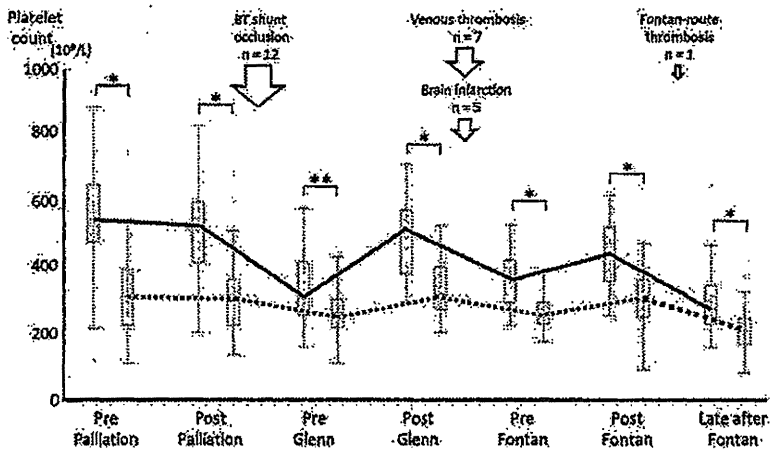

Fig. 2. Sequential changes of platelet counts in Group A (solid line) and Group B (dashed line) at the seven therapeutic stages of cardiac catheterizations and the timing (arrows) of thromboembolic events. The box plots represent medians with 25–75th centile boxes, minimums and maximums still in the 1.5 interquartile range bars, and outliers. The median platelet counts in Group A were significantly higher than those in Group B at any of the seven stages of cardiac catheterizations (* $p<0.0001$, ** $p=0.0002$).

multivariate logistic regression analyses were used to determine the relative contribution of various factors to the risk of thromboembolic events. P values of less than 0.05 were considered significant. All statistical operations were performed by using the JMP 8 statistical software package (SAS Institute. Inc. Cary. NC).

4. Results

The time course of the platelet counts and thromboembolic events are shown in Fig. 2. The median platelet counts in Group A were consistently higher than those in Group B at any of the seven stages of cardiac catheterizations (557×10^9/L vs. 333×10^9/L, $p<0.0001$; 543×10^9/L vs. 349×10^9/L, $p<0.0001$; 313×10^9/L vs. 254×10^9/L, $p=0.0002$; 524×10^9/L vs. 304×10^9/L, $p<0.0001$; 310×10^9/L vs. 248×10^9/L, $p<0.0001$; 391×10^9/L vs. 278×10^9/L, $p<0.0001$; and 336×10^9/L vs. 207×10^9/L, $p<0.0001$, respectively). The most frequent thromboembolic event was the BT shunt malfunction in the infant period (large arrow). The other occasional thromboembolic events were venous thrombosis and cerebral infarction after Glenn operation.

The incidence of thromboembolic events was also higher in Group A than in Group B (28% vs. 10%, $p=0.0302$, Fig. 3). Furthermore, patients in Group A were associated with increased incidences of all four types of thromboembolic complications: BT shunt malfunction; cerebral infarction; venous thromboembolism; and Fontan-rout thrombosis.

There were significant correlations between platelet counts of individual patients at each other stage of cardiac catheterization ($p<0.05$, Table 2). On the basis of the correlations, we investigated the possible risk factors for thromboembolic events including platelet count at the first cardiac catheterization as one of the representative values of thrombophilic predisposition.

The univariate logistic regression analysis indicated that patients who had a platelet count of more than 550×10^9/L at the first cardiac catheterization (Odds ratio 3.17; $p=0.023$), or underwent BT shunt (Odds ratio 2.47; $p=0.044$) were at a higher risk of thromboembolic complications. The multivariate logistic regression analysis selected only the platelet count of more than 550×10^9/L as a risk factor of thromboembolic events ($p=0.046$). No other clinical variables including birth weight, BT shunt size, pulmonary vein obstruction, ejection fraction of the systemic ventricle, atrioventricular valve regurgitation, hemoglobin concentration, and activated partial thromboplastin time at the first cardiac catheterization were associated with thromboembolic events (Table 3).

5. Discussion

This is the first report that demonstrated the presence of persistent thrombocytosis and its association with thromboembolic complications in patients with asplenia syndrome. Patients with asplenia syndrome were reported to have poor outcome. Pulmonary vein obstruction, arrhythmias associated with twin atrioventricular nodes, and susceptibility

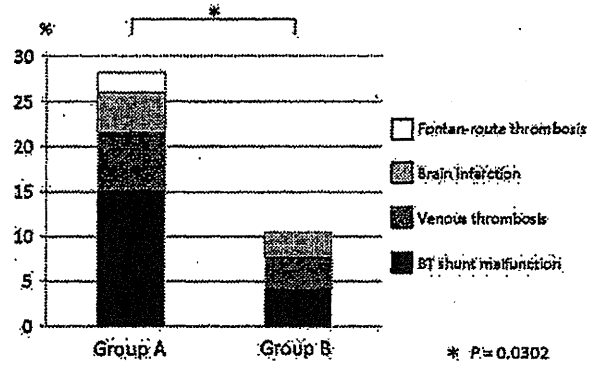

Fig. 3. Incidence of thromboembolic complications in Group A and Group B. The incidence of thromboembolic complications is significantly higher in Group A than in Group B. BT shunt: Blalock–Taussig shunt.

Table 2
Correlation and coefficient among platelet counts at each stage of cardiac catheterization.

		Pre palliation	Post palliation	Pre Glenn	Post Glenn	Pre Fontan	Post Fontan	Late after Fontan
Pre palliation	R^2		0.2724	0.2179	0.2203	0.1466	0.1266	0.1059
	p Value		<0.0001	<0.0001	<0.0001	0.0002	<0.0001	0.0292
Post palliation	R^2			0.1268	0.1923	0.1489	0.1577	0.2205
	p Value			0.0003	<0.0001	0.0002	0.0002	0.0011
Pre Glenn	R^2				0.2545	0.2018	0.1736	0.3165
	p Value				<0.0001	<0.0001	0.0001	0.0002
Post Glenn	R^2					0.3978	0.2406	0.4329
	p Value					<0.0001	< 0.0001	<0.0001
Pre Fontan	R^2						0.1578	0.318
	p Value						<0.0001	<0.0001
Post Fontan	R^2							0.238
	p Value							<0.0001
Late after Fontan	R^2							
	p Value							

to pneumococcal infections were reported to be the reasons of unfavorable result [5]. The present study suggests that thrombocytosis may be the other risk factor of morbidity and mortality in patients with asplenia syndrome.

The spleen plays a major role in platelet regulation, as it is the primary site of destruction of platelets [3]. In post-splenectomy patients, platelet count rises steeply with a peak value at 7 to 12 days and usually subsides only for the next 2 to 3 months. Thromboembolic complications reportedly occurred in only 1.6% in patients with these reactive thrombocytosis, compared with 12.4% of patients with primary thrombocytosis [9]. Our study showed that patients with asplenia syndrome have higher platelet counts than other Fontan candidates having spleen throughout the surgical stages. The platelet counts were especially high in infancy, when patients have the possibility of BT shunt occlusion, and after Glenn operation, when they often experience cerebral infarction. It is possible that this persistent thrombocytosis due to asplenia may augment the predisposition to developing thromboembolism in patients with a single ventricle.

Platelet adhesion and aggregation are key early events in the development of thromboembolism. Exposed substances from the subendothelium (collagen, tissue factor, von Willebrand factor, and so on) and increased shear force all lead to platelet activation, adhesion, and finally aggregation, which subsequently may cause luminal obstruction or embolization into the microcirculation [10]. Splenectomized patients are at a different risk of thromboembolism according to the kinds of underlying diseases. Nevertheless, Soyer et al. reported that post-splenectomy platelet counts in patients with portal vein thrombosis were higher than those in patients without thrombosis (804×10^9/L vs. 465×10^9/L) [11]. A recent prospective study demonstrated that platelet count of more than 650×10^9/L was significantly associated with post-splenectomy thromboembolic complications [12]. These reports suggest the importance of platelet count as a risk factor of thromboembolic complications. In the present study, more than 550×10^9/L of the platelet count at the first catheterization was selected as the most potent predictor for thromboembolic complications in the multivariate analysis. We should pay more attention to the platelet counts in patients with asplenia syndrome, especially when the platelet count is more than 550×10^9/L at the first cardiac catheterization.

Table 3
Univariate and multivariate analyses of factors influencing thromboembolic complications.

Variable	No. (%)	Odds ratio (95%CI)	p Value	
			Univariate	Multivariate
Platelet count>550×10^9/L [a]	32 (20)	3.17 (1.18–8.30)	0.023	0.046
BT shunt	94 (58)	2.47 (1.02–6.62)	0.044	0.077
Moderate to severe AVVR [a]	32 (20)	1.85 (0.51–6.12)	0.132	0.365
Age>7y	80 (50)	1.72 (0.76–4.04)	0.178	0.607
Birth weight<2500 g	26 (16)	1.59 (0.42–5.10)	0.468	0.188
APTT<40 s [a]	72 (45)	1.29 (0.27–6.02)	0.743	0.166
Systemic ventricular EF<50% [a]	71 (44)	1.15 (0.19–5.17)	0.839	0.370
Pulmonary vein obstruction	11 (7)	1.11 (0.16–4.64)	0.898	0.760
Hemoglobin>15 g/dL [a]	37 (23)	1.05 (0.27–3.35)	0.932	0.841
BT shunt size<3.5 mm [b]	21 (22)	1.68 (0.53–5.01)	0.367	–

BT shunt: Blalock–Taussig shunt, AVVR: atrioventricular valve regurgitation, APTT: activated partial thromboplastin time, EF: ejection fraction.
[a] At the first catheterization.
[b] BT shunt size was investigated among all patients having BT shunt (n=94).

Our result also showed a significantly higher incidence of thromboembolic complications in patients with asplenia syndrome than in patients with other functionally single ventricles. Patients with asplenia syndrome are often associated with pulmonary vein obstruction. They occasionally underwent a smaller size of the Blalock–Taussig shunt because inherent atrioventricular valve regurgitation may worsen with too much pulmonary blood flow. It seems possible that this pulmonary vein obstruction or smaller shunt size may be another predisposing factor to thromboembolic complications. However, our results showed that only platelet count at the first cardiac catheterization was significantly associated with thromboembolic complications. This result emphasized the clinical importance of elevated platelet count in patients with asplenia syndrome during the treatment course. Antithrombotic therapy should be optimized in patients with asplenia syndrome showing high platelet count.

The limitation of the present study was the lacked assessment of the precise coagulation profile, including protein C, protein S, antithrombin activity, and lupus anticoagulant. Further prospective study is needed to compare the coagulation states in single ventricle patients with and without asplenia syndrome.

6. Conclusion

Our study demonstrated the presence of persistent thrombocytosis as a risk factor of thromboembolism in patients with asplenia syndrome. Precautious monitoring of the platelet count and aggressive antithrombotic or anticoagulation therapy for patients with thrombocytosis

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may be beneficial to decrease the incidence of thromboembolic complications in patients with asplenia syndrome.

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

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Dear Sirs,

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

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

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

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

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

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

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

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

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

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

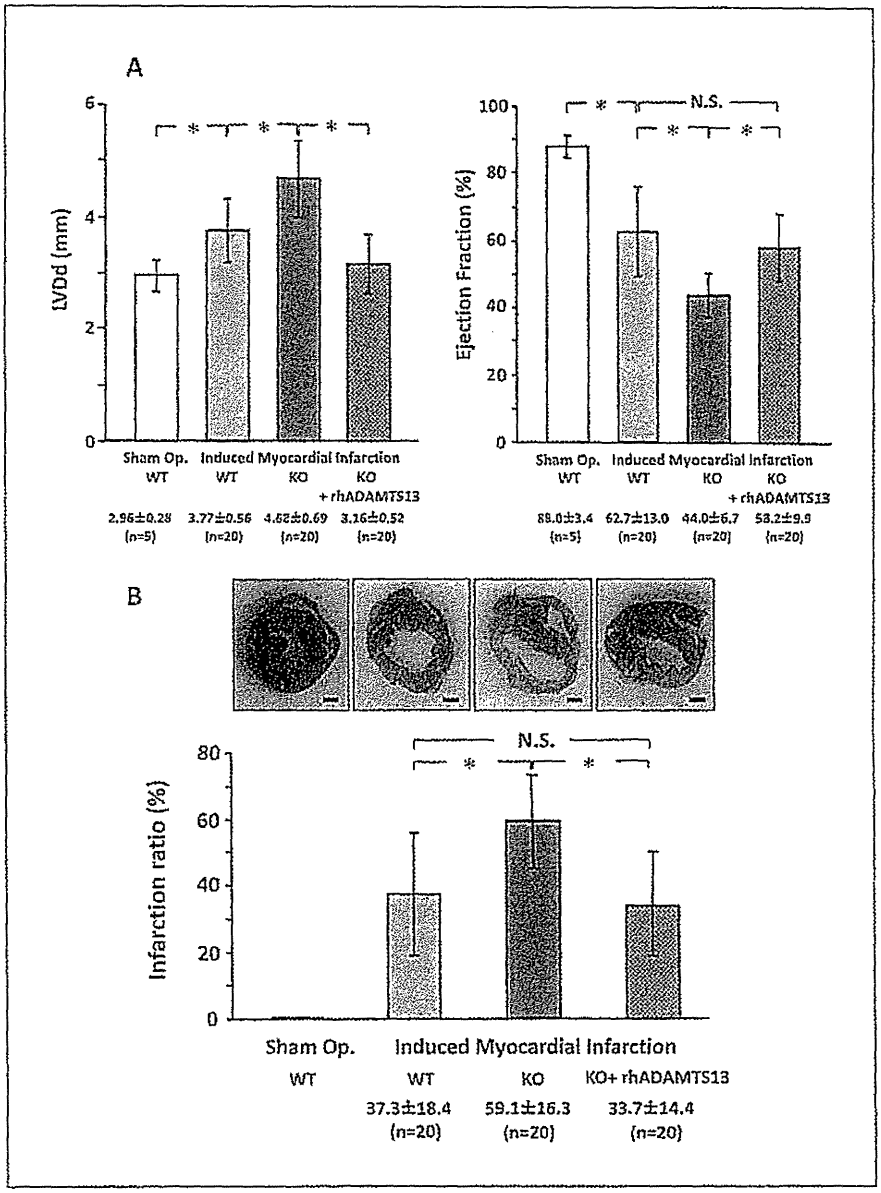

Figure 1: Evaluation of cardiac functions by echocardiography and histological evaluation in wild-type (WT) or ADAMTS13 KO mice with induced myocardial infarction. Acute myocardial infarction (AMI) was successfully induced in 20 WT mice (9 male, 11 female) and 20 KO mice (10 male, 10 female). In another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

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Conflicts of interest
None declared.

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Phenprocoumon and acenocoumarol treatment in paediatric patients

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Dear Sirs,

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

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

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ORIGINAL ARTICLE

Global haemostasis and point of care testing

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

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

Introduction

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

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

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

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

Thrombin generation assay

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

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

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

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

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

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Whole blood thromboelastometry

The method

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

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

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

Complementary additional information on overall haemostatic capacity

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

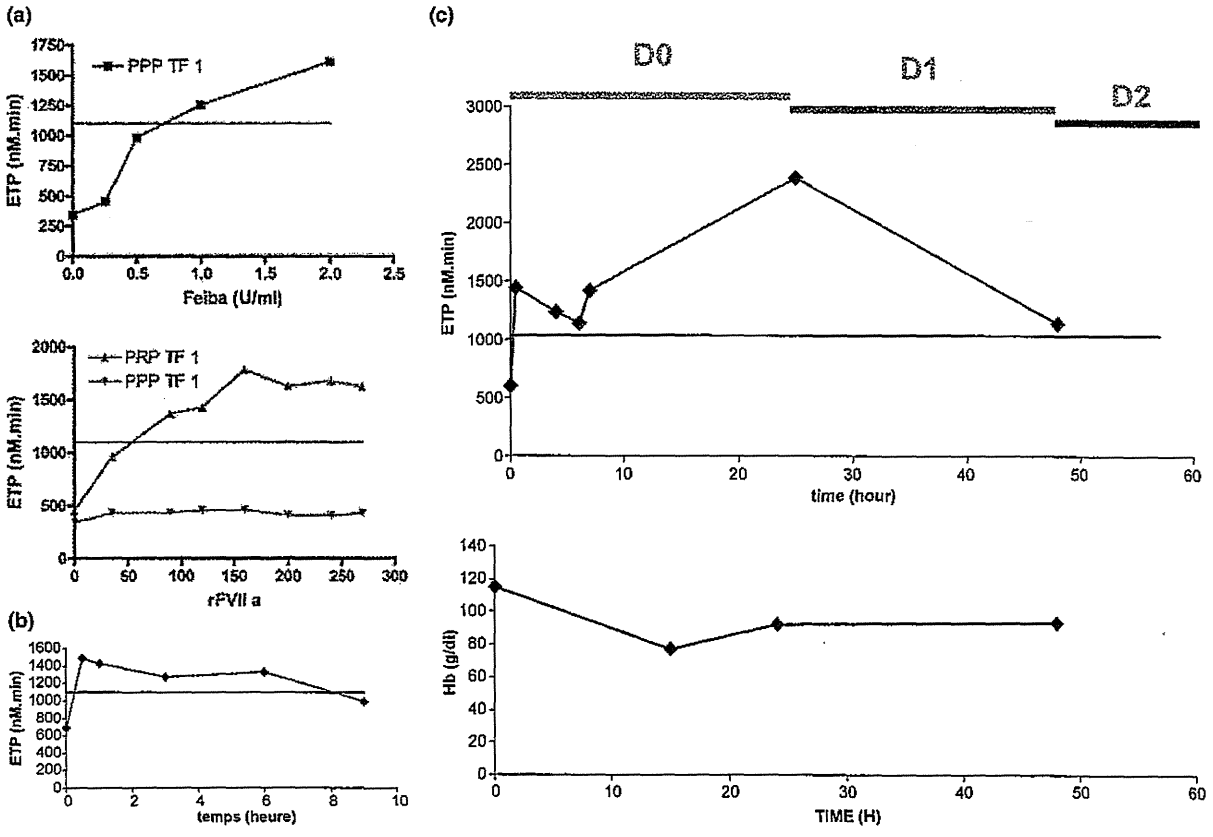

Fig. 1. A representative case illustrating the use of the three-step protocol.

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