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Intensive Plasma Exchange Increases ADAMTS-13 Activity And Reverses Organ Dysfunction In Children With Thrombocytopenia-Associated Multiple Organ Failure

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Abstract

Thrombocytopenia-associated multiple organ failure (TAMOF) is a poorly understood syndrome in critically ill children. ADAMTS-13, formerly known as von Willebrand factor (VWF) cleaving protease, is decreased in adults with VWF mediated thrombotic microangiopathy, and intensive plasma exchange (PEX) both replenishes ADAMTS-13 and improves outcome in these patients. We hypothesized that children with TAMOF syndrome have decreased ADAMTS-13 activity which can be replenished with PEX. In the first of two consecutive studies, relationships between platelet count, VWF multimers, ADAMTS-13 activity, plasminogen activator inhibitor-1 (PAI-1) and prothrombin time (PT) were analyzed in children with and without TAMOF. In the second study, children with severe TAMOF (platelet counts $<100,000/\text{mm}^3$ and ≥ 3 organ failure) were randomized to PEX or standard therapy. In the first study, children with TAMOF ($n=28$) had decreased ADAMTS-13 activity, but similar PAI-1 activity and PT compared to children with MOF without thrombocytopenia ($n=9$) ($p<0.05$). All non-survivors ($n=7$) had TAMOF, reduced ADAMTS 13 activity, and VWF-rich microvascular thromboses at autopsy. In the second study, PEX ($n=5$, median 12 days, 4–28 days) restored ADAMTS-13 activity and organ function, compared to standard therapy ($n=5$) ($p<0.05$). Children with TAMOF syndrome can have VWF mediated thrombotic microangiopathy. Similar to adult experience, PEX can replenish ADAMTS-13 activity and reverse organ failure in these children.

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Keywords

ADAMTS-13; von Willebrand factor; thrombocytopenia; multiple organ failure; plasma exchange; thrombotic microangiopathy

Introduction

New onset thrombocytopenia is an independent risk factor for development of MOF and death in critically ill patients.(1–8) It remains unknown whether new onset thrombocytopenia is an innocent marker of disease severity, or an important part of the pathogenic process which contributes to outcome in critically ill patients with MOF.

Investigators have examined the role of ADAMTS-13 (A Disintegrin And Metalloprotease with ThromboSpondin motifs), formerly known as von Willebrand factor cleaving-protease, in the pathophysiology of primary and secondary thrombotic microangiopathy syndromes. ADAMTS-13 regulates microvascular thrombosis by cleaving the large thrombogenic von Willebrand factor (VWF) multimers into smaller less hemostatically active forms. The pathophysiology of thrombotic thrombocytopenic purpura (TTP), a primary thrombotic microangiopathy heralded by new onset thrombocytopenia associated with microvascular ischemic injury to brain, kidney, and other organs, has been ascribed to deficiency of ADAMTS-13 levels to <10% of normal activity and an inability to cleave high molecular weight VWF.(9–11) Platelets adhere to uncleaved ultra-large VWF (ULVWF) multimers and form microvascular aggregates on endothelial cells, resulting in microvascular occlusions that are rich in platelets and VWF, and organ dysfunction. Autopsies of patients with TTP show microthrombi that are distinctively rich in VWF and platelets; whereas autopsies in patients with disseminated intravascular coagulation or hemolytic uremic syndrome show microthrombi that are distinctively rich in fibrin.(12–15) Unrecognized or untreated TTP carries an 80–90% mortality rate; however, survival can be as high as 90% with intensive plasma exchange therapy.(16;17) Plasma exchange is believed to be efficacious because it replenishes ADAMTS-13, removes ADAMTS-13 inhibitors, and removes thrombogenic ULVWF multimers.(10;18) Investigators have also reported decreased ADAMTS-13 activity in adults with new onset thrombocytopenia and secondary thrombotic microangiopathy syndromes associated with systemic infection/sepsis, cancer, transplantation, or autoimmune disease.(19–21) Most of these patients had moderate deficiency of ADAMTS-13 activity (10–57% of control) but some had severe deficiency (<10 % of control).(19) Some patients also had circulating ADAMTS-13 inhibitors or antibodies. Reduced ADAMTS-13 activity in these patients correlated with the degree of thrombocytopenia and increased VWF antigen levels. (19) These authors speculated that secondary thrombotic microangiopathy was caused in part by endothelial activation mediated release of higher levels of ULVWF multimers in the presence of reduced ADAMTS-13 activity. There are a number of reports describing improved outcomes in adults with various forms of secondary thrombotic microangiopathy after treatment with centrifugation-based plasma exchange therapy.(22–27)

In our present study, we reasoned that if thrombocytopenia associated MOF in children is an unrecognized VWF mediated secondary thrombotic microangiopathy, then similar to adult reports 1) these patients should have decreased ADAMTS-13 activity < 57% of normal(11) 2) the degree of ADAMTS-13 activity reduction should correspond to the degree of thrombocytopenia and increased VWF antigen release,(19) and 3) the autopsy specimens from patients who died with reduced ADAMTS-13 activity should show microvascular VWF thrombi. We further hypothesized that intensive plasma exchange therapy should restore ADAMTS-13 activity and facilitate organ failure resolution as measured by reduction in the

Pediatric Multiple Organ Dysfunction (PEMOD),(28) Pediatric Logistic Organ Dysfunction (PELOD),(29) and Organ Failure Index (OFI)(30) scores over 28 days.

Patients and Methods

First study period: Patients

The study was performed in two consecutive periods and approved by the Institutional Review Board at Children's Hospital of Pittsburgh. Inclusion criteria in the first study period were an Organ Failure Index (OFI) ≥ 2 . Exclusion criteria were clinical suspicion of TTP, or unwillingness of parents to pursue aggressive therapies, or to sign informed consent. In the first study period, consecutive children with MOF defined as an OFI ≥ 2 , and 5 critically ill children without MOF were enrolled. For purposes of analysis, children were divided into three groups; those without MOF, those with MOF without thrombocytopenia (platelet counts $\geq 100,000/\text{mm}^3$) and those with MOF and thrombocytopenia (platelet counts $< 100,000/\text{mm}^3$). Plasma was collected on day one in citrate and frozen at -70°C for batch analysis.

Second study period: Patients

Inclusion criteria in the second study were restricted further to patients with platelet counts $< 100,000/\text{mm}^3$ and OFI ≥ 3 (with oliguric renal failure defined as serum Creatinine $> 1\text{ mg/dl}$ and urine output $< 1\text{ mL/kg/h}$ despite diuretics including aminophylline). This identified patients with an 80% mortality risk. Exclusion criteria were extended in this study period to infants $< 5\text{ kg}$ because the cell separator used is not approved for patients below this weight, and the presence of inclusion criteria for more than 30 hours. In the second study period, consecutive children were randomized to receive standard therapy or an intensive plasma exchange protocol. Plasma was collected on days 1, 3, and 7 in citrate and frozen at -70°C for batch analysis. Some patients had additional convenience sampling.

Plasma exchange protocol

Plasma exchange was performed with a centrifugal cell separator (SPECTRA, Gambro BCT, Lakewood, CO) using a whole blood/anticoagulant ratio of 14:1. The inlet flow rate (whole blood removal rate) ranged between 10–50 mL per minute depending on patient weight, tolerance of volume changes, and citrate load. Ionized calcium levels were monitored at 15 minute intervals. Calcium chloride 10% solution, dosage 10 mg/kg, was given for citrate-mediated hypocalcemia symptoms or ionized calcium levels less than 0.9 mmol/L. In order to avoid a rapid decrease in circulating red cell volume in small children, a red blood cell prime consisting of 1 unit of leuko-reduced packed red blood cells diluted with normal saline to a hematocrit no less than 30% was used for patients who weighed 5–22 kilograms with an estimated total blood volume between 500–1750 mL (31).

The patients randomized to intensive plasma exchange received a 1.5 plasma volume exchange (based on weight-based nomogram and patient hematocrit) on day one followed by either 1 volume exchange daily $\times 14$ days or until resolution to an OFI < 3 for 48 hours, whichever was shorter in duration. If the OFI recrudesced again to be ≥ 3 within the initial 14 day period, then plasma exchange therapy was reinstituted. The decision to continue plasma exchange therapy after the 14 day point was left to the primary attending physician's discretion.

Controls for the VWF multimer measurements and ADAMTS-13 activity—The VWF multimer measurements and ADAMTS-13 activity were normalized as percent of reference values obtained in pooled healthy adult plasma in the clinical coagulation laboratory (The Institute for Transfusion Medicine, Pittsburgh, PA).

VWF multimeric pattern analysis: VWF antigen, and ULVWF multimers—Size distribution of VWF multimers were analyzed by 1% sodium dodecyl sulphate agarose gel electrophoresis under non-reducing conditions, and visualized by using a rabbit anti-human polyclonal VWF antibody (DAKO, Carpinteria CA) with a secondary ¹²⁵I-labeled donkey anti-rabbit IgG antibody (Amersham Pharmacia Biotech, NJ, USA).(32) Using scanning densitometry, the total VWF antigen was determined as the density of all bands in a sample expressed as the percentage of all bands density in pooled normal control on the same gel. The presence of ULVWF multimers was determined by the appearance of the VWF bands larger than the largest band seen in pooled normal control on the same gel.(33)

ADAMTS-13 Activity Static Assay—ADAMTS-13 activity was determined by the generation of dimers of the 176 kD and the 140 kD fragments from exogenous VWF substrates. Purchased human VWF (Haematologic Technologies Inc., VT, USA) with a concentration of ~500 mcg/ml was exposed to guanidine (1.5 mol/liter) and was diluted to a concentration of 15–25 mcg/ml to be used as substrate for ADAMTS-13. The VWF dimers of 176 kD and the 140 kD fragments were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The fragments were visualized by immunoblotting using the rabbit anti-human polyclonal VWF antibody (DAKO, Carpinteria CA) labeled with the secondary ¹²⁵I-labeled donkey anti-rabbit IgG antibody as mentioned above (Amersham Pharmacia Biotech, NJ, USA).(11) Using scanning densitometry, the density difference in the generated 176 kD fragments in the sample reaction mixtures with and without ethylene-diamine-tetra-acetic acid (EDTA) represented the proteolytic fragments cleaved by ADAMTS-13. The ADAMTS-13 activity was determined by the cleaved 176 kD proteolytic fragments from patient plasma expressed as the percentage of the cleaved 176 kD fragments from pooled control sample on the same gel. Abnormal ADAMTS-13 activity was defined as <57% of control plasma.(11)

Inhibitor titers of ADAMTS-13 activity—A modified Bethesda method that was originally used to determine the inhibitors titers for factor VIII was used to measure the ADAMTS-13 inhibitor titers.(18;34) Plasma samples were incubated at 56° Celsius for 60 minutes to inactivate ADAMTS-13 activity. The patient samples were serially diluted and added to an equal volume of pooled control plasma. ADAMTS-13 activity was measured by the above described method after incubating at 37° Celsius for 60 minutes. Serially diluted pooled control plasma incubated at 56° Celsius for 60 minutes was also added to pooled control plasma to serve as negative control. The ADAMTS-13 activity of the pooled control plasma was defined as 100%. 1 unit/ml inhibitor was defined as the amount inhibiting 50% of ADAMTS-13 activity. Only the ADAMTS-13 activity between 35% and 75% was used for calculation of the inhibitor titers.(18) Inhibitor levels were routinely measured only in patients in the second study period on days 1 and 3.

Plasminogen activator inhibitor-1 activity (PAI-1)—PAI-1 activity in plasma was measured by using a chromogenic assay kit. Abnormal PAI-1 activity was defined as >23.3 U/mL (Spectrolyse/pL, Chromogenic Assay Kit for plasminogen activator inhibitor activity, Biopool International, Ventura, CA).

Interleukin–6 levels—Interleukin–6 levels were measured in plasma using ELISA kits purchased from R&D (Minneapolis, Minnesota).

Platelet counts, Prothrombin time (PT), and Schistocytes—Platelet counts, prothrombin time (PT) and schistocytes were measured by the hospital clinical laboratory. Schistocytes were quantified by the hospital clinical hematopathologist based on review of high power microscopic fields (hpf) using the following schema: <1% (<1–2 cells/hpf); 1–2.5% (2–5 cells/hpf); and >2.5% (>5 cells/hpf). Thrombocytopenia was defined as platelet

counts $<100,000/\text{mm}^3$. Elevated PT was defined as 1.5 times the upper normal hospital standard PT measured in seconds. The normal range for PT in our hospital clinical laboratory was 8.1–10.2 seconds. We chose elevated PT as 1.5 times the upper normal hospital standard because 1) the consensus definition of Disseminated Intravascular Coagulation (DIC) put forth by the International Society on Thrombosis and Hemostasis used prolonged PT (> 6 seconds of normal) as one of the 4 criteria to meet the definition of DIC, and 2) the OFI used PT 1.5 times normal as one of the 3 criteria to defined hematologic failure. (30;35)

Immunohistochemistry of autopsy specimens—Microvascular VWF thrombi in paraffin embedded autopsy tissue specimens from kidneys, brain, and lungs were analysed with VWF antibodies (rabbit polyclonal, DAKO A0082, Carpinteria CA) and DAKO Envision + System HRP (K4011) Rabbit, with heat retrieval. Diaminobenzidine was the chromogen and hematoxylin was the counterstain. Negative control slides were processed as above with the substitution of Universal Negative Control Rabbit Serum (DAKO NP001) in place of specific VWF antibody. Each specimen was graded for the presence or absence of microvascular VWF thrombi. The histology was blinded to the diagnosis of autopsy specimens from twenty patients, including seven from the present study population.

Statistical analysis—In the first study period, differences between ordinal variables among control patients and patients with MOF; and MOF patients with and without thrombocytopenia were analyzed using t-test or Mann Whitney rank Sum test depending on parametric or non-parametric distribution of the data. Differences in categorical data were analyzed using Fisher's exact tests. Relationships between continuous variables were analyzed using linear regression or Spearman correlation depending on parametric or non-parametric distribution of the data. Logistic regression analysis was performed to determine any relationship between ADAMTS-13 activity and liver disease.

In the second study period, the sample size calculation predicted the need for 20 subjects to show a 50% treatment effect on resolution of organ dysfunction defined by reduction in PELOD, PEMOD, and OFI scores over 28 days ($p<0.05$). Differences over time between groups were measured using 2 Factor ANOVA (SAS, Cary NC). Interim analysis was planned at ten-patient enrollment with pre hoc determined cessation of the study if a robust nested treatment effect ($p<0.001$) negative or positive, was found in differences in organ dysfunction over 28 days evaluated by PELOD, PEMOD, and OFI scores between treatment groups.

Results

First Study Period

In the first study period (13 months), thirty-seven consecutive children identified with an OFI ≥ 2 were enrolled with the following diagnoses; previously healthy with sepsis as the only diagnosis (n=8), post-transplant organ dysfunction (n=12), end stage liver failure (n=6), post-bone marrow transplant (n=4), and miscellaneous (end-stage heart failure, hypoplastic left heart syndrome s/p Glenn procedure, ALL, pancytopenia, Wilm's tumor, chronic granulomatous disease [n=7]). All of these 37 patients with MOF met the clinical definition of sepsis (36), and all were on antibiotics for suspected infection. There were 17 males and 20 females. The age range was from 9 days to 23 years (72 months, [11,180]; median, [25%ile, 75%ile]). Seventy six per cent (n=28) of these children had thrombocytopenia (platelet counts $<100,000/\text{mm}^3$). Five additional critically ill children without MOF were also enrolled with varied diagnoses (one each with clonidine ingestion, tricyclic antidepressant ingestion, and three status post tonsillectomy and adenoidectomy); ages ranged from 20 months to 5 years of age. None had thrombocytopenia.

Figure 1A shows that the children with MOF who survived (n=30), resolved thrombocytopenia by 14 days, whereas non-survivors (n=7) did not resolve thrombocytopenia by 14 days ($p<0.05$, 2 Factor ANOVA). Figure 1B shows in representative fashion VWF-rich microvascular thrombi observed in the brain (6/6 available specimens), kidney (4/7), and lung (4/7). All seven non-survivors in our study had thrombocytopenia, decreased ADAMTS-13 activity, MOF, and VWF-rich microvascular thrombi at autopsy. Four out of seven non-survivors had ULVWF multimers in circulation. In comparison, VWF-rich microvascular thrombi were not seen in the control specimens without TAMOF.

Compared to children without MOF (n=5), those with MOF (n=37) had reduced platelet counts ($62,000/\text{mm}^3$ [40,000; 99,000] vs $283,000/\text{mm}^3$ [273,000; 313,000], median, [25%ile, 75%ile], $p<0.05$, Mann-Whitney Rank Sum), and ADAMTS-13 activity ($39.4\% \pm 23.3$ vs $79.4\% \pm 5.6$, mean $\pm$ S.D., $p<0.05$, t-test). They also had increased VWF antigen levels ($172\% \pm 48$ vs $106\% \pm 12$, mean $\pm$ S.D., $p<0.05$, t-test), PAI-1 activity ($46 \text{ U/ml} \pm 36.6$ vs $5 \text{ U/ml} \pm 7.6$, mean $\pm$ S.D., $p<0.05$, t-test), and PT (16.3 seconds [14.5; 19.7] vs 12.5 seconds [11.8; 12.9], median [25%ile; 75%ile]) ($p<0.05$ t-test, Mann-Whitney Rank Sum).

Compared to the children who had MOF without thrombocytopenia (n=9), those with thrombocytopenia associated MOF (n=28) had reduced platelet counts ($51,000/\text{mm}^3$ [37,000; 75,000] vs $204,000/\text{mm}^3$ [162,000; 293,000], median, [25%ile, 75%ile]), $p<0.05$, Mann-Whitney Rank Sum) and ADAMTS-13 activity ($33.5\% \pm 18.3\%$ vs $57.7\% \pm 27.52\%$, mean $\pm$ S.D., $p<0.05$, t-test), but no significant differences in VWF antigen levels ($175\% \pm 47\%$ vs $161\% \pm 53\%$, mean $\pm$ S.D.), PAI-1 activity ($44.9 \text{ U/ml} \pm 37.34$ vs $50 \text{ U/ml} \pm 37.74$, mean $\pm$ S.D.), or PT (17.3 seconds [14.2, 20.7] vs 15.5 seconds [14.7, 16.6], median [25%ile, 75%ile]).

A greater proportion of children with thrombocytopenia associated MOF had a reduced ADAMTS-13 activity ($< 57\%$) ($25/28$ [89%] vs $3/9$ [33%]) (Fisher's exact $p<0.05$) compared to children with MOF without thrombocytopenia (Figures 2A,B). Children with thrombocytopenic associated MOF also tended to be more likely to have circulating ULVWF multimers ($15/28$ [54%] vs $3/9$ [33%]) (Fisher's exact $p=0.057$) compared to children with MOF without thrombocytopenia. In children with thrombocytopenia associated MOF, circulating ULVWF multimers were only seen in those with decreased ADAMTS-13 activity. In the 3 children with MOF without thrombocytopenia who had ULVWF multimers, only 1 child had decreased ADAMTS-13 activity. There was no difference in the proportion of patients with increased PAI-1 activity and PT in the two groups. ADAMTS-13 deficiency was not related to liver failure (logistic regression $p > 0.5$)

Analysis of all three groups of patients together (n=42) showed that platelet counts correlated directly with ADAMTS-13 activity ($r=0.61$, $p<0.05$), but inversely with VWF antigen levels ($r=-0.43$, $p<0.05$) and PT ($r=-0.34$, $p<0.05$). Platelet counts had no relationship with PAI-1 activity. Figure 3 shows that, similar to reports in adults with secondary thrombotic microangiopathy, ADAMTS-13 activity quartiles (19;21) correlated inversely with VWF antigen levels (figure 2A) and, directly with platelet counts (figure 2B) (Linear Regression, $p<0.05$). ADAMTS-13 activity showed no relationship to PT or PAI-1 activity. Interleukin-6 levels correlated directly with VWF antigen levels ($r_s 0.217$, $p<0.05$) and the presence of ULVWF multimers ($r_s 0.269$, $p<0.05$). Interleukin-6 levels were 21 pg/ml [7, 91] in patients without circulating ULVWF vs 79.5 pg/ml [13–242] (median [25%ile, 75%ile]) in patients with circulating ULVWF multimers ($p<0.05$ Mann Whitney Rank Sum Test). Interleukin-6 levels were not related to ADAMTS-13 activity measured under static condition.

Second Study Period

Twenty additional patients met criteria for inclusion into the therapeutic plasma exchange protocol randomized controlled trial. Primary physicians and parents agreed to enrollment in only 10 of the 20 eligible patients. The fifty per cent enrollment rate stayed unchanged throughout the study period; however, the reasons for declining enrollment changed over time. In the initial phase of the study, primary physicians who declined enrollment did not want their patients randomized to the treatment arm. After the initial study phase, however, primary physicians who declined enrollment wanted their patients to get plasma exchange without randomization.

Although not part of the specific entry criterion, all ten patients enrolled were being treated with antibiotics for culture-positive sepsis. In the plasma exchange treatment arm the ages ranged from 1 year to 16 years with 3 females and 2 males, and the underlying diagnoses included unrecognized chronic granulomatous disease, liver and small bowel failure awaiting transplant, status-post bone marrow transplant, and two patients with meningococcal purpura fulminans sepsis. In the standard treatment arm the ages ranged from 3 years to 18 years with 5 males, and the diagnoses included bone marrow transplant, heart transplant, and three cases of primary sepsis. All ten patients had elevated lactate dehydrogenase (LDH) levels but none had >1% schistocytes on peripheral smear.

Interim analysis was performed as planned after ten patients completed the protocol. Patients randomized to the treatment arm received a median of 12 days of therapy. There was no difference in Pediatric Risk of Mortality Score (PRISM), (37) PELOD, PEMOD, or OFI scores between randomized groups at the time of initiation of the trial. There was a robust reduction over the 28-day study period in OFI, PEMOD, and PELOD score in patients randomized to the treatment arm compared to those given standard therapies (2 Factor Repeated Measures ANOVA $p < 0.001$, Figure 4A) which activated the stop rule. A statistically significant difference in organ severity between groups was observed after the eighth day of the study. There was also an improved 28-day survival in the treatment arm (1/5 with standard therapy, 5/5 with plasma exchange protocol $p < 0.05$ Fisher's exact test).

All ten patients received culture and sensitivity directed antibiotic therapy and American College of Critical Care Medicine recommended Hemodynamic Support Guideline therapies (38) for reversal of septic shock before randomization to treatment arm. In the plasma exchange arm, one patient required extracorporeal membrane oxygenation (ECMO) and continuous renal replacement therapy (CRRT). In the standard therapy arm two patients required CRRT. Patients in the plasma exchange arm received calcium infusions for citrate-mediated hypocalcemia. In addition, they required increased inotrope/vasopressor infusions used for hypotension, and adjustment of sedation/analgesia medications used to treat awakening, because these drugs are removed during plasma exchange. (Figure 4B) ADAMTS-13 activity increased during the first seven day cycle of plasma exchange therapy in the plasma exchange arm compared to standard therapy arm (Day 1; 44% vs 25%, Day 3; 69% vs 9%, Day 7; 56% vs 17%; 2 Factor ANOVA, $p < 0.05$) as did platelet counts (Day 1; 48,000 vs 42,000/mm³, Day 3; 54,000 vs 38,000/mm³, Day 7; 87,000 vs 34,000/mm³; 2 Factor ANOVA, $p < 0.05$). Plasma exchange therapy also tended to decrease PAI activity, PT, and VWF antigen levels over the first seven days (2 Factor ANOVA, $p < 0.1$). ADAMTS-13 inhibitor levels were 0.00 units $\pm$ 0.1 in the five patients from study period one without MOF, 1.09 units $\pm$ 0.22 in the patients in the plasma exchange arm, and 0.55 units $\pm$ 0.22 in the patients in the standard treatment arm at the time of study entry. Plasma exchange reduced inhibitor levels over three days compared to standard therapy ($p < 0.05$, 2 Factor Repeated Measures ANOVA group $\times$ time).

Three of the five plasma exchange patients recrudesced to having ≥ 3 OFI when plasma exchange therapy was stopped. These patients subsequently experienced a reduction in

ADAMTS-13 activity with increased organ dysfunction scores, ADAMTS-13 inhibitor levels, and VWF antigen levels. Plasma exchange was reinstituted in two patients (for 14 and 28 days) who survived with subsequent restoration of ADAMTS-13 activity and organ function. The ADAMTS-13 activity never recovered in the one patient who did not have plasma exchange reinstituted. This child died after 28 days with thrombocytopenia associated MOF. Figures 5–7 show VWF multimeric analyses, available autopsies with VWF immunohistochemistry staining, and coagulation parameter measures of selected patients in the second study period.

Discussion

The observed relationship between decreased ADAMTS-13 activity, decreased platelet counts and increased VWF antigen was similar to that previously seen in adults with systemic endotheliopathy syndromes associated with infection/sepsis, transplantation, cancer and autoimmune disease.(19;21) Similar to these adult findings, some children had increased ADAMTS-13 inhibitor levels and some had ADAMTS-13 activity <10%; however, most children had ADAMTS-13 activity between 10% and 57%. In contrast to the adults, our children did not have high schistocyte counts. ULVWF multimers were not seen in children with >57% ADAMTS-13 activity and thrombocytopenia associated MOF. In addition, all seven autopsies from children who died with thrombocytopenia associated MOF and decreased ADAMTS-13 activity, showed VWF-rich thrombi in the microvasculature of brain, lung, and kidney. These findings are consistent with the hypothesis that many children with thrombocytopenia associated MOF have a secondary thrombotic microangiopathy associated with decreased ADAMTS-13 activity.

ULVWF multimers were seen in 15/25 children with low ADAMTS-13 activity and thrombocytopenia associated MOF. The exact mechanism of why we did not observe ULVWF multimers in the other 10/25 children brings into the question of whether the effect of low ADAMTS-13 activity is in fact mediated through its effect on VWF cleavage. The exact level of ADAMTS-13 activity required to regulate the hyper-adhesiveness ULVWF multimers is not the scope of this observational study. However, we postulate that ADAMTS-13 activity of 10–57% might still be adequate for ULVWF multimers cleavage. ADAMTS-13 activity <10% is associated with TTP. All 4 children with ADAMTS-13 activity level < 10% in our study had the presence of ULVWF multimers and thrombocytopenia associated MOF. Another explanation could be that ULVWF multimers were being consumed in forming vaso-occlusions, and therefore, were not available to be detected in the plasma. In the children with MOF without thrombocytopenia, there were 2/9 children with the presence of ULVWF multimers with ADAMTS-13 activity > 57%. One of the limitations of our study was using a widely accepted and less cumbersome static technique of measuring ADAMTS-13 activity (11). However, it has been shown by other investigators that measuring ADAMTS-13 activity under flowing conditions could detect additional mechanisms of acquired ADAMTS-13 deficiency. It is postulated that ADAMTS-13 is required to anchor onto the endothelium under flowing conditions for efficient ULVWF cleavage (10;39). Another explanation could be that the mechanism of MOF in these children without thrombocytopenia might not be mediated through VWF mediated thrombotic microangiopathy.

We also found that intensive plasma exchange therapy restored ADAMTS-13 activity in children with thrombocytopenia associated MOF. The importance of plasma exchange in restoration of ADAMTS-13 is evidenced both by comparison of the two arm trial, and the observation that three of the children experienced a reduction in ADAMTS-13 activity to <57% when plasma exchange was stopped. The two children who had plasma exchange reinstituted restored ADAMTS-13 activity, and the one child who did not have plasma exchange reinstituted had persistently decreased ADAMTS-13. Plasma exchange is documented to be more effective than plasma infusion alone for restoring ADAMTS-13 activity in adults with

primary thrombotic microangiopathy (TTP) because it both replenishes ADAMTS-13 and removes ADAMTS-13 inhibitors.(17) We observed a reduction in ADAMTS-13 inhibitor levels with plasma exchange. Similar to adult studies of plasma exchange therapy for primary and secondary thrombotic microangiopathies, we also found that intensive plasma exchange was associated with resolution of organ dysfunction in children with thrombocytopenia associated MOF.(17;22;24)

The mechanism for reduced ADAMTS-13 activity in the children with thrombocytopenia associated MOF is unknown. Genetic polymorphisms can influence enzyme activity in patients with TTP(40); however, our patients were not genotyped. ADAMTS-13 is synthesized in the liver and endothelium, and potentially could be decreased with hepatic or vascular dysfunction.(41;42) Although the logistic regression analysis showed no relationship between liver disease and reduced ADAMTS-13 activity in our patients, we had no measure of ADAMTS-13 synthesis *per se*. The activity of the enzyme can also be decreased by circulating inhibitors. In TTP patients these inhibitors are generally observed at >2 u/mL. For the most part, our children with thrombocytopenia associated MOF had inhibitor levels <2 u/mL. Several inhibitors have been characterized. ADAMTS-13 antibodies occur in patients with TTP(11); however, we did not measure antibodies in our inhibitor assay. Products of hemolysis can inhibit ADAMTS-13.(43) Although our patients did not have increased schistocytes, they did have increased LDH consistent with low grade hemolysis. More recently, investigators have reported an *in vitro* link between inflammatory cytokines and inhibition of ADAMTS-13.(44) Interleukin-6 inhibits proteolysis of ULVWF multimers under conditions of flow. Although the assay we used to measure ADAMTS-13 activity was done under static conditions and showed no relationship to IL-6 levels, the children who had circulating ULVWF multimers had higher IL-6 levels. Further work is required to address these multi-factorial mechanistic issues.

Although children with MOF and thrombocytopenia had reduced ADAMTS-13 compared to those without thrombocytopenia, and plasma exchange therapy restored ADAMTS-13 activity and resolved organ failure, it is still unknown whether decreased ADAMTS-13 is an innocent bystander or the pathogenic cause of organ failure. Ono et al., recently reported that severe ADAMTS-13 deficiency (defined as <20% normal) in adult patients with sepsis-induced DIC was associated with development of acute renal failure.(20) This finding suggests a role for ADAMTS-13 deficiency in the development of organ failure. Experimental research using models of toxin-induced systemic endotheliopathy in ADAMTS-13-knockout mice is under way to determine any role of ADAMTS-13 in the development of secondary thrombotic microangiopathy and organ failure.(45;46)

Further clinical investigation of the role of plasma exchange therapy in improving outcomes in children with thrombocytopenia associated MOF is also needed. Our randomized controlled therapeutic plasma exchange study was not designed to determine outcome and therefore should not be interpreted as evidence that plasma exchange therapy will improve outcome in these children. Darmon and colleagues recently reported that an average of nine days of centrifugation based plasma exchange improved organ failure resolution and survival in critically ill adults with secondary thrombotic microangiopathy syndromes.(24)

Continued study of the ability of plasma exchange and ADAMTS-13 replacement therapy to improve outcomes in children with thrombocytopenia associated MOF is warranted.

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Fig 1A

Fig 1B

Figure 1. Platelet counts recovered in children with multiple organ failure who survived compared to those who did not survive.[1–8] Von Willebrand Factor (VWF)-rich thrombi are seen in the small blood vessels of the brains, lungs, and kidneys of non-survivors with thrombocytopenia-associated multiple organ failure

(A) Black circles represent platelet counts (mean $\pm$ S.D.) in children with multiple organ failure who survived ($n = 30$). White circles represent platelet counts in children who did not survive ($n = 7$). Patients who survived recovered their platelet counts compared to children who did not survive (2 Factor ANOVA $p < 0.05$). (B). Brain (I, IV), kidney (II, V), and lung (III, VI) tissue from a representative patient stained for VWF (brown color) (polyclonal rabbit anti-human VWF antibody). Panels I, II, and III serve as negative controls from the same

patients as in Panels **IV**, **V**, and **VI** respectively, labeled with isotype antibody (non-specific rabbit anti-human IgG). The secondary antibody was conjugated with horse radish peroxidase (brown color).

Fig 2A. Thrombocytopenia associated MOF (n = 28)

Fig 2B. MOF without Thrombocytopenia (n = 9)

Fig 2C. No MOF (Critically Ill Controls) (n = 5)

Figure 2. ADAMTS-13 activity, presence of ULVWF multimers and prothrombin time (PT) in critically ill patients with thrombocytopenia associated MOF, MOF without thrombocytopenia, and no MOF

There were a total of 37 MOF and 5 non-MOF critically ill patients enrolled in the first study period. (A) There were 28 MOF patients with thrombocytopenia. 89% (25/28), 53% (15/28) and 46% (13/28) of these patients had low ADAMTS-13 activity, the presence of ULVWF multimers, and elevated PT respectively. All thrombocytopenic MOF patients with ULVWF multimers had low ADAMTS-13 activity. Only 4% (1/28) of thrombocytopenic MOF had normal ADAMTS-13 activity, normal PT, and no ULVWF multimers. (B) There were 9 MOF patients without thrombocytopenia. All 9 of these patients had normal PT. 67% (6/9) of patients had normal ADAMTS-13 of whom 33% (2/6) had the presence of ULVWF multimers. 33% (3/9) patients had decreased ADAMTS-13 of whom 11% (1/9) patients had the presence of ULVWF multimers. (C) There were 5 non-MOF critically ill controls. These patients were not thrombocytopenic and had normal ADAMTS-13 activity, normal PT, and no ULVWF multimers.

Figure 3. Relationships between quartiles of ADAMTS-13 activity and VWF antigen levels, and platelet counts are similar to those reported in adults with secondary thrombotic microangiopathic syndromes.[16;18]

White circles represent critically ill children without thrombocytopenia (platelet counts $\geq$ 100K) and without multiple organ failure ($n = 5$). Black circles represent children with multiple organ failure ($OFI \geq 2$) ($n = 37$). ADAMTS-13 activity in quartiles 1, 2, 3, and 4 are defined respectively as 0–24%, 25–49%, 50–74%, 75–100% relative to healthy adult donor plasma activity. Similar to adult reports, ADAMTS-13 activity correlated inversely with VWF antigen levels (**A**), and positively with platelet counts (**B**) (Linear Regression, $p < 0.05$).

Fig 4A

Fig 4B

Figure 4. Plasma exchange replenishes ADAMTS13, and reduces organ failure

Plasma exchange patients are represented by black circles ($n = 5$), and Standard therapy patients are represented by white circles ($n = 5$). **(A)** There was a robust reduction ($p < 0.001$ 2 Factor Repeated Measures ANOVA group $\times$ time) over the 28 day study period in PELOD scores in patients randomized to the plasma exchange arm compared to those given standard therapies. **(B)** There was a significant increase in ADAMTS-13 activity with plasma exchange ($44 \pm 10\%$ on day 1 increased to $56 \pm 11.5\%$ on day 7; mean $\pm$ SEM) compared to standard therapy ($25 \pm 11\%$ on day 1 decreased to $17 \pm 11\%$ on day 7; mean $\pm$ SEM) ($p < 0.05$, 2 Factor ANOVA group $\times$ time).

Day in MOF	1	3	5	Control
ULVWF	(+)	(+)	(+)	(-)
ADAMTS-13 Activity (%)	23	12	21	100
VWF Antigen (%)	238	338	320	100
Platelet Cts (x 1,000/mm ³)	52	29	37	
PT (seconds)	16	39	23	
Inhibitor (unit/ml)	0.74	1.12		

Figure 5. A 3 y.o. male with gram negative sepsis and thrombocytopenia associated multiple organ failure who had decreased ADAMTS-13 activity with ultra-large VWF multimers and increased PT and PAI-1 activity which did not resolve with randomization to standard therapy
(A) Ultra-large VWF multimers were present on day 1 and increased over a 5-day period. C represented healthy control sample. Autopsy showed VWF-rich microvascular thrombi. The brain **(B)**, and kidney **(C)** are labeled with VWF (brown color) (polyclonal rabbit anti-human VWF antibody). ADAMTS-13 activity was persistently low. VWF antigen levels were more than 200% of normal control. Platelet count decreased and ADAMTS-13 inhibitors and PT (seconds) increased over time.

Figure 6. A 16 y.o. female with unrecognized chronic granulomatous disease, aspergillus infection, P. aeruginosa pneumonia, and thrombocytopenia associated multiple organ failure who had reduced ADAMTS 13 activity with circulating ultra-large VWF multimers and ADAMTS 13 inhibitors was randomized to the plasma exchange arm

Ultra-large VWF multimers, VWF antigen level, and ADAMTS-13 inhibitor levels decreased and ADAMTS-13 activity, and platelet counts increased with three days of plasma exchange. When three organ failure had resolved for 48 hours, plasma exchange was stopped on day four. Thereafter, an increase in VWF antigen and ADAMTS-13 inhibitor levels and a reduction in ADAMTS-13 activity were observed. Plasma exchange therapy was subsequently restarted on day 8 for a total of 14 days of therapy with eventual resolution of thrombocytopenia associated multiple organ failure. PT (seconds) was normal throughout. This child survived.

	Plasma Exchange			Plasma Exchange Not Reinstigated							
Day	1	3	7	14	20	23	25	39	40	45	Control
ULVWF	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
ADAMTS-13 Activity (%)	41	63	60	11	11	7	39	49	40	27	100
VWF Ag (%)	181	100	135	266	317	300	215	195	166	275	100
Platelet Cts (x1,000)	45	18	89	54	105	94	83	44	44	33	
PT (seconds)	12	12	12.4	11.3	12	12.2	12	12.2	13.8	13.7	
Inhibitor (unit/ml)	1.65	0.93							0.8		

Figure 7. A 7 y.o. male wilt ALL s/p bone marrow transplant with *E. faecalis* sepsis. The patient developed thrombocytopenia associated multiple organ failure with decreased ADAMTS-13 activity and increased VWF antigen. Clinical and laboratory abnormalities resolved with randomization to plasma exchange therapy but recrudesced after cessation of the protocol (A) Plasma exchange was associated with restoration of ADAMTS-13 activity, reduced VWF antigen and ADAMTS-13 inhibitor levels, reduced LDH, and increasing platelet count. Cessation of plasma exchange at 7 days when the OFI was < 3 for 48 hours was associated with a recrudescence of coagulation abnormalities. ADAMTS-13 activity decreased with a persistence of ADAMTS-13 inhibitor levels. Thrombocytopenia worsened and VWF antigen increased. At the request of the primary service, plasma exchange therapy was never reinstituted after the 14 day study period. The child recrudesced and died after 45 days with unremitting thrombocytopenia associated multiple organ failure and an ADAMTS-13 level that was 27% of normal. (B) Autopsy showed VWF-rich (brown color) (polyclonal rabbit anti-human VWF antibody) microvascular thrombi in the brain.