Devised in collaboration with...
complicated falciparum malaria in comparison to healthy controls and assessed the relationships with disease severity.

**Material and methods**

**Patients and study design**

Patients enrolled in the study were recruited from Chittagong Medical College Hospital in Chittagong, Bangladesh. Adult non-pregnant patients (≥16 years) with slide-confirmed falciparum malaria were included. Written informed consent was obtained from the patient or its attending relative. Patients were classified as having severe malaria when one or more of the following criteria were met: Glasgow Coma Scale (GCS) score <11, acute respiratory distress syndrome (ARDS), severe anaemia or jaundice (haematocrit < 20 %, bilirubin > 3.0 mg/dl, combined with parasite counts > 100,000/μl), renal failure (serum creatinine > 3 mg/dl), hypoglycaemia (blood glucose < 40 mg/dl), shock (systolic blood pressure < 80 mm Hg with cool extremities), hyperparasitemia (peripheral asexual stage parasitaemia ≥ 10 %), hyperlactatemia (venous plasma lactate > 4 mM), or acidemia (venous plasma bicarbonate < 15 mM) (12). A total of 30 severe malaria and 12 uncomplicated malaria falciparum patients were included in the study. Fourteen healthy Bangladeshi volunteers, who were known to have no medical illness that could affect haemostasis and were not on any medication, served as a control group.

On admission a full history and physical examination were carried out. Venous blood samples were obtained for haemoglobin, haematocrit, parasitaemia, platelet count, white blood cell count, plasma lactate levels, glucose levels and full biochemistry. Venous blood samples for coagulation parameters were collected into 3.2% sodium citrate tubes (Vacutainer® Blood Collection Tube, Becton Dickinson, Piscataway, NJ, USA). Samples were centrifuged at 3,000 rpm at 4°C for 10 minutes and plasma was stored immediately at –80°C until assays were performed. The primary outcome measure of the study was the difference in ADAMTS13 activity between different groups of disease severity. To explore the mechanisms associated with ADAMTS13 deficiency, VWF antigen and VWF propeptide were measured, and VWF multimer analysis was performed. Furthermore free haemoglobin and interleukin 6 (IL-6) levels were assessed. The study was part of ongoing research projects approved by the Ethics Committee of the Bangladeshi Ministry of Health and the Oxford Tropical Research Ethics Committee.

### Table 1: Baseline characteristics of patients diagnosed with severe and uncomplicated falciparum malaria

<table>
<thead>
<tr>
<th></th>
<th>Severe malaria (n=30)</th>
<th>Uncomplicated malaria (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>37 (25–51)</td>
<td>28 (22–47)</td>
<td>0.16</td>
</tr>
<tr>
<td>Male gender, no. of</td>
<td>24 (80)</td>
<td>10 (83)</td>
<td>0.88</td>
</tr>
<tr>
<td>patients (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasite count /μl</td>
<td>31998 (14228–17961)</td>
<td>52521 (670–41132)</td>
<td>0.04</td>
</tr>
<tr>
<td>Platelet count, x10^11 cells/μl</td>
<td>30 (14–50)</td>
<td>49 (23–125)</td>
<td>0.17</td>
</tr>
<tr>
<td>WBC count, x10^9 cells/μl</td>
<td>9.9 (7.0–13.1)</td>
<td>6.3 (3.7–7.6)</td>
<td>0.003</td>
</tr>
<tr>
<td>Haematocrit, %</td>
<td>32.2 (25.4–36.1)</td>
<td>37.4 (33.0–39.6)</td>
<td>0.04</td>
</tr>
<tr>
<td>Haemoglobin, g/dl</td>
<td>10.6 (8.8–12.4)</td>
<td>12.1 (10.7–12.9)</td>
<td>0.16</td>
</tr>
<tr>
<td>GCS score</td>
<td>6 (4–11)</td>
<td>15 (15–15)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>49.0 (25.0–76.5)</td>
<td>16.1 (10.6–26.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>2.2 (1.1–3.1)</td>
<td>1.0 (0.9–1.2)</td>
<td>0.02</td>
</tr>
<tr>
<td>Total serum bilirubin, mg/dl</td>
<td>4.0 (2.1–9.4)</td>
<td>1.2 (0.9–1.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>ASAT, U/l</td>
<td>72 (49–127)</td>
<td>31 (22–35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALAT, U/l</td>
<td>21 (15–42)</td>
<td>20 (17–28)</td>
<td>0.64</td>
</tr>
<tr>
<td>AP, U/l</td>
<td>78 (51–118)</td>
<td>91 (77–105)</td>
<td>0.34</td>
</tr>
<tr>
<td>Serum bicarbonate, mM</td>
<td>15.2 (12.9–19.7)</td>
<td>21.9 (20.4–24.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>117 (88–149)</td>
<td>133 (110–191)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

**Laboratory procedures**

ADAMTS13 activity was determined in the Academic Medical Center (AMC) in Amsterdam, the Netherlands, using the rapid fluorescence resonance energy transfer (FRETs-VWF73) assay (13). Pooled plasma collected from more than 200 healthy Dutch volunteers was used as standard. The reference range for ADAMTS13 activity was 54% – 150% (standard deviation [SD] 26.5%) and the inter-assay variation 5.4% (above the 50% level) and 6.4% (below the 50% level). VWF antigen and VWF propeptide levels were assessed by ELISA using commercial antibodies (DAKO, Denmark and Sanquin, the Netherlands, respectively). The multimeric pattern of VWF was analysed using 2% agarose gel electrophoresis, followed by immunoblotting according to Raines and others (14). Free haemoglobin was measured in plasma by absorption spectrophotometry using a cyanide-free haemoglobin reagent (Cell Dyn 4000, Abbott Diagnostics) and IL-6 concentrations were determined using a commercially available human IL-6 ELISA kit (Sanquin, the Netherlands).
Statistical analysis

Baseline variables of patients diagnosed with severe and uncomplicated falciparum malaria were described as median and interquartile range (IQR). To assess the difference in baseline characteristics between groups, Mann Whitney U test was used. Differences in ADAMTS13 activity, VWF antigen and VWF propeptide were determined by ANOVA, as variables had a near Gaussian distribution. For comparison of ADAMTS13 activity, VWF antigen and propeptide levels between groups, student’s t-test was used, and where appropriate a correction for multiple comparisons between individual groups was made using Fisher’s LSD post-hoc test. Linear regression was used to describe the association between ADAMTS13 activity, VWF antigen, VWF propeptide, platelet count and haemoglobin. Differences in IL-6 concentrations between groups were assessed by Mann Whitney U test. Data were analysed using SPSS software package for Windows version 14.0 (SPSS Inc, Chicago, IL, USA). Graphics were constructed using GraphPad Prism, version 4 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Clinical characteristics

A total of 30 patients with severe falciparum malaria, 12 with uncomplicated falciparum malaria and 14 healthy controls were enrolled in the study. Baseline characteristics are shown in Table 1. Mortality in the patients with severe malaria was 23%, compared to 0% in patients with uncomplicated malaria. Of the 30 patients diagnosed with severe malaria, 21 had a GCS score < 11 and seven patients had acute renal failure, defined as serum creatinine > 3 mg/dl.

ADAMTS13 activity

The results for ADAMTS13 activity in uncomplicated and severe malaria as well as normal controls are shown in Figure 1. There was a significant difference in ADAMTS13 activity between the three groups (p < 0.001). ADAMTS13 levels were evidently decreased in patients with severe malaria (mean ADAMTS13 activity: 23.2%, 95% confidence interval [CI] 20.1 to 26.4), compared to both patients with uncomplicated malaria and normal controls (p <0.001). ADAMTS13 activity in patients with uncomplicated malaria (mean 56.0%, 95%CI 46.9 to 65.1) and healthy controls (mean 63.9%, 95%CI 55.4 to 72.5) were not significantly different (p = 0.64). Although ADAMTS13 activity correlated with overall disease severity, which may include features such as coma, renal failure and thrombocytopenia, there was no association between ADAMTS13 and GCS score, serum creatinine, admission platelet count or mortality within the group of severe malaria. In the severe malaria patient group there was a weak, but significant association between ADAMTS13 and haemoglobin concentration (r = 0.31, p = 0.05). Parasite count, however, was not associated with low ADAMTS13 (r = 0.15, p = 0.35), nor did associations between ADAMTS13 and VWF antigen or VWF propeptide reach a level of significance (r = 0.25, p = 0.10 and r = 0.29, p = 0.07, respectively).

VWF antigen and VWF propeptide

VWF antigen and VWF propeptide levels (Figs. 2 and 3) significantly differed between study groups (p < 0.001). There was a stepwise increase in VWF antigen concentrations from healthy controls to uncomplicated malaria to severe malaria. VWF propeptide was higher in malaria patients compared to controls, but did not significantly differ between patients with severe and uncomplicated malaria. Elevated VWF antigen and propeptide levels were associated with low admission platelet count (r = 0.38, p < 0.02 for VWF antigen and r = 0.49, p = 0.002 for VWF propeptide). Associations between VWF antigen and propeptide concentrations and haemoglobin were not significant. VWF multimer analysis was performed in both patients with severe and uncomplicated malaria as well as in healthy controls. No clear difference in VWF multimer pattern could be demonstrated between groups, since ultralarge (UL)-VWF multimers appeared to be present in some, but not all, severe as well as uncomplicated malaria patients, and also in some normal Bangladeshi controls (data not shown).
Free haemoglobin (Hb)

Free Hb was measured in a selection of samples most likely to give a positive result based on the appearance, where a pink discoloration was believed to reflect a high free Hb level. As a control, some plasma samples without discoloration were also included. Free Hb levels were low and did not exceed the reference range of 5.0 – 28.0 μM in any of the 11 samples measured, including eight samples of severe malaria patients (free Hb [mean ± SD] 5.2 ± 1.9 μM) and three of healthy controls (5.2 ± 3.0 μM). Based on these results the decision was made not to measure free Hb in the remaining samples.

Interleukin 6 (IL-6)

IL-6 levels were measured in all subjects included in the study. In healthy controls IL-6 concentrations were below 5 pg/ml (median [IQR]: 0 [0–0.25]) which is within reference range (< 20 pg/ml). In patients however, both severe as well as uncomplicated malaria, IL-6 levels were nearly all elevated (median [IQR]: 53 (34–176) in severe malaria and 61 (16–439) in uncomplicated malaria). Comparison of these groups showed that there was no significant difference in IL-6 values between severe and uncomplicated disease (p-value 0.87).

Discussion

Our results show that plasma ADAMTS13 activity levels are markedly decreased in severe falciparum malaria patients, but not in patients with uncomplicated falciparum malaria, compared to healthy controls. VWF antigen and propeptide levels are increased in both severe and uncomplicated malaria, although most pronounced increases were observed in severe malaria. These results are largely in agreement with two recently published studies, performed by de Mast et al. and Larkin et al. respectively, who also observed an association between malaria and both elevated VWF and decreased ADAMTS13 levels (15, 16).

There is a growing notion that cytoadherence of parasitised red blood cells in falciparum malaria is not a passive process, but causes endothelial activation with a wide variety of secondary events (4). Recent data suggest that exocytosis of WPBs from endothelial cells is part of the endothelial activation in malaria, resulting in the acute release of UL-VWF multimers stored in WPBs. This occurs in the early phase of adult experimental human infection before levels of pro-inflammatory cytokines are high enough to cause clinical disease (5) and has also been described by different groups in adult and paediatric patients with severe malaria (15–18). Both desmopressin infusion and endotoxin-induced systemic inflammation have previously shown to result in a pronounced increase in VWF concentrations together with a reciprocal decrease in ADAMTS13 activity (7, 8). Secondary ADAMTS13 deficiencies have been reported in various diseases, including in-
flammatory states and severe sepsis (9, 10, 19–21) and it has been suggested that the ratio of VWF antigen level and ADAMTS13 activity is more important for the identification of highly prothrombotic states than the VWF levels and VWF multimer analysis alone (21). The presence of reduced ADAMTS13 activity in combination with elevated VWF concentrations as observed in the current study could lead to insufficient cleavage and inactivation of prothrombotic UL-VWF multimers. Although others have recently demonstrated the presence UL-VWF multimers in malaria patients (15, 16), we could not confirm this observation in our study population. However, if we assume that UL-VWF multimers are indeed present in (some of the) severe malaria patients that have low ADAMTS13 activity and high VWF concentrations these UL-VWF multimers could subsequently cause platelet adhesion and aggregation in the microvasculature. Furthermore the relative deficiency of ADAMTS13 could explain the accumulation of platelets in the proximity of the vessel wall of the cerebral microcirculation as observed in paediatric autopsy series (22). This platelet accumulation in turn could play an important role in cytoadherence of parasitized red cells: recently a direct role of platelets serving as a bridge between the infected red cell ligand and the endothelial receptor has been proposed (23).

Taken together, there are several pathways how decreased ADAMTS13 activity could contribute to the organ dysfunction defining severe malaria. Coma is an important presentation of severe malaria and its pathogenesis remains largely unknown. It should be mentioned that in post mortal autopsy series in adults with cerebral malaria a thrombotic microangiopathy is not a prominent finding, but the extent of sequestered red blood cells in the cerebral microcirculation correlates with premorbid coma depth (24). Fragmented red cells in a blood smear are also a rare finding in severe malaria. In contrast, in paediatric cerebral malaria in addition to red cell sequestration, intravascular accumulation of mononuclear cells, platelets and fibrin strands are more commonly observed (25). Larkin et al. recently described the presence of reduced ADAMTS13 levels, increased UL-VWF levels and enhanced VWF functionality in Ghanian children with severe or cerebral malaria falciparum infection (16). In the current study, in which we compared ADAMTS13 activity levels between patients with severe and uncomplicated malaria falciparum, ADAMTS13 was shown to correlate with overall disease severity, which may include coma. Within the group of severe malaria there was no significant association present between reduced ADAMTS13 and lower GCS scores or acute renal failure. Thus, within this group of patients with severe disease low ADAMTS13 activity does not seem to define those with more severe coma or renal failure. However, considering the small sample size sure statements cannot be made.

Another prominent finding in severe and cerebral malaria of which the underlying pathophysiological mechanism is incompletely understood is thrombocytopenia. The average platelet lifespan in severe malaria is decreased from 7–10 days to 2–4 days (26). Splenic pooling, platelet activation, antibody-mediated mechanisms, and platelet adhesion to erythrocytes have all been described as contributing factors (27). Our study suggests that the presence of low ADAMTS13 activity combined with high VWF concentrations, inducing platelet adhesion and aggregation, can be another important mechanism contributing to thrombocytopenia. Low platelet count was not significantly associated with ADAMTS13 activity, although a trend could be observed (r = 0.30, p = 0.07), but was significantly associated with elevated VWF levels. These results are in agreement with the data presented by De Mast et al. (15).

Until now it is poorly understood which factors regulate ADAMTS13 function. In TTP ADAMTS13 deficiency can be caused by mutations in the ADAMTS13 gene (inherited TTP) or by circulating antibodies (acquired TTP). Other factors that have been proposed as possible inhibitors of ADAMTS13 activity include free Hb, inflammatory cytokine IL-6, leukocyte elastase, thrombin, activated coagulation factor X and plasmin (28–30). However, most data pointing to an inhibitory function of these proteins on ADAMTS13 activity are obtained in vitro and for free Hb and IL-6 supra-physiologic concentrations were used. In a recently published study high levels of Hb in sickle cell patients are described to inversely correlate with low proteolytic activity of ADAMTS13 (31). This is suggested to be caused by competitive binding of free Hb to VWF thereby blocking its proteolysis. In the current study we measured free Hb levels in a selection of samples, including eight samples of severe malaria patients and three of healthy controls. Since free Hb levels were low and within reference range in all of the samples measured, it seems highly unlikely that extracellular Hb plays a role in the reduced ADAMTS13 activity levels observed in severe malaria in this study. To rule out a possible inhibitory effect of IL-6 on ADAMTS13 activity, IL-6 concentrations were measured in all study subjects. In healthy controls values were normal, but in nearly all patients, both with severe and uncomplicated disease, IL-6 concentrations were elevated. Two remarks should be made to support our argument that elevated levels of IL-6 cannot explain the low ADAMTS13 activity levels observed in the severe malaria patients. First of all, IL-6 concentrations were elevated in both severe and uncomplicated disease with no difference between the groups, while ADAMTS13 activity was significantly decreased only in severe malaria. Secondly, in the experiments previously pointing to an inhibitory function of IL-6 on ADAMTS13 function supra-physiologic concentrations of IL-6 were used. Compared to the highest IL-6 value measured in our study population the IL-6 concentration used in most of the in-vitro experiments was still 150-fold higher. In agreement with our results Larkin et al. also reported increased levels of IL-6 in children with cerebral or severe malaria falciparum infection, but again these levels did not approach those previously reported necessary to inhibit ADAMTS13 activity (29). The same group also performed plasma mixing studies demonstrating the presence of an unidentified inhibitor of ADAMTS13 function which could possibly explain the observed reduction in ADAMTS13 levels in the malaria patient groups. In contrast to these results, however, no inhibitors or ADAMTS13 auto-antibodies were found in the Plasmodium falciparum and Plasmodium vivax malaria patients studied by De Mast et al. (15). Furthermore the ADAMTS13 gene mutation studies performed by this group were also negative. The role of the remaining factors that have been proposed as possible

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inhibitors of ADAMTS13 function in severe malaria is still unknown. Further research is needed to identify what protein(s) regulate(s) ADAMTS13 function in vivo and in severe malaria.

An interesting observation is that ADAMTS13 activity levels measured in healthy Bangladeshi controls in this study were markedly lower than ADAMTS13 concentrations in a healthy Western population. The mean activity level in the Bangladeshi normal controls was 64% (reference range 34 to 94%) in comparison to a mean activity level of 100% with reference range 54 to 150% in a healthy Dutch population. A difference in ADAMTS13 antigen level between healthy Caucasians and Chinese people has previously been observed by others with a similar difference of approximately 35% in favour of Caucasian individuals (32). Although a true population-based difference in ADAMTS13 activity between people from Bangladesh and the Netherlands should be considered, the limited sample size precludes a definite conclusion and larger studies are needed to address this question.

ADAMTS13 activity was evidently lower in patients with severe malaria compared to patients with uncomplicated malaria and normal controls; however, to identify causal relationships between reduced ADAMTS13 activity level and features complicating severe malaria more research will be required. Low ADAMTS13 activity levels in non-immune individuals may identify a risk group more prone to develop severe malaria once infected, which could be subject to future studies. It would be interesting to investigate whether the administration of exogenous (recombinant) ADAMTS13 in patients with severe malaria will be beneficial for the improvement of vital organ dysfunction in severe malaria.

In conclusion, our results show that plasma activity levels of ADAMTS13 are markedly decreased in patients with severe malaria falciparum, but not in uncomplicated falciparum malaria infection compared to healthy controls. The presence of reduced ADAMTS13 activity in combination with significantly increased VWF concentrations may contribute to the pathophysiological changes underlying thrombocytopenia, and possibly coma and renal failure, which are important complications of severe falciparum malaria. Further research is needed to identify the cause and clinical relevance of this relative ADAMTS13 deficiency in severe malaria which could lead to novel therapeutic strategies.

Acknowledgements

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References


What is known about this topic?

- Mechanical obstruction in the microcirculation, caused by cytotoxicity and sequestration of parasitised erythrocytes, seems to be central in severe falciparum malaria; however, the pathogenesis of many complications, including coma, renal failure and thrombocytopenia, remains incompletely understood.
- Deficiency of ADAMTS13 is crucial in the pathogenesis of thrombotic microangiopathies such as thrombotic thrombocytopenic purpura (TTP) and recently secondary deficiencies have been reported in other disease states including severe sepsis and inflammatory states.
- The disease manifestations of severe malaria are also key features of TTP, and it may well be that ADAMTS13 deficiency is important in the pathogenesis of microvascular failure and organ dysfunction in patients with severe malaria.

What does this paper add?

- Plasma activity levels of ADAMTS13 were markedly decreased in patients with severe malaria falciparum but not in uncomplicated falciparum malaria infection compared to normal controls.
- Von Willebrand factor (VWF) antigen and propeptide concentrations were significantly elevated in both, severe and uncomplicated malaria patients, in comparison to healthy controls.
- The results suggest that the presence of low ADAMTS13 activity in combination with high VWF concentrations may contribute to the pathophysiological changes underlying important complications of severe falciparum malaria, including thrombocytopenia, coma and renal failure.