Factor VIII expression and regulation in health and disease

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Chapter 5

Von Willebrand factor propeptide in severe malaria: evidence of acute endothelial cell activation

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ABSTRACT

The pathogenicity of Plasmodium falciparum is thought to relate to the unique ability of infected erythrocytes to adhere to and subsequently activate the vascular endothelium. To study the state of endothelial activation during falciparum malaria, we measured plasma levels of both von Willebrand factor (VWF) and its propeptide, indices respectively of chronic and acute endothelial cell perturbation. Results were correlated with clinical and biochemical markers of disease severity, including plasma lactate. Our data show that acute endothelial cell activation is a hallmark of malaria in children, indicated by a significant rise in VWF and the VWF propeptide. The highest VWF and propeptide levels were seen in cerebral and severe malaria cases, and associations found between VWF propeptide level and lactate (P < 0.001). Mean VWF propeptide levels (nmol/L) were in cerebral malaria 33.4, severe malaria 26.3, mild malaria 22.1, non malaria febrile illness 10.2, and controls 10.1. Differences between patient and control groups were highly significant (P < 0.005). Follow up samples in 26 cerebral malaria cases showed levels of VWF propeptide, but not of VWF fell by 24 hours, following the clinical course of disease and recovery. These novel findings potentially implicate acute, regulated exocytosis of endothelial cell Weibel-Palade bodies in the pathogenesis of Plasmodium falciparum malaria.

INTRODUCTION

Severe Plasmodium falciparum malaria is a major cause of death in young children in sub-Saharan Africa. Severity is worse in children under the age of five years in holoendemic areas, prior to development of partial immunity. Although the underlying mechanisms leading to severe P. falciparum malaria are still not completely understood, it is unlikely this will be due to a single pathophysiological process. However adhesion of infected erythrocytes to host vascular endothelium appears important, and is associated with severity, particularly when this occurs in the brain, as in cerebral malaria (1). Cerebral malaria is a distinct clinical syndrome, characterised by a diffuse encephalopathy, classically with seizures and loss of consciousness, which if treated early is usually reversible within 2-3 days of onset.

Sequestration of parasitized red blood cells in the microvasculature is mediated by various endothelial cell adhesion molecules, such as intercellular cell adhesion molecule-1 (ICAM-1) (2), CD36 (3), thrombospondin (4), platelet/endothelial cell adhesion molecule (PECAM) (5), P-selectin (6) and E-selectin (7). In addition to P. falciparum-infected erythrocytes, sequestered cells such as leukocytes and platelets have been described in patients with cerebral malaria (8-12). Most of the endothelial surface receptors are inducible, including, ICAM-1, VCAM-1 and E-selectin. In P. falciparum infected patients the expression of these receptors at post mortem is increased in several different tissues, including the brain, and the distribution of sequestered parasitized erythrocytes co-localizes with these receptors (12). It therefore seems likely that endothelial cell activation
plays an important role in the pathogenesis. Consistent with this view is the observation that increased levels of soluble forms of these receptors are present in patients infected with *P. falciparum* (13). Endothelial activation, up-regulation of cell adhesion receptors, and increased adheriveness for leukocytes and platelets may result from the systemic release of pro-inflammatory cytokines induced by the parasite, which are known to activate endothelial cells (14-16). For instance, levels of the cytokine tumour necrosis factor (TNF-α) are increased in *falciparum* malaria and correlate with the severity of the disease (15,16). In addition plasma interferon γ, interleukin (IL)-1β, IL-6 and IL-8 are elevated in severe malaria (1).

Despite numerous data suggesting that activation of endothelial cells plays an important role in the pathogenesis of cerebral malaria, little is known about the molecular events associated with endothelial cell activation and subsequent expression of cell adhesion receptors. One of the critical mechanisms determining the development, nature and site of resulting lesions is the state of endothelial cell activation. Endothelial cells can undergo different patterns of activation, each associated with increased adheriveness for leukocytes. For instance, the expression of ICAM-1, E-selectin or VCAM-1, results from *de novo* synthesis induced by cytokines such as TNF-α and IL-1. Following stimulation of endothelial cells with these agonists there is an increase in adheriveness for leukocytes over 4-24 hours. On the other hand, P-selectin differs from other endothelial adhesion molecules in that it is stored in endothelial cell-specific storage vesicles, so-called Weibel-Palade bodies. P-selectin, and other Weibel-Palade body contents, are translocated to the cell surface within minutes of endothelial cell activation by exposure of endothelial cells to agonists, such as thrombin, histamine or endotoxemia. This response is independent of protein synthesis. In addition, P-selectin can be transcriptionally upregulated upon stimulation by cytokines and subsequently expressed at the cell surface by exocytosis of Weibel-Palade bodies (17). Clearly, endothelial cells may undergo different activation stages, and the kinetics and diversity of the expression of adhesion receptors is highly dependent upon both type and quantity of endothelial cell agonists. In addition the expression of certain receptors will vary within and between organs, for example CD36 is poorly expressed in cerebral endothelial cells, yet appears to be an important receptor for the adhesion and sequestration of infected erythrocytes.

The aim of this study was to assess the state of excitation of endothelial cell activation in *falciparum* malaria directly in peripheral venous blood samples. To measure the degree of vascular involvement in this disorder we measured the concentrations of both plasma von Willebrand factor (VWF) and VWF-propeptide at various stages of the disease. Both VWF and its propeptide are stored in Weibel-Palade bodies in equimolar concentrations and, like P-selectin, are rapidly released upon endothelial cell activation. Previously we and others have shown that because of its rapid turnover, the propeptide level returns to its baseline value much faster after termination of the vascular challenge than the VWF concentration (18-20). This knowledge has previously permitted discrimination between chronic and acute endothelial activation in patients with documented acute and chronic vascular disease, such as thrombotic thrombocytopenic
purpura (TTP) and diabetes mellitus respectively (20). We reasoned that this approach could also be of help to more clearly document the extent of endothelial cell activation in *P. falciparum* malaria during acute episodes and treatment of the disease. We carried out a prospective study of young Ghanaian patients with cerebral, severe and mild malaria and of uninfected controls. This data was correlated with clinical and laboratory markers of disease severity.

**PATIENTS AND METHODS**

*Patients*

This study was carried out at Komfo Anokye Teaching Hospital in Kumasi, Ghana. Both outpatients and inpatients in this hospital were included in the study. Children between the ages 6 months – 6 years were recruited for both the control and index cases. The ethical committee of Liverpool School of Tropical Medicine and Komfo Anokye Teaching Hospital, Kumasi, Ghana, approved this proposal. Consent was obtained from immediate relatives before sample collection. Clinical details were taken for all cases at the time of blood collection, and all subsequent analysis were carried out blind to these details. All patients received standard anti-malarial treatment. For children with cerebral malaria (*n* = 26), follow-up samples were also collected at 24 and 72 hours following admission. Three malaria groups, all with *P. falciparum* parasites seen on thick blood film, were defined:

**Cerebral malaria:** Blantyre coma score of 2 or less in a child without any other cause of coma (e.g. hypoglycaemia, or meningitis (26 patients)).

**Severe malaria:** Children admitted with *P. falciparum* and one or more complications of severe malaria as defined by standard WHO criteria (ref: 21) (73 patients).

**Mild malaria:** Febrile illness not requiring admission with positive blood film, without other explanation for the fever, and with none of the criteria for severe malaria, (44 patients).

As controls we included two groups:

**Non-malaria febrile illness:** Children not requiring admission, presenting with febrile illness and having no malaria parasites seen on blood film (74 patients).

**Non febrile, well children:** These were recruited from children attending for immunisation (25), admitted for elective surgery (6) or well outpatient surgical review cases (5).

*Methods*

For the measurement of plasma concentrations of VWF and VWF-propeptide, 1.2 ml of venous blood samples were collected (on admission, in clinic, or for surgical controls, on cannulation in theatre) into 3.2% citrate (1:9 vol/vol), and immediately placed on ice. After centrifugation at 3.000 g for 20 min at 4°C, plasma was aliquoted and stored at –80°C until assessment. Samples were transported on dry ice to Amsterdam and Liverpool for analysis. Propeptide and mature VWF concentrations were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (18). Normal plasma from a pool of
40 adult Caucasian donors served as standard. The plasma pool contained 6.3 nmol/L VWF propeptide and 50 nmol/L of VWF. Plasma lactate (a marker of disease severity) was measured by SYSMEX KX-21N analyser (YSI Inc, Ohio). A latex-enhanced immunoturbidimetric assay was used to determine levels of C-reactive protein (CRP). EDTA blood was used for preparing a thick and thin Giemsa-stained blood film for the analysis for the number of parasites, for measuring haemoglobin and platelet count.

**Statistical analysis**

Data are presented as the mean ± SD, apart for parasite count which are presented as geometric mean and 95% confidence interval. The means in plasma or serum levels of various proteins were compared by Student’s t-test. Comparison of groups was by ANOVA. The Pearson correlation coefficient was used as a measure of linear association between two variables.

**Table 1. Clinical and laboratory findings on admission in various subsets of children with *Plasmodium falciparum* malaria at baseline.**

<table>
<thead>
<tr>
<th></th>
<th>Cerebral malaria</th>
<th>Severe malaria</th>
<th>Mild malaria</th>
<th>Non-malaria febrile illness</th>
<th>Non-febrile controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (months) *</td>
<td>35.2 ± 22.1</td>
<td>23.1 ± 15.1</td>
<td>37.3 ± 18.5</td>
<td>24.8 ± 16.6</td>
<td>25.8 ± 22.5</td>
</tr>
<tr>
<td>Parasitemia (x10^9/L) #</td>
<td>169.337</td>
<td>58.458</td>
<td>43.382</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(49,631- 577,763)</td>
<td>(32,501- 105,145)</td>
<td>(19,801- 95,043)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dL) *</td>
<td>7.0 ± 1.7</td>
<td>6.5 ± 2.3</td>
<td>9.4 ± 1.6†</td>
<td>10.5 ± 1.3†</td>
<td>-</td>
</tr>
<tr>
<td>Platelets (x10^9/L) *</td>
<td>77.5 ± 54.8</td>
<td>101.7 ± 72.1</td>
<td>157.7 ± 73.7†</td>
<td>332.0 ± 128.5†</td>
<td>-</td>
</tr>
<tr>
<td>Lactate (mmol/L) *</td>
<td>5.2 ± 4.4</td>
<td>3.9 ± 3.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CRP (mg/L) *</td>
<td>142.5 ± 120.5</td>
<td>64.8 ± 97.8</td>
<td>102.9 ± 80.5§</td>
<td>30.7 ± 48.7</td>
<td>7.7 ± 11.0</td>
</tr>
</tbody>
</table>

Results are as * mean ± SD, # geometric mean (95% CI); †) n = 9, ‡) n = 24, §) n = 41, n.d., not detectable

**RESULTS**

The clinical and laboratory findings of the different groups of patients with *falciparum* malaria, with non-malarial febrile illness, and controls are summarized in table 1. All patients admitted with cerebral malaria had a Blantyre coma score of 2 or lower (21), while only 3 of the patients admitted with severe malaria had a coma score lower than 3 (all improved rapidly on giving glucose). Differences in geometric mean parasitaemia between groups were not significant (P = 0.1). Platelet count differed significantly between the non-malarial febrile group and all malaria groups (P < 0.001). Platelet counts were significantly lower in patients with cerebral malaria and severe malaria than in mild malaria (P = 0.002
and 0.03 respectively). The haemoglobin level was also significantly lower in the patients with cerebral malaria and severe malaria compared to mild malaria and non malaria groups ($P < 0.001$). Two patients died, one with cerebral malaria and one with severe malaria; all other patients recovered to be discharged home.

Table 2. VWF and propeptide levels in patients with *Plasmodium falciparum* malaria.

<table>
<thead>
<tr>
<th></th>
<th>Cerebral malaria</th>
<th>Severe malaria</th>
<th>Mild malaria</th>
<th>Non-malaria febrile illness</th>
<th>Non-febrile controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n = 26$</td>
<td>$n = 73$</td>
<td>$n = 44$</td>
<td>$n = 74$</td>
<td>$n = 36$</td>
</tr>
<tr>
<td>VWF (nmol/L)</td>
<td>193.3 ± 73.0</td>
<td>171.2 ± 44.7</td>
<td>168.5 ± 79.0</td>
<td>79.8 ± 40.8</td>
<td>68.7 ± 42.5</td>
</tr>
<tr>
<td>Propeptide (nmol/L)</td>
<td>33.4 ± 16.8</td>
<td>26.3 ± 11.6</td>
<td>22.1 ± 13.3</td>
<td>10.2 ± 5.8</td>
<td>10.1 ± 7.5</td>
</tr>
</tbody>
</table>

Results are the mean ± SD

Table 2 summarises the results of the VWF and propeptide assays. Plasma concentrations of VWF and propeptide of the well control group were 68.7 ± 42.5 and 10.1 ± 7.5 nmol/L respectively, similar to normal values for Caucasians (18,19), and allowed us to define Ghanaian children’s local normal ranges for analysis. In the children with non-malarial febrile illness the mean VWF and propeptide levels were 79.8 ± 40.8 and 10.2 ± 5.8 nmol/L respectively, values that are also within the normal range of healthy Caucasian subjects. Mean concentration of VWF at the time of admission was significantly elevated ($P < 0.005$) in all three malaria groups when compared with controls. These values were 2 to 3 fold higher than plasma levels of febrile patients without *P. falciparum* infection. Similarly, the mean propeptide level in the different subgroups of malaria patients was significantly elevated (2 to 4 fold increase, $P < 0.005$). Propeptide levels were significantly higher in patients with cerebral malaria than in severe malaria ($P < 0.05$), and in cerebral malaria compared with mild malaria ($P < 0.005$). The difference in mean VWF level between cerebral and severe malaria and severe and mild malaria was not significant ($P = 0.07$ and 0.8 respectively). Also the difference in mean propeptide level between severe and mild malaria was not significant ($P = 0.07$). The individual VWF and propeptide data for each of the patients and the control groups are shown in Fig.1.

Plasma concentration of propeptide and VWF at the time of admission were related to lactate as a marker of malaria severity in the 99 cerebral and severe malaria patients combined. In children with a lactic acidosis (23 out of 99 patients, lactate > 5 mmol/L) propeptide levels were significantly higher (mean 34.6 ± 11.6 nmol/L; $P = 0.008$) than in children with normal lactate levels (< 5 mmol/L; mean 26.2 ± 13.5 nmol/L). Similarly, lactate and propeptide levels were correlated ($R = 0.35$, $P < 0.001$) whereas VWF levels and lactate were not ($P = 0.3$). These findings suggest an association between the plasma level of propeptide and the illness severity when assessed by this biochemical marker. Also mean lactate levels were higher in cerebral malaria (5.2 mmol/L) compared to severe malaria (3.9 mmol/L), but this did not reach significance ($P = 0.09$). However malaria
parasite count (another marker of disease severity) was not closely associated with VWF propeptide level between clinical groups \( P = 0.8 \).

Figure 1. Plasma concentration of VWF and VWF propeptide in various subsets of children with *Plasmodium falciparum* malaria. CM, cerebral malaria; SM, severe malaria; MM, mild malaria; NMFI, non-malaria febrile illness; NFC, non-febrile controls. Dotted lines indicate normal levels in adult Caucasians (18-20). *) \( P < 0.05 \); **) \( P < 0.005 \). Bars, mean values.

As VWF is an acute phase reactant we sought associations to check that we were not purely observing this in our VWF results. CRP, a sensitive marker of inflammatory responses and tissue damage did not correlate with concentrations of VWF \( (P = 0.4, R = 0.18) \). Also propeptide concentrations appeared not to be associated with CRP concentration \( (P = 0.3, R = 0.08) \). The 26 patients with cerebral malaria were also studied prospectively. We related propeptide and VWF levels at the time of admission to plasma levels of these proteins respectively 24 h and 3 days after receiving treatment. Following therapy propeptide levels on day 1 were significantly lower than baseline levels \( (21.6 \text{ and } 31.6 \text{ nmol/L respectively, } P < 0.003) \) and in almost 50 % of the patients had reached normal levels by day 3 \( \text{mean level } 12.6 \text{ nmol/L, } P < 0.003 \) \( \text{(Fig.2)} \). On the other hand, mean VWF level on day 1 after treatment slightly decreased from 193.1 to 169.4 nmol/L. The difference was not significant \( (P = 0.06) \). Mean VWF concentration in samples collected at 3 days \( (130.8 \text{ nmol/L}) \) was significantly lower than those collected at
admission or 1 day thereafter ($P < 0.003$). In the majority of cases VWF levels remained elevated during the 3 day observation period (Fig.2).

![Figure 2](image_url)

**Figure 2.** Plasma concentration of VWF and propeptide in patients with cerebral *Plasmodium falciparum* malaria during treatment. Dotted lines indicate local Ghanaian normal mean levels (non-febrile control group). *) $P < 0.003$. Bars, mean values.

**DISCUSSION**

We selected 5 groups of patients to allow comparison of VWF and VWF propeptide responses between mild and severe malaria. Furthermore severe malaria was compared with a subgroup, cerebral malaria. Cerebral malaria has a short 1-3 day presentation, which is more likely due to a common pathophysiology. As normal values for VWF and VWF propeptide in young Ghanaian children had not been defined, we determined these, and found them to be similar to those for Caucasians (Table 2, refs.18-20). In addition we wanted to determine whether elevated levels in malaria were specific to this disease, and therefore potentially relevant to pathogenesis. We therefore needed to also determine levels in non-malarial febrile illness. Our results indicate that in malaria there is an acute and specific endothelial perturbation, with markedly elevated VWF propeptide levels, associated with severity of disease, which fall coincident with recovery in cerebral malaria.

The concept that endothelial cells may undergo specific patterns of activation upon exposure to changes of their environment has been generally accepted, but the significance of specific endothelial cell perturbation in disease pathology, in particular...
malaria, has, to our knowledge, not been evaluated before. The primary aim of the present study was to answer the question whether *P. falciparum* infection is associated with exocytosis of Weibel-Palade bodies and regulated secretion of previously stored molecules, and whether the degree of this mode of endothelial cell activation can be assessed in peripheral venous samples *in vivo* directly. We have shown this to occur as circulating concentrations of both VWF and its propeptide, typical residents of Weibel-Palade bodies, are elevated in patients with *P.falciparum* infection. Raised VWF and propeptide is most notable in the patients with cerebral and severe malaria, but it is also seen in mild cases not apparently requiring admission when assessed. This finding is consistent with fulminant, acute endothelial cell activation (19,20) occurring in *P. falciparum* malaria. The observation that recovery was associated with a significant decrease of propeptide levels towards control normal values for cases with cerebral malaria (Fig.2) is also in agreement with this conclusion. Thus, a new aspect of this study is that, in terms of regulated secretion and subsequent translocation of Weibel-Palade body contents, the presence, nature and degree of endothelial cell activation in *P. falciparum* is directly assessed.

Propeptide and VWF levels were unusually high in the majority of patients with mild, severe and cerebral malaria compared with patients who had fever without malaria or control subjects (Fig.1). In some patients propeptide and VWF levels reached peak levels (more than 5-fold elevated) that were even higher than the levels published for documented cases of fulminant vascular disease, such as TTP and septicemia (20). This observation suggests that measurement of both propeptide and VWF are useful in monitoring the degree of vascular involvement in *falciparum* malaria. As propeptide levels more rapidly decline after successful treatment than VWF (Fig.2), (probably because of differences in clearance rate), this suggests that propeptide levels are a more reliable marker of endothelial activation and Weibel-Palade exocytosis in these patients than VWF. Pertinent to this point is the observation that propeptide and lactate levels, a marker of malaria disease severity, were correlated whereas VWF levels and lactate were not. Plasma or serum concentrations of soluble (s) forms of adhesion receptors expressed on endothelial cells, including sICAM-1, sE-selectin and sVCAM-1, which are also raised in *P. falciparum* malaria, are probably less specific in this respect. The release of these molecular species, *e.g.* by shedding from the endothelial cell surface or alternative mRNA splicing, are secondary events and may be considered as a surrogate index of endothelial cell perturbation. Indeed, reports on the assessment of soluble adhesion molecules as a quantitative parameter of disease severity are controversial (13,22-24).

We should consider whether elevated propeptide and VWF levels could have been derived, at least in part, from consumed or activated platelets, a common feature in *falciparum* infections (1) (*cf. Table 1*). However, the amount of propeptide and VWF stored in the α-granules of platelets is not sufficient to account for the increased levels (18-20,25). Rather vascular endothelial cells are the major source of circulating propeptide and VWF in malaria patients examined in this study. This view is supported by our observation that propeptide (and VWF) levels and platelet number do not correlate.
Despite highly statistically significant correlations, it is important to note that each patient group studied was associated with a wide distribution of observed propeptide and VWF levels. These distributions overlapped considerably (Fig.1). This overlap may partly reflect differences in time of infection to the onset of symptoms, and presentation to hospital with malaria, and that severe malaria is unlikely to be homogeneous, with a single underlying pathophysiology. One of the key aspects of this study is that elevated levels of VWF and propeptide in severe and mild malaria were not proportional to parasitaemia, reflecting the heterogeneity of this disease. For example, severe malarial anaemia may have a chronic, haemolytic course, compared to cerebral malaria, with an acute fulminant presentation, where peripheral parasitaemia may or may not be marked due to sequestration in the tissues. In addition, as massive endothelial cell activation seems to be occurring in severe and cerebral malaria, exhaustion of storage pools of VWF and propeptide could potentially confound relationships between severity of the disease and plasma concentrations of these proteins.

As to the agonists responsible for inducing regulated secretion we can only speculate. In cultured endothelial cells, exocytosis of Weibel-Palade bodies can be achieved by a number of naturally occurring agonists, including thrombin, histamine or leukotrienesm (26). These agonists probably don’t play a significant role in falciparum malaria (1). There is compelling evidence that cytokines known to be increased in P. falciparum malaria, including TNF-α, IL-1β, IL-6. IL-8 and TNF-α, are able to activate endothelial cells at the transcriptional level and upregulate synthesis and subsequent expression of adhesive surface receptors. However, there is less evidence to support a role for cytokines in enhancing secretion through the regulated pathway, independent of de novo protein synthesis, although a recent study demonstrated that TNF-α could be effective in this respect (27). It is also possible that adhesion of infected erythrocytes to the vascular endothelium itself elicits regulated secretion. We also considered whether elevated propeptide and VWF levels were a reflection of non-specific responses to P. falciparum infections. As the plasma concentrations of these proteins did not correlate with CRP levels, an acute-phase reactant and typical non-specific marker of inflammation and tissue damage (28), this possibility seems less likely.

A corollary of our findings is that it is to be expected that besides VWF and its propeptide other Weibel-Palade body contents, including IL-8, eotaxin-3 and P-selectin are at the same time delivered to the surface of the perturbed endothelial cell (29). Therefore, rapid, stimulus-induced accumulation of these chemoattractants and leukocyte adhesion receptor could play a significant role at an early stage in a “cascade” leading to focal adhesion. Pertinent to this point is the observation that P-selectin translocated to the cell surface in response to agonists not only promotes rapid leukocyte adhesion to the endothelial cell surface but may also anchor newly released hyperactive VWF multimers (30). This in turn may allow recruitment of CD36, expressed by adherent platelets, as seen in patients with P. falciparum infections (11). This may be important as CD36 is poorly expressed on the cerebral endothelial cell, and paediatric isolates of P. falciparum appear to have a phenotype which binds predominantly to CD36 (31). Release of VWF, and the
subsequent recruitment of platelets to the endothelial wall may therefore be a component in the pathophysiology of infected erythrocyte adhesion and sequestration in *falciparum* malaria (32).

In conclusion, we have demonstrated elevated levels of plasma VWF and VWF propeptide in malaria, which suggest significant Weibel-Palade exocytosis is occurring. The degree of endothelial cell perturbation is associated with disease severity as measured both by plasma lactate and clinical disease. This specific response in *falciparum* malaria is indicative of massive endothelial activation. VWF propeptide may therefore be a useful marker of endothelial activation in *in vivo* studies, and suggests components of the Weibel-Palade body are important in the pathogenesis of malaria. Further *in vitro* studies will be required to determine whether VWF or VWF propeptide have specific roles in the pathophysiology of severe malarial disease.

**ACKNOWLEDGEMENTS**

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