Seizures in children with acute falciparum malaria: risk factors, mechanisms of neuronal damage and neuro-protection
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Axonal and astrocyte injury markers in the cerebrospinal fluid of Kenyan children with severe malaria

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ABSTRACT

A retrospective study of cerebrospinal fluid (CSF) levels of markers of brain parenchymal damage was conducted in Kenyan children with severe falciparum malaria. Two markers were analysed by immunoassays: the microtubule-associated protein tau for degenerated axons; and S-100B for astrocytes. The level of tau proteins in the CSF was significantly elevated in children with cerebral malaria compared with either malaria with prostration or malaria with seizures but normal consciousness (p<0.001). Elevated tau was also found to be associated with impaired delivery of oxygen (severe anaemia), severe metabolic acidosis manifesting as respiratory distress (increased respiratory rate and deep acidotic breathing) and at higher parasite densities. Elevated S-100B in children was associated with an increased risk of repeated seizures. This study provides evidence that axonal injury is associated with malaria coma and identifies the potential role of severe anaemia, acidosis and hyperparasitaemia to causing brain parenchymal damage in children with malaria.
INTRODUCTION

Falciparum malaria is arguably one of the most common causes of non-traumatic coma in children in the world. It kills over a million people each year, most of whom are children living in sub-Saharan Africa[1]. Cerebral malaria is the most severe neurological complication of falciparum malaria, manifesting as coma with frequent seizures[2]. It causes death in 18.6% of children, and 11% of the survivors have residual neurological deficits on discharge[3]. Furthermore up to 24% of children have neurological and cognitive impairments when carefully assessed more than 3 years after discharge[4]. Despite these findings, the pathophysiology of cerebral malaria, in particular the pathogenesis of neurocognitive sequelae is incompletely understood.

Biomarkers in cerebrospinal fluid (CSF) have been used to help delineate pathophysiological mechanisms, predict and monitor neurological outcomes and develop and evaluate new therapeutic strategies in human neurological disease (reviewed in [5]). Measurement of CSF biomarkers is a particularly useful tool for studies of pathological processes in malaria endemic countries, where post mortem brain tissue samples are rarely available and neuroimaging and neurophysiology facilities are limited. In this study we have examined the levels of two markers of brain damage, the tau protein (tau) and S-100B, in the CSF of Kenyan children with malaria.

Tau is a phosphorylated microtubule-associated protein, considered to be important for maintaining the stability of axonal microtubules involved in the mediation of fast axonal transport or synaptic constituents. S-100B is an acidic calcium-binding protein synthesised in astrocytes in all parts of the central nervous system. Increased CSF levels of these proteins have been observed in the CSF of patients with neurological symptoms and often reflect neurological outcome, disability and death[6, 7]. In a recent study[8], the levels of tau and S-100B were examined in the CSF of adult Vietnamese patients with severe malaria. Tau levels were found to be associated with duration of coma and S-100B was associated with seizures. Concentrations of these markers were also associated with vital organ dysfunction. In multivariate analysis, the correlations with coma and seizures were independent of vital organ dysfunction suggesting multiple mechanisms of CNS impairment in severe malaria. To gain further insight into pathogenesis of cerebral complications of malaria infection we studied a different population, African children, in whom the clinical and pathological presentation of severe and complicated malaria differs significantly with South East Asian adults (reviewed in [2, 9]).

We have examined CSF levels of tau and S-100B on admission in children from Kenya with differing levels of neurological severity of malaria. We have investigated the
systemic metabolic effects of malarial disease on the levels of tau and S-100B in CSF and compared the findings with those previously reported in the CSF of adult Vietnamese patients with severe malaria.

**MATERIALS AND METHODS**

**Case selection and sample collection**

A random sample of stored CSF from children admitted to Kilifi District Hospital located on coastal Kenya, between 1999 and 2004 was used to measure levels of tau and S-100B. Lumbar puncture was performed when clinically indicated as part of the investigation of children admitted with impaired consciousness prostration and/or seizures, to exclude infectious causes in particular bacterial meningitis[10].

The CSF was collected in plastic tubes and stored –20°C within 1 hour of being obtained and then frozen at –80°C and later transported to the UK on dry ice. All samples were obtained with permission from patients or relatives, and the National Ethical Research Committee of the Kenya Medical Research Institute approved sample collection protocols. CSF samples were used with ethical approval from the Oxford Tropical Research Ethics Committee (OXTREC).

A total of 143 CSF samples from children were studied. Patients were classified into 3 main groups according to the level of consciousness at admission and further subdivided on the basis of whether they had malaria or not:

a) **Coma** (Blantyre coma score [BCS] ≤2) [11]
   i. *Cerebral malaria* (CM): 38 children had a clinical diagnosis of CM (unable to localise a painful stimulus or BCS ≤2 on admission, asexual forms of *P. falciparum* parasitaemia, no improvement after treatment for hypoglycaemia and no evidence of meningitis in the CSF sample – CSF WBC<10/μl [12]
   ii. *Non-cerebral malaria coma*: - 7 children with coma due to other causes (no *P. falciparum* parasitaemia)

b) **Prostration** (inability to sit upright or breast feed) [12] but not comatose
   i. *Malaria with prostration* - this group was made up of 25 children with *P. falciparum* parasitaemia admitted either with mild impairment of consciousness (BCS 3 and 4 - able to localise pain) or with full consciousness (BCS 5) but unable to sit upright or breast feed.
ii. Non-malaria with prostration – This group included 7 children without P. falciparum parasitaemia and admitted with either mild impairment of consciousness or with full consciousness but unable to sit upright or breast feed.

c) Fully alert patients
   i. Children with malaria and seizures but no coma or prostration – 54 patients
   ii. Children with febrile seizures but no malaria, coma or prostration – 12 patients

Measurement of tau and S-100B using a solid phase sandwich ELISA
Both markers are stable in CSF and do not degrade readily at room temperature or following multiple rounds of freeze-thaw [13, 14]. Total human tau (both phosphorylated and nonphosphorylated) and S-100B were measured using commercially available ELISA kits (BioSource International, Nivelles, Belgium and DAKO, Ely, UK, respectively) using a solid phase sandwich ELISA according to the manufacturer’s instructions. The reported intra-assay precision for tau is 2.7-5.2% and the inter-assay precision is 4.3-7.8%. The lower limit of sensitivity is 12pg/mL. There is no cross reactivity with human β-amyloid 1-42, α-synuclein, or β-synuclein. The reported intra-assay precision for S-100B is 1.3-2.5% and the inter-assay precision is 1.5-2.2%. The lower limit of sensitivity is ≤10ng/L.

Clinicopathological correlations
To investigate the significance of the CSF levels of markers of brain parenchymal damage in severe malaria, we performed a detailed clinicopathological correlation with a number of clinical and biochemical parameters. These included a history, number and duration of seizures, markers of metabolic acidosis, the level of consciousness, haemoglobin, blood glucose, the parasite density and CSF proteins.

STATISTICAL ANALYSES
Statistical analysis was carried out using Stata 9 (StataCorp, College Station, TX) programme. Normally distributed continuous variables were compared between groups using unpaired Student’s t-test; data that were not normally distributed were compared using the Kruskall-Wallis test. Correlations between continuous variables were determined nonparametrically using Spearman’s rho. No adjustments for multiple comparisons were made, though for the purposes of interpretation and discussion p < 0.01 was regarded as significant.
RESULTS

Of the 143 children, 72 were female and 71 male. The ages ranged from 1 - 98 months (median [IQR] 21 [12-36] months). One hundred and seventeen children had malaria and the remaining 26 had other diagnosis. These included 12 children with febrile seizures mainly associated with respiratory tract infections, 9 with suspected viral meningoencephalitis, 2 with seizures and developmental delay, and a child each with septicaemia, pyogenic meningitis and epilepsy.

**Tau and S100B levels in the CSF of Kenyan children admitted to hospital**

Cerebrospinal fluid Tau levels were available for 138 patients of who 54 (39.1%) had high (>300 pg/ml) levels while S100B levels were available for 142 patients 7 of who had high (>1ng/ml) levels. There was no significant difference between the median CSF levels of tau or S100B in the malaria group compared with the non-malaria group (p=0.87 and p=0.83 respectively). Within the malaria sub-groups, median tau levels were significantly higher in children with cerebral malaria (coma) compared with either malaria with prostration or malaria with seizures but normal consciousness, p<0.001 (Kruskall Wallis test, figure 1). A similar trend was observed with S100B but the differences were not statistically significant (p=0.089) (table I). Among children with non-malarial illnesses, the median CSF levels of tau did not vary with the level of consciousness (table I).

<table>
<thead>
<tr>
<th>Clinicopathological correlations and markers of cerebral damage</th>
<th>Coma</th>
<th>Prostration</th>
<th>Normal consciousness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria N=38 Non malaria n=7</td>
<td>Malaria n=25 Non malaria n=7</td>
<td>Malaria n=54 Non malaria n=12</td>
<td></td>
</tr>
<tr>
<td>Median (IQR) duration of illness, days</td>
<td>2 (1-3)</td>
<td>5 (3-7)</td>
<td>3 (2-4.5)</td>
</tr>
<tr>
<td>History of seizures, (%)</td>
<td>36 (94.7)</td>
<td>6 (85.7)</td>
<td>19 (76.0)</td>
</tr>
<tr>
<td>Median (IQR) number of seizures</td>
<td>2 (1-3)</td>
<td>1 (0-2)</td>
<td>1 (0-3)</td>
</tr>
<tr>
<td>Deep breathing, (%)</td>
<td>15 (39.5)</td>
<td>3 (42.9)</td>
<td>8 (32.0)</td>
</tr>
<tr>
<td>Severe anaemia, (%)*</td>
<td>4 (10.8)</td>
<td>2 (28.6)</td>
<td>7 (28.0)</td>
</tr>
<tr>
<td>Hypoglycaemia, (%)</td>
<td>6 (15.8)</td>
<td>1 (14.3)</td>
<td>1 (4.0)</td>
</tr>
<tr>
<td>Median (IQR) CSF Tau levels, pg/ml‡</td>
<td>346.8</td>
<td>218.9</td>
<td>266.1</td>
</tr>
<tr>
<td>(204.5 - 793.9)</td>
<td>(7.4 - 420.3)</td>
<td>(159.6 – 494.8)</td>
<td>(132.3-1159.3)</td>
</tr>
<tr>
<td>Median (IQR) CSF S100 levels, pg/ml†</td>
<td>344.0</td>
<td>310.7</td>
<td>245.8</td>
</tr>
<tr>
<td>(184.1-480.8)</td>
<td>(266.8-1654.9)</td>
<td>(174.0-361.7)</td>
<td>(69.0-848.3)</td>
</tr>
</tbody>
</table>

*n=140; ‡=138; †=142
Relationship between tau and S100 with neurological signs and other complications of falciparum malaria

Cerebrospinal fluid levels of tau and S100B were categorized as normal or high and compared with complications of falciparum malaria that have been associated with poor outcomes. Tau was found to correlate with several complications of malarial infection (see Table II). In contrast S100B did not correlate with any of these markers.

Seizures

Overall 116 (81%) patients were admitted with a history of seizures. The median (IQR) CSF levels of S100B was not different in patients who presented with and without seizures, (p=0.879). However, patients with high levels of S100B were more likely to have recurrence of seizures during the course of the admission, (5/7 [71.4%] vs 32/135 [23.7%], p=0.005). A similar but weak association was observed with high tau levels; (19/54 (35.2%) patients with high tau levels had seizure recurrence compared to 18/84 [21.4%], p=0.075). The median tau and S100B levels did not correlate with the number of seizures.

Severe anaemia

The overall mean (SD) haemoglobin level was 6.7 (2.3) g/dl in patients with high tau and 8.1 (1.8) g/dl in those with normal levels, p<0.001. Thirteen children with malaria and 3 children with non-malarial illnesses had life threatening severe anaemia (haemoglobin levels <5g/dL). There was a strong correlation between levels of tau and severe anaemia:

Table II Levels of tau and features of disease severity

<table>
<thead>
<tr>
<th>Makers of disease severity</th>
<th>Malaria</th>
<th></th>
<th>P value</th>
<th>Non – malaria</th>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (SD) in months</td>
<td>High Tau N=42</td>
<td>Normal tau N=71</td>
<td>0.147</td>
<td>High tau N=12</td>
<td>Normal tau N=13</td>
<td>0.272</td>
</tr>
<tr>
<td>Median (IQR) number of seizures</td>
<td>21.8 (18.8)</td>
<td>26.8 (22.8)</td>
<td></td>
<td>20.9 (15.7)</td>
<td>29.0 (19.7)</td>
<td></td>
</tr>
<tr>
<td>Multiple seizures (&gt;2 seizures, %)</td>
<td>1 (0-2)</td>
<td>2(1-3)</td>
<td>0.180</td>
<td>1 (0-2)</td>
<td>1 (0-2)</td>
<td>0.870</td>
</tr>
<tr>
<td>Mean (SD) temperature, °C</td>
<td>37.8 (1.1)</td>
<td>38.2 (1.5)</td>
<td>0.104</td>
<td>37.4 (1.2)</td>
<td>37.9 (1.4)</td>
<td>0.416</td>
</tr>
<tr>
<td>Mean (SD) respiratory rate, per min</td>
<td>44.2 (13.3)</td>
<td>35.5 (11.7)</td>
<td>&lt;0.001</td>
<td>38 (10)</td>
<td>38 (10)</td>
<td>0.986</td>
</tr>
<tr>
<td>Deep breathing, (%)</td>
<td>14 (33.3)</td>
<td>8 (11.3)</td>
<td>0.004</td>
<td>2 (16.7)</td>
<td>2 (15.4)</td>
<td>0.930</td>
</tr>
<tr>
<td>Hypoglycaemia, (%)</td>
<td>4 (9.5)</td>
<td>16 (22.5)</td>
<td>0.080</td>
<td>1 (8.3)</td>
<td>4 (30.8)</td>
<td>0.161</td>
</tr>
<tr>
<td>Mean (SD) haemoglobin, g/L</td>
<td>64 (21)</td>
<td>81 (18)</td>
<td>&lt;0.001</td>
<td>77 (8)</td>
<td>84 (20)</td>
<td>0.461</td>
</tr>
<tr>
<td>Severe anaemia, (%)</td>
<td>10 (24.4)</td>
<td>2 (2.8)</td>
<td>&lt;0.001</td>
<td>3 (25.0)</td>
<td>0 (0)</td>
<td>0.075</td>
</tr>
<tr>
<td>Geometric (95% CI) mean parasite density x 10³/µl</td>
<td>83.3 (45.5-152.3)</td>
<td>32.9 (20.7-52.3)</td>
<td>0.017</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean (SD) CSF glucose, mmol/L</td>
<td>2.7 (1.1)</td>
<td>2.8 (1.6)</td>
<td>0.832</td>
<td>3.3 (1.2)</td>
<td>2.5 (1.6)</td>
<td>0.182</td>
</tr>
<tr>
<td>Mean (SD) CSF protein,</td>
<td>0.40 (0.23)</td>
<td>0.20 (0.09)</td>
<td>&lt;0.001</td>
<td>0.29 (0.14)</td>
<td>0.74 (1.37)</td>
<td>0.266</td>
</tr>
<tr>
<td>Neurological deficits, (%) †</td>
<td>2 (4.8)</td>
<td>3 (4.2)</td>
<td>0.893</td>
<td>0 (0)</td>
<td>2 (15.4)</td>
<td>0.157</td>
</tr>
</tbody>
</table>

† Includes 3 children who had neurological deficits before admission and 4 with new onset neurological deficit
the median tau level in the cases with severe anaemia was 1135.8 (IQR 424.1 – 4416 pg/ml) compared to 192.8 (92.5 – 482.8 pg/ml) in patients without severe anaemia. The association between high levels of tau and severe malaria anaemia (adjusted OR 9.4 [95%CI 1.9 – 50]) was independent of the presence of severe acidosis (deep breathing, adjusted OR 3.0 [95%CI 1.1 – 8.6]). The effect of severe anaemia on tau levels was also not limited to children with malaria since median levels in children with malaria (1085.3 [441.2-2901.9] pg/ml) and that in children with other non-malarial illnesses (1159.3 [383.7-3430.0] pg/ml) were similar.

**Metabolic acidosis**
Respiratory distress, in particular deep breathing, is a feature of severe metabolic acidosis in African children with severe falciparum malaria [15, 16]. There was a strong correlation between CSF tau level and respiratory rate (p=0.0002, r=0.3) and with deep breathing. The median (IQR) of tau in children with malaria and deep breathing was 499.3 (153.2-1250.7) pg/ml compared to 190.2 (94.6-472.4) pg/ml in those without deep breathing (p=0.003). This relationship was not observed with S100B.

**Level of consciousness**
Among children with malaria, the CSF levels of tau increased with decreasing level of consciousness (figure 1). Coma was particularly associated with high levels of CSF tau: 20/37 (54.1.6%) children with coma had high levels of tau compared to 22/76 (29.0%) of those who presented without coma, (p=0.01). This relationship was not observed with S100B (p=0.983).

![Figure 1: Cerebrospinal fluid levels of tau and level of consciousness in children with malaria](image)
In addition, coma was significantly associated with deep breathing, (39.5 vs 11.4%, p<0.001) but not severe anaemia (p=0.926). On logistic regression analysis (stepwise entry), the association between level of consciousness and high levels of tau (adjusted OR [95%CI], 2.4[1.1 – 5.3], p=0.026), was independent of deep breathing (adjusted OR [95%CI], 29.9 [3.9 – 230], p=0.001).

Hypoglycaemia
Unlike patients with severe anaemia or severe metabolic acidosis, children presenting with hypoglycaemia (blood glucose <2.2 mmol/L) had lower median CSF tau levels compared to those who presented without hypoglycaemia (120.9 vs 217.2 pg/ml, p=0.005). Their median S100B level was also lower (154.5 vs 283.8 pg/ml, p=0.001).

Parasite density
The geometric mean parasite density in children with high levels of tau was about two and half times that in patients with normal levels (table II). A similar association was not observed between parasite density and high S100B levels.

Outcome
One child died and apart from the 3 children with neurological deficits on admission (one with epilepsy and two with delayed development), 4 other children had neurological deficits at discharge. This included one with continuing epileptic seizures discharged on phenytoin, a child with quadriparesis and 2 with generalised hypotonia. Three of the four children with deficits had malaria. The child who died had coma and a severe lower respiratory tract infection. She had a very high tau (2554 pg/ml) level. The median [IQR] CSF levels of tau in the 3 children with malaria who had neurological sequelae at discharge was higher (1010.4 [126.2-3580.6] pg/ml) compared to those discharged well (209.0 [109.0-539.4] pg/ml) although this difference was not statistically significant (p=0.197). Similarly, although the median levels of S100B in children with deficits were higher, the differences were not significant, (p=0.507).

DISCUSSION
This study set out to examine the CSF levels of the proteins tau and S-100B, biomarkers of cerebral damage, to help understand the pathogenesis of neuronal involvement and damage in children with severe falciparum malaria. We found that CSF levels of tau inversely correlated with the level of consciousness and other complications of malaria infection such as severe anaemia and acidosis but not seizures. In contrast, children admitted with high levels of S-100B had an increased risk of repeated seizures.
There is a hierarchy of susceptibility of brain parenchymal elements to neurological insult with axons and astrocytes at either end of the spectrum. Axons are the most vulnerable because they often extend for great distances from their cell bodies of origin, and may possibly depend on local production of ATP to maintain ion gradients and sustain energy-consuming functions. They are therefore susceptible to ischaemic or toxic damage in several different vascular territories, which is not the case for neuronal cell bodies or glia. It was therefore not unexpected that we have found more significant elevations and clinical correlation with tau compared with S-100B. The differences in the findings between both markers may also reflect the timing of release of tau and S-100B following injury. This can only be confirmed through the measurement of these biomarkers in serial CSF samples, a procedure that would be unethical in this group of patients.

The findings of this study suggest that in children with severe falciparum malaria, impaired consciousness and coma are associated with axonal injury. This is consistent with previous work showing that axonal damage is associated with clinically reversible coma or coma followed by a fatal outcome in both trauma and Vietnamese adults with severe malaria (reviewed in [17]). Post-mortem analysis of brain tissue from Vietnamese adults showed that frequency and extent of axonal injury was more severe in patients with cerebral malaria than in those with no cerebral involvement [18]. However, a CSF study showed that injury resulting in degeneration of axons did not correlate with coma depth but, rather, coma duration [19]. In the present study, elevated levels of CSF tau were associated with depth of coma in children with malaria. Multiple regression analysis was performed to determine if the association between tau and coma was due to systemic complications. Tau remained a significant independent predictor of coma. Median tau levels in children with cerebral malaria were 3-fold greater compared with our previously reported findings in adults although median tau levels do not differ between children and adults when all malaria patients are considered in the analysis (see [8]). These findings imply that axons are more susceptible to damage during malaria coma in children. Previous studies have also detected differences in the neurophysiological milieu of the CSF of African children and Vietnamese adults with severe malaria suggesting intrinsic age-related differences in the ability to regulate brain function in response to a pathological insult [20]. High quality magnetic resonance may provide further insights into the extent and distribution of axonal injury.

Systemic complications of severe malaria in adult South East Asian patients, such as severe anaemia, acidosis and renal failure[21], are recognised in paediatric African patients[22-24]. Whether systemic disease may cause or exacerbate damage to the brain, was investigated in this study. Axonal damage was not significantly associated
with seizures or hypoglycaemia, but tau was significantly elevated in patients with impaired delivery of oxygen (severe anaemia), those with severe metabolic acidosis manifesting as respiratory distress and at higher parasite densities. These systemic complications can alter cerebral blood flow, oxygen extraction or metabolite levels. Clearly, metabolic derangements such as acidosis could have a profound impact on the internal milieu of axons that are particularly susceptible to pH and oxygen stress. Although similar correlations have been found in adults with severe malaria[8, 18], this is the first finding of axonal injury in association with severe malaria anaemia. The brain is vulnerable to anaemia-induced injury (reviewed in [25]). Associations between neurological sequelae and severe malarial anaemia in children have been reported[26]. When compensatory mechanisms directed at maintaining cerebral oxygen delivery have become overwhelmed, hypoxia ensues. Markedly high levels of tau (>1000pg/ml) like those reported in children with progressive and lysosomal disease with abnormal white matter[27] were observed in children with severe anaemia and in the small group of patients with a bad outcome.

No clinical or biochemical parameter analysed correlated with S-100B levels so the mechanism for the elevation in the CSF of malaria patients is unclear. In our post-mortem study of adults with severe malaria, the presence of astrocyte degeneration, clasmatoendrosis, did not correlate with coma, but there was a significant correlation with intracerebral haemorrhage[18]. Petechial haemorrhages are a pathological feature of severe malaria and in other clinical settings, intracerebral haemorrhage are associated with epileptic seizures in children[28]. Irrespective of the inducing factor there are numerous lines of evidence that implicate a role for astrocytes and S-100B with seizure activity. This data suggests that seizures after admission are a manifestation of brain damage, rather than a cause of brain damage. In severe falciparum malaria, multiple seizures are associated with increased mortality and neurological deficits[11, 29, 30]. The small number of children with a fatal outcome (n=1) and neurological deficits (n=4) in this study does not allow us to determine the potential usefulness of this marker as an indicator of poor neurological outcome.

CONCLUSIONS

Our study provides evidence to support the view that several mechanisms may be involved in the causation of brain parenchymal damage in children with malarial infection. Axonal injury is associated with coma, hyperparasitaemia and metabolic disturbances both in paediatric and adult populations from different geographical regions. In addition, anaemia-induced impairment of oxygen delivery also plays a role in brain damage in children with malaria. Levels of CSF tau are greater in children...
with coma compared with previous findings in adults and elevated levels of S-100B in children are associated with an increased risk of repeated seizures. Whether axonal and astrocyte dysfunction or degeneration are the pathological correlates of neurological sequelae and multiple seizures in children with malaria should be investigated further.

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