

# Metabolites of the Kynurenine Pathway of Tryptophan Metabolism in the Cerebrospinal Fluid of Malawian Children with Malaria

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**A retrospective study of 100 Malawian children (87 with malaria and 13 with a diagnosis other than malaria) was conducted to determine the relationship between levels of metabolites of the kynurenine pathway in cerebrospinal fluid (CSF) and disease outcome. Three metabolites were measured: quinolinic acid (QA), an excitotoxin; kynurenic acid (KA), a neuroprotective receptor antagonist; and picolinic acid (PA), a proinflammatory mediator. Elevated levels of QA and PA in CSF were associated with a fatal outcome in Malawian children with cerebral malaria (CM). QA was associated with a history of convulsions. An increase in the QA:KA ratio, which favors neurotoxicity, was observed only in the 3 patients with tuberculosis meningitis. Compared with Vietnamese adults with malaria, Malawian children with malaria had higher concentrations of KA. Elevated levels of KA in children with CM may serve to contain injury in the developing brain, which is more susceptible to excitotoxic damage than is the adult brain.**

Cerebral malaria (CM) is a serious complication of *Plasmodium falciparum* infection. It is characterized by neurological manifestations, such as convulsions and coma, which can, in many patients, resolve completely [1]. The clinical presentation of severe and complicated malaria, in general, differs significantly between different patient populations (e.g., Southeast Asian adults vs. Malawian children). Although CM does occur in

Southeast Asian adults with falciparum malaria, the incidence of convulsions is lower, and the involvement of other organs is more common, compared with that in Malawian children [2]. Indeed, CM as an isolated clinical presentation in the absence of systemic acidosis, renal failure, or other combination organ failure is relatively rare among adults in Southeast Asia. Pathological data from Malawi and Vietnam also indicated differences between the incidence of intracerebrovascular leukocytes, cerebral parenchymal inflammatory responses, and blood-brain barrier breakdown [3, 4], with more striking responses found in Malawian children [5]. However, both populations have as common features significant parasitized erythrocyte sequestration in the cerebral vasculature and hemorrhage formation within the central nervous system.

The contribution of excitatory mediators to the initiation and maintenance of seizures and neurodegeneration during CM is currently being studied in both human populations and mouse models [6–8]. Of particular interest in the context of malaria infection are 3 metabolites of the kynurenine (KYN) pathway—

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quinolinic acid (QA), kynurenic acid (KA), and picolinic acid (PA) [9–11]. Glutamate and aspartate are the classic excitatory neurotransmitters that act through N-methyl-D-aspartate (NMDA) receptors and have been implicated in excitotoxic mechanisms. Significant decreases in these neurotransmitters have been observed in mice with CM, compared with control mice [8]. In contrast, elevations in the levels of the excitotoxin QA have been observed in the mouse model [8]. QA is an excitotoxic NMDA receptor agonist and may be involved in a range of neurodegenerative and convulsive diseases [12]. KA is an antagonist of NMDA and other excitatory amino-acid receptors. It has been proposed that increased levels of KA in the brain could protect against the neurotoxic effects of QA and other excitotoxins, whereas decreased levels of KA could promote excitotoxicity [12]. PA can provide some protection against QA neurotoxicity [9] and can act as a costimulus for the activation of macrophage effector functions [13, 14].

In a recent study [7], the levels of metabolites of the KYN pathway in the cerebrospinal fluid (CSF) of adult Vietnamese patients with severe malaria were examined. In those patients, levels of QA that were comparable to, or even higher than, those reported elsewhere for other neurologic or inflammatory diseases were found [12, 15]. Similar elevations were found for PA but not for KA. The levels of QA and PA were highly predictive of a fatal outcome, but there were no associations with neurological complications. However, the levels of QA and PA were strongly associated with renal failure, which suggests that, rather than being a reflection of increased cerebral production, the measurements of KYN metabolites in the CSF may have been partly or wholly due to spillover of KYN metabolites from the blood.

To gain further insight into the role of KYN metabolites in the cerebral complications of malaria infection, we studied a different population, Malawian children with CM. Renal failure is rare in this group, but convulsions are more common, occurring in 40% of children with CM, compared with <10% Southeast Asian adults with CM. A study by Dobbie et al. [6] showed increases in levels of QA in CSF of 97 Kenyan children with CM, compared with those in a reference population. We extended these studies to Malawian children by looking at other metabolites of the KYN pathway, because increases in CSF QA can occur without seizure development and neurodegeneration if accompanied by increased production of KA.

## MATERIALS AND METHODS

**Case selection and sample collection.** CSF samples were selected randomly from archived samples obtained from pediatric patients admitted to the research ward in the Department of Paediatrics, Queen Elizabeth Central Hospital, Blantyre, Malawi. Ethical approval for this study was given by the University of

Malawi's College of Medicine Research Committee. CSF samples were collected at the time of admission, frozen at  $-20^{\circ}\text{C}$  within 1 h of collection, and stored at  $-80^{\circ}\text{C}$  until use. Control samples from UK adults ( $n = 20$ ) were collected from the Departments of Bacteriology and Biochemistry of the Oxford Radcliffe Hospital, Oxford, United Kingdom. These samples, which remained from diagnostic medical tests, subsequently proved to be normal. Control samples were used with ethical approval by the Central Oxford Research Ethics Committee.

**High-performance liquid chromatography (HPLC) analyses: quantification of KA.** KA was analyzed as described elsewhere [6]. One hundred microliters of CSF was mixed with an equal volume of 0.1 mol/L perchloric acid, and supernatants were retrieved by centrifugation (8000 g for 5 min at  $4^{\circ}\text{C}$ ). Supernatants (10–50  $\mu\text{L}$ ) were subjected to reverse-phase HPLC, by use of Pharmacia LKB 2248 HPLC pumps and an LC-18 column (7.5  $\times$  4.6 mm; Supleco) with a guard column (2-cm cartridge; Supelco), and were eluted at 1.0 mL/min with 50 mmol/L sodium acetate containing 4.5% (vol/vol) acetonitrile (pH 6.2). Zinc acetate (0.5 mol/L) was delivered post-column, at a flow rate of 1.0 mL/min. The reaction mixture was passed through a fluorescence detector (LC 240; Perkin Elmer) equipped with a 7- $\mu\text{L}$  flow cell, with excitation and emission wavelengths set at 344 and 398 nm, respectively. Quantification of KA was done by area comparison with authentic standard.

**Gas chromatography/mass spectrometry (GC/MS): quantification of QA and PA.** QA and PA were analyzed as described elsewhere [7, 16]. Fifty-microliter CSF samples were placed in screw-capped glass tubes, dried, treated with 1,1,1,3,3,3 hexafluoro-2-propanol and trifluoroacetic anhydride (Sigma-Aldrich), to esterify, and then dried again. The derivatized samples were dissolved in toluene (0.5–1 mL; Univar AR grade; Lab Supply). The toluene solution was washed with 5% (wt/vol)  $\text{NaHCO}_3$  (1 mL) and water (1 mL), dried over anhydrous sodium sulfate ( $\sim 300$  mg), transferred to autosampler vials (11 mm; Alltech Associates), and sealed with a teflon-lined cap. A 1- $\mu\text{L}$  sample was injected into the GC/MS via an Agilent Technologies 7683 Autosampler.

GC/MS was performed on a Hewlett-Packard 6890 gas chromatograph interfaced to a Hewlett-Packard 5973 mass selective detector. Chromatographic separations were performed in splitless mode by use of a Hewlett-Packard 5MS capillary column with the following temperature program:  $50^{\circ}\text{C}$  constant for 1 min, then  $30^{\circ}\text{C}/\text{min}$  to  $180^{\circ}\text{C}$ . The GC-MS interface heater, the ion source, quadrupole, and injection port temperatures were maintained at  $280^{\circ}\text{C}$ ,  $150^{\circ}\text{C}$ ,  $106^{\circ}\text{C}$ , and  $240^{\circ}\text{C}$ , respectively. All analyses were performed with the mass spectrometer operating in electron-capture, negative-ion mode, using methane as reagent gas (Ultrapure grade; Matheson Gas Products), and a methane flow control setting of 40 was used.

**Statistical analyses.** Statistical analysis was done with the Stata 6 program (StataCorp). Normally distributed continuous variables and variables log-transformed toward normality were compared between groups using unpaired Student's *t* test; data that were not normally distributed were compared using the Kruskal-Wallis test. Correlations between continuous variables were determined nonparametrically using Spearman's  $\rho$ . No adjustments for multiple comparisons were made, although  $P < .01$  was regarded as significant for the purposes of interpretation and discussion.

## RESULTS

A total of 100 children (49 girls and 51 boys; age range, 4–139 months [median, 30 months]) were studied; 21 died, 1 survived with sequelae, and 78 made a full recovery. At admission, 93 were obtunded, with a Blantyre coma score of  $\leq 2$ ; 82% had a history of convulsions, and 23% had convulsions. Of the 100 patients, 82 had a clinical diagnosis of CM (i.e., Blantyre coma score of  $\leq 2$  for at least 2 h after admission, *P. falciparum* parasitemia, no improvement after treatment for hypoglycemia, and no evidence of meningitis in the CSF sample obtained at admission). Of the remaining 18 patients, 5 had uncomplicated malaria, 3 were anemic (packed red blood cell volume  $< 15\%$ ), and 10 had a diagnosis other than malaria, including acute hepatitis, drug intoxication, sepsis, tuberculosis (TB) meningitis (defined either at autopsy or by clinical evidence [i.e., elevated white blood cells in the CSF with a lymphocyte predominance, positive Mantoux test, and/or response to anti-TB therapy]), and convulsions of unknown etiology.

**Levels of KYN pathway metabolites in the CSF of Malawian children admitted to hospital.** A summary of the levels of KYN metabolites in Malawian children is shown in table 1. Of the Malawian children admitted to this study, 66% had levels of QA higher than median UK control levels, and 5% had levels

higher than the UK reference range (figure 1). For KA, QA:KA, and PA, 74% (41%), 29% (7%), and 72% (37%) of Malawian children had higher levels in the CSF than median (reference range) UK control levels, respectively (figure 1). When only patients with malaria were taken into consideration, these percentages were 72% (2%) for QA, 77% (43%) for KA, 24% (6%) for QA:KA, and 74% (38%) for PA. Only 4% of Malawian children with malaria showed levels of QA in the micromolar range, which is the range associated with neurodegeneration [11]. None of the UK control samples had QA levels  $> 1 \mu\text{mol/L}$ .

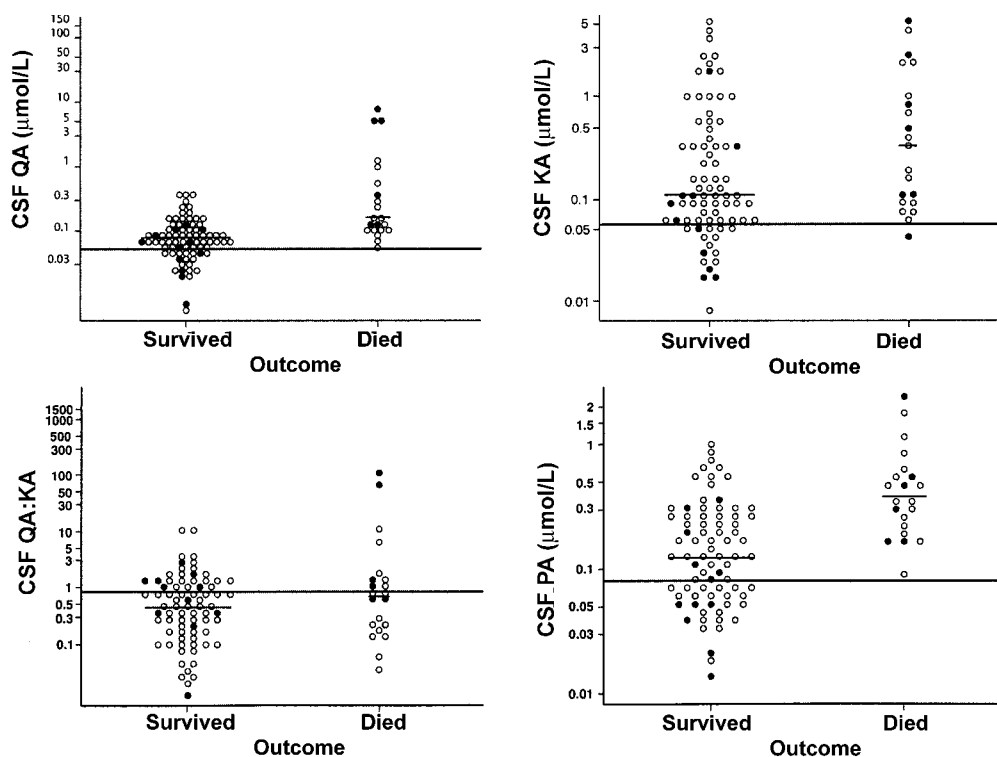
**Relationship between KYN metabolites, outcome, and neurological signs.** Levels of QA and PA in the CSF of Malawian children were strongly correlated with a fatal outcome in the entire group ( $P < .0001$ ) and in those with CM ( $P < .002$  for QA;  $P < .0003$  for PA). There were no associations between outcome and KA, QA:KA, or QA:PA. There was a statistically significant association between levels of QA in the CSF of Malawian children and a history of convulsions ( $P < .01$ ). However, there were no associations between the levels of KYN metabolites and other clinical neurological parameters (convulsions at admission, convulsion duration, convulsions after admission, Blantyre coma score at admission, and diagnosis of CM). There were no statistical correlations between levels of KYN metabolites and time from admission to death.

**Relationship between KYN metabolites and complications of severe malaria.** There was a correlation between PA and the creatinine concentration at admission ( $P < .01$ ) and a trend toward an association between QA and creatinine concentration at admission ( $P = .02$ ). QA ( $P < .01$ ), KA, and PA ( $P < .001$ ) also were associated with fever duration. Levels of both KA ( $P < .01$ ) and PA ( $P < .001$ ) negatively correlated with hematocrit. The QA:KA ratio was found to correlate with parasitemia at admission ( $P < .01$ ). Levels of both KA and PA correlated with levels of aspartate aminotransferase ( $P = .01$  and  $P = .006$ , respectively) but not with levels of alanine aminotrans-

**Table 1. Levels of kynurenine pathway metabolites in the cerebrospinal fluid (CSF) of Malawian children with cerebral malaria, uncomplicated malaria, and nonmalarial disease.**

Group	No. of subjects	QA	KA	PA	QA:KA	QA:PA
Cerebral malaria	82	0.09 (0.07–0.11)	0.21 (0.15–0.29)	0.18 (0.14–0.22)	0.41 (0.30–0.55)	0.50 (0.41–0.61)
Uncomplicated malaria	5	0.08 (0.04–0.16)	0.05 (0.01–0.25)	0.06 (0.02–0.26)	1.64 (0.50–5.43)	1.23 (0.35–4.28)
Convulsions	5	0.03 (0.01–0.13)	0.05 (0.02–0.10)	0.05 (0.02–0.09)	0.70 (0.26–1.93)	0.70 (0.13–3.76)
Acute hepatitis	1	0.11	0.31	0.30	0.36	0.36
Anemia	3	0.10 (0–2.37)	1.23 (0.18–8.40)	0.38 (0.11–1.26)	0.08 (0–9.64)	0.26 (0.02–3.46)
Drug intoxication	1	0.15	0.10	0.28	1.53	0.53
Sepsis	2	5.60	5.0	2.47	1.12	2.25
TB meningitis	2	6.10 (0.37–100.9)	0.07 (0.0–34.1)	0.17 (0.12–0.23)	92.5 (2.96–2888)	36.2 (1.6–833.6)

**NOTE.** Data are geometric mean  $\mu\text{mol/L}$  (95% confidence interval). The group with convulsions includes results from the CSF of 4 patients with convulsions of unknown etiology and 1 patient with atypical febrile convulsions. The diagnosis of uncomplicated malaria generally results when a child is admitted to the hospital in a coma and reaches a Blantyre coma score of  $\geq 3$  within 2 h of admission. KA, kynurenic acid; PA, picolinic acid; QA, quinolinic acid; TB, tuberculosis.



**Figure 1.** Levels of kynurenine pathway metabolite in cerebrospinal fluid (CSF) of Malawian children admitted to the Department of Paediatrics, Queen Elizabeth Central Hospital, Blantyre, Malawi. *White circles*, patients with malaria; *black circles*, patients with a diagnosis other than malaria; *small horizontal bars*, median of the patient groups that survived or died; *large horizontal bars*, median levels in UK control samples. KA, kynurenic acid; PA, picolinic acid; QA, quinolinic acid.

ferase ( $P > .3$ ) at admission. There were no correlations with the levels of KYN metabolites and jaundice, bilirubin levels, blood urea nitrogen, or glycemic status at admission.

## DISCUSSION

The present study has examined levels of 3 metabolites from the KYN pathway in the CSF of Malawian children with severe malaria, to determine whether changes in potentially neurotoxic or neuroprotective mediators reflects the prognosis of the disease. In addition, we compared these parameters with the same ones in Malawian children with diagnoses other than malaria and with those previously reported in the CSF of adult Vietnamese patients with severe malaria (table 2) [7].

**Levels of proexcitotoxic QA in Malawian children with malaria are associated with a fatal outcome.** In the group of Malawian children with malaria, there was a strong association between elevated levels of QA and death, and, in contrast to that in the Vietnamese adults with severe malaria, this association was independent of renal function [7]. However, only 5 of the 100 Malawian children in the present study had plasma creatinine levels above the normal range (0.7–1.5 mg/dL). Of these 5 children, 3 had CM.

Despite the association with fatal outcome, the levels of QA

in the CSF of the Malawian group with malaria were considerably lower than those detected in the 3 Malawian children with TB meningitis or sepsis (table 1) and were lower than those in Vietnamese adults with malaria [7]. We found that 44% of Vietnamese patients had levels of QA  $>1 \mu\text{mol/L}$ , concentrations that would be compatible with neurotoxicity, whereas only 4% of the Malawian children had levels of QA in the micromolar range. The most likely explanation is that malaria in adults from Southeast Asia often is accompanied by multiorgan dysfunction, a development that is rare in children with malaria. Elevated levels of QA are seen in the CSF of patients with renal failure and liver disease [15, 17]. Both Malawian children and Vietnamese adults with malaria had elevated levels of PA. PA has several biological properties involving activation of the immune system [13, 14], and the relationship between levels of PA and a number of complications of severe malaria (e.g., fever duration and hematocrit) suggests that the elevated levels of PA may be a general response to malaria parasite infection.

**Elevated levels of KA in the CSF of Malawian children with malaria may be protective.** A major difference between Malawian children and Vietnamese adults with malaria was their levels of KA in the CSF. In the Vietnamese adults, we found no elevation in levels of KA, compared with those in UK control

**Table 2. Similarities and differences between cerebrospinal fluid kynurenine (KYN) metabolism in Malawian children and that in Vietnamese adults.**

Group	Elevated KYN metabolites			Correlation between KYN metabolites and neurological complications	Correlation between KYN metabolites and death	Dependence on renal function
	QA	KA	PA			
Malawian children	–	+	+	–	+	–
Vietnamese adults	+	–	+	–	+	+

**NOTE.** KA, kynurenic acid; PA, picolinic acid; QA, quinolinic acid; +, present; –, absent.

samples, whereas 41% of Malawian children had elevated levels of KA in CSF relative to the UK reference range. The developing brain is thought to be more susceptible to excitotoxic damage as a result of enhanced expression of glutamate receptors, the NMDA receptors in particular. These receptors are highly expressed in the developing brain [18] and are critical in seizure discharges and hypoxic ischemic neuronal injury [19]. Several reports have shown that the developing brain can rapidly mobilize KA in response to injurious stimuli [20, 21]. In the case of neonatal asphyxia in rats, levels of KA are elevated within 5 min of hypoxia and can increase to 302% of baseline levels within 20 min, whereas levels of other KYN metabolites with excitotoxic properties are decreased [20]. This phenomenon is thought to be a mechanism to protect the developing brain from excitotoxic damage during a period of heightened susceptibility.

Although KA is best known for its neuroinhibitory, anti-convulsant, and neuroprotective properties, which serve to contain the spread of excitotoxic activity, KA also antagonizes the NMDA receptors involved in cognitive functions. Therefore, changes in KA metabolism may represent a pathogenic factor in neuronal dysfunction that causes cognitive deterioration [10, 22, 23]. Cognitive sequelae have been observed in Kenyan children after recovery from *P. falciparum* infection [24, 25].

In summary, there was considerable elevation and dysregulation of levels of KYN metabolites in the CSF of Malawian children with malaria. Dysregulation of CSF KYN metabolism has become a recognized feature of malaria infection [6–8]. Our data demonstrate a difference between Malawian children and Vietnamese adults with malaria: elevated concentrations of CSF QA are independent of renal function in Malawian children, and concentrations of KA are higher in the CSF of Malawian children with CM than in Vietnamese adults with severe malaria. These differences may reflect the different neurophysiological milieu of the pediatric brain, in which there is a relative overexpression of receptors for excitatory neurotransmitters. The functional effect of elevations in KA may be to contain the spread of excitotoxic activity in the developing brain. In both patient populations, elevated levels of QA and PA in CSF predicted a fatal outcome, although the nature of any causal mechanisms remains to be established. The difference between

KA concentrations in Malawian children and those in Vietnamese adults suggests that there may be a fundamental difference in the response of the pediatric brain to malaria.

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