

The Clinical Significance of Cerebrospinal Fluid Levels of Kynurenine Pathway Metabolites and Lactate in Severe Malaria

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A retrospective study of 261 Vietnamese adults with severe malaria was conducted to determine the relationship between cerebrospinal fluid (CSF) levels of metabolites of the kynurenine pathway, the incidence of neurologic complications, and the disease outcome. Three metabolites were measured: the excitotoxin quinolinic acid (QA); the protective receptor antagonist kynurenic acid (KA); and the proinflammatory mediator picolinic acid (PA). These measurements were related prospectively to CSF lactate levels. QA and PA levels were elevated, compared with those of controls. There was no difference in the levels of KA between these groups. Although >40% of malaria patients had QA CSF concentrations in the micromolar range, there was no association with convulsions or depth of coma. Levels of QA and PA were associated significantly with death, but a multivariate analysis suggested that these elevations were a consequence of impaired renal function. CSF lactate remained an independent and significant predictor of poor outcome.

Severe *Plasmodium falciparum* malaria may present itself as a diffuse encephalopathy, termed "cerebral malaria" (CM), associated with potentially reversible neurological manifestations, such as convulsions and coma [1]. Pathologic studies indicate that these cerebral complications may result from several coexisting pathological processes in the same individual, including hypoxia, hypoglycemia, cerebral swelling, hemorrhage, and inflammation. The contribution of systemic processes to cerebral dysfunction, such as renal or hepatic failure, acidosis, or systemic cytokine release, has not been determined.

One clue to the cause of neurologic damage in CM is revealed by examination of mediators released into the cerebrospinal fluid (CSF). Although this gives a limited temporal and structural

reflection of processes occurring in the brain parenchyma, it has the advantage over postmortem studies of allowing comparisons between patient groups during life [2]. Recent studies of Kenyan children with CM have proposed that the imbalances of excitatory mediators may be implicated in the initiation and maintenance of seizures and neurodegeneration and might contribute to neurological symptoms and deficit in CM [3]. An imbalance in the production of excitotoxic and neuroprotective tryptophan metabolites produced from the kynurenine (KYN) pathway has also been observed in a murine model of CM in which these pathological processes are prominent [4].

Two metabolites of the KYN pathway, which were shown previously to have major roles in its excitotoxic effects, are quinolinic acid (QA) and kynurenic acid (KA). QA is an agonist of N-methyl-D-aspartate (NMDA)-type excitatory amino acid receptors, and KA is a protective receptor antagonist [5, 6]. An imbalance, with QA production predominating, would predispose to convulsions and coma, whereas a balanced production of QA and KA should protect the brain from toxicity. Other metabolites of the pathway, including picolinic acid (PA), can also provide some protection against QA neurotoxicity [7] and can act as a costimulus for the activation of macrophage effector functions [8, 9].

In the current study, we have investigated whether excitotoxicity, resulting from disturbances in the production of KYN metabolites, contributes to clinical neurologic symptoms and outcome in adult patients with severe malaria. Levels of KYN metabolites in CSF samples, taken from Vietnamese patients with severe *P. falciparum* malaria, were measured and correlated

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All samples from patients with malaria were obtained with permission from patients or relatives, and sample-collection protocols were approved by the Ethical and Scientific Committee of the Centre for Tropical Diseases, Ho Chi Minh City, Vietnam. The use of control cerebrospinal fluid from patients in the United Kingdom was approved by the Central Oxford Research Ethics Committee.

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with a panel of clinical and biochemical parameters considered important in maintaining the balance of neurotransmitter function in the brain. In addition, we related these measurements to CSF and plasma lactate concentrations, which are established markers of poor outcome in severe malaria.

Methods

Study site and patients. The study was carried out at the Centre for Tropical Diseases, Ho Chi Minh City, Vietnam, an infectious disease hospital that acts as a referral center for much of southern Vietnam. Patients admitted to the severe malaria ward were diagnosed as having malaria on the basis of a positive peripheral blood film for *P. falciparum* and exclusion of other possible diagnoses. The definition of severe malaria was based on a modification of World Health Organization criteria, as published [10]. Clinical details of these patients have been published previously [10, 11].

CSF and plasma sampling. CSF samples for measurement of lactate, QA, KA, and PA were obtained by sterile lumbar puncture. Lumbar puncture was performed when clinically indicated as a routine part of the investigation of impaired consciousness, causes of which in the Vietnamese population include viral encephalitis, bacterial meningitis, and malaria. The CSF was stored at -80°C until use. Whole-blood samples for assessment of lactate levels were obtained and put into a cooled 10% (wt/vol) perchloric acid solution as close as possible to the time of lumbar puncture (<1 h in all cases). The perchloric acid samples were stored at -80°C and transported on dry ice to Oxford, United Kingdom, where lactate and pyruvate were measured enzymatically by use of a centrifugal analyzer (COBAS FARA; Roche Products).

Control samples were obtained from the Department of Bacteriology, Oxford Radcliffe Hospital, Oxford, United Kingdom. The CSF was stored at -80°C until use. These samples were the residue of diagnostic samples from adult patients ($n = 20$; mean age, 46.5 years [range, 14–77 years]) and were assessed by the researchers blinded to the patients' identities. CSF samples were from patients who received a lumbar puncture for clinical exclusion of central nervous system infection or hemorrhage and also from drainage of CSF shunts in patients with idiopathic intracranial hypertension. Samples were used as controls when subsequent clinical course excluded any pathology, and biochemical and microbiological assessment of the CSF was normal.

High-performance liquid chromatography (HPLC) quantification of KA. KA was analyzed as described elsewhere [4, 12]. In brief, KA was determined by HPLC, using Pharmacia LKB 2248 HPLC pumps and an LC-18 column (7.5×4.6 mm) with a Supelco guard column, with a 2-cm cartridge eluted with 50 mM sodium acetate, plus 4.5% (vol/vol) acetonitrile, pH 6.2 (adjusted with glacial acetic acid), at 1.0 mL/min. Zinc acetate (0.5 mM) was delivered after the column at a flow rate of 1.0 mL/min. A high-pressure mixer was used, and an additional mixing loop with a volume of 0.2 μL was installed before a fluorescence detector (LC 240; Perkin-Elmer; 7- μL flow cell) was used to detect KA. The detector was set at an excitation wavelength of 344 nm and an emission wavelength of 398 nm.

Preparation of CSF samples for HPLC. For determination of KA, 100 μL of CSF was mixed with an equal volume of 0.1 M

perchloric acid. Supernatants were retrieved by centrifugation at 8000 g for 5 min at 4°C . All samples were stored at 4°C until analyzed. Sample (10–50 μL) was injected into the HPLC system.

Gas chromatography–mass spectrometry (GC-MS) quantification of QA and PA. GC-MS was performed on a gas chromatograph (Hewlett-Packard 6890) interfaced to a mass spectrometer (5973 MSD; Hewlett-Packard). Chromatographic separations were performed in splitless mode using a capillary column (HP-5MS; Hewlett-Packard; 27–30 m \times 0.25 mm internal diameter with 0.25- μm stationary-phase film thickness) at 50°C constant for 1 min and then a 30°C increase/min to 180°C . The GC-MS interface heater, ion source, quadrupole, and injection port temperatures were maintained at 280°C , 150°C , 106°C , and 240°C , respectively. All analyses were performed with the mass spectrometer operating in electron capture–negative ion mode, using methane (Ultrapure grade, Matheson Gas Products) as reagent gas; a methane flow-control setting of 40% was used. The on-column limit of quantification was <1 fmol for each of the analytes at a signal-to-noise ratio of better than 10:1. The precision and accuracy of the assays for QA and PA were $>99\%$ at concentrations ranging from 10 fmol to 1 pmol [13].

Preparation of CSF samples for GC-MS. CSF samples (50 μL) were placed in 13 \times 100-mm screw-capped glass tubes with Teflon-lined caps. Samples were then heated at 70°C for 15–20 min to deactivate contaminating virus. ($^2\text{H}_4$)-Picolinic acid was prepared by the oxidation of ($^2\text{H}_7$)-3-picoline with potassium permanganate, and 20 pmol of ($^2\text{H}_4$)-picolinic acid and 200 pmol of ($^3\text{H}_3$)-quinolinic acid (Le Research) were added to the samples for use as internal standards in the calibration curves. Samples were dried and treated with 1,1,1,3,3,3-hexafluoro-2-propanol and trifluoroacetic anhydride (Sigma-Aldrich) to esterify, and they were then dried again. The derivatized samples were dissolved in 0.5–1 mL toluene (Univar AR grade; Lab Supply). The toluene solution was washed with 5% (wt/vol) NaHCO_3 (1 mL) and water (1 mL), dried over anhydrous sodium sulfate (~ 300 mg), and then transferred to 11-mm autosampler vials (Alltech Associates) and sealed with a Teflon-lined cap. The sample was then ready for injection into the GC-MS system via an autosampler (7683; Agilent Technologies). A 1- μL volume of sample was injected into the interfaced chromatography system.

Statistical analyses. Statistical analysis was carried out using the Stata 6 (StataCorp) program. 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 ρ . Multivariate analyses were carried out using either multiple linear regression (after transformation of variables, where appropriate) or logistic regression, depending on the nature of the dependent variable. No adjustments for multiple comparisons were made, although, for the purposes of interpretation and discussion, $P < .01$ was regarded as significant.

Results

A total of 261 adults (mean age, 33 years; range, 15–79 years) with severe *P. falciparum* malaria were studied: 38 (14.6%) died and 223 (85.4%) survived. On admission, 14.7% had convulsions

and 208 (79.7%) had a clinical diagnosis of CM [10]. This left a group of 53 patients (20.3%) with severe non-CM. Severe malaria in this population is a multisystem disease, and, on admission, 6% of the population had hemodynamic shock (systolic blood pressure <80 mmHg), 24.5% had acute renal failure (plasma creatinine >3 mg/dL), 19.4% had anemia (hematocrit <20% plus parasitemia >100,000 μ L), 51% had jaundice (bilirubin >2.5 mg/dL), and 6.1% had hypoglycemia (plasma glucose <2.2 mM). None of the patients suffered long-term sequelae after recovery. In fatal cases, the mean time to death after admission was 36 h (interquartile range, 12–52.75 h).

This group of patients is a subset of individuals entered into a randomized double-blind trial of artemether versus quinine in the treatment of severe malaria [10, 11]. Further clinical and biochemical findings from this group of patients have been reported elsewhere by Day et al. [11]. Patients were treated with a loading-dose regimen of intramuscular quinine (20 mg/kg quinine dihydrochloride salt followed by 10 mg/kg every 8 h) or intramuscular artemether (4 mg/kg immediately followed by 2 mg/kg every 8 h). To determine whether drug treatment modulated the levels of KYN metabolites or lactate, we compared log-transformed data from both groups, using an unpaired Student's *t* test. There was no association between the levels of the excitotoxic receptor antagonist KA or the proinflammatory mediator PA and drug treatment (KA, *P* = .69; PA, *P* = .15). The quinine group had a tendency for higher excitotoxin QA levels, but this was not statistically significant. Of note is the finding in the same group of patients that treatment had no significant effect on mortality or fever clearance time; however, parasite clearance was more rapid in the artemether group [10].

Elevated levels of KYN pathway metabolites in the CSF of patients with severe malaria. All KYN metabolites, with the exception of the neuroprotective KA, were elevated in the CSF of patients with severe malaria, relative to control subjects in the United Kingdom (PA, *P* = .0002; QA, *P* < .0001; QA:KA, *P* < .0001; KA, *P* = .42; see table 1). Of all malaria patients, 43.9% had QA levels >1 μ M, which is in the range associated with neurodegeneration (reviewed in [6]). The proportion of patients with CM and QA levels >1 μ M was not significantly different (38%) from the proportion of the whole group of patients with severe malaria (43.9%). In contrast, none of the control patients had QA levels >1 μ M.

Relationship between KYN metabolites and complications of severe malaria infection. As determined by univariate analysis, increased levels of QA, QA:KA, and PA were strongly predictive of a fatal outcome (figure 1). However, 0 of the KYN pathway metabolites were associated with convulsions, coma score, a diagnosis of CM, CSF protein, or time until death after admission. Raised levels of CSF QA, QA:KA, and PA were all associated with a clinical diagnosis of hypoglycemia (*P* < .0001), renal failure (*P* < .0001), jaundice (*P* < .0001), shock (QA, *P* < .0001; QA:KA, PA, *P* < .01), and anemia (QA, *P* < .0001; QA:KA, *P* < .001; PA, *P* < .01). QA was also positively associated with hyperlactatemia (>5 mM; *P* < .01), but the QA:KA ratio was not. Figure 2A shows the strong correlation between admission plasma creatinine levels and the QA:KA ratio (*P* = .67). In contrast, there was no correlation between the admission Glasgow coma score and QA:KA ratio (figure 2B). In addition to the clinical and biochemical parameters described above, PA was associated with hyperparasitemia (*P* < .001) and pulmonary edema (*P* < .01). KA was not positively associated with any clinical or biochemical parameters, with the exception of anemia (*P* > .003).

PA. CSF levels of PA were also significantly increased, as determined by univariate analysis. PA has been implicated in control of local CNS inflammatory responses because of its effects on monocyte/macrophage activation and recruitment via both chemokine and NO release [8]. We therefore assessed leukocyte recruitment to the CNS by examining CSF total white cell and monocyte counts. CSF PA levels were not significantly associated with CSF total white blood cell counts or monocyte counts. Of interest, CSF PA levels were significantly associated with hyperparasitemia (*P* < .01).

Levels of pro-excitotoxic KYN metabolites reflect renal impairment and are not independently associated with a fatal outcome. In the univariate analyses, levels of QA and PA and the QA:KA ratio were strongly positively associated with mortality (figure 1). To determine whether these KYN metabolites were associated independently with a fatal outcome, we performed several multivariate logistic regression analyses with outcome as the dependent variable and combinations of creatinine, bilirubin, lactate, parasite count, CSF pressure, hemoglobin, and blood glucose as the independent variables. Using these models, it was evident that 0 of the KYN metabolites was associated in-

Table 1. Levels of kynurenine pathway metabolites in the cerebrospinal fluid of patients with severe malaria and in controls from the United Kingdom.

Group	QA level, μ M	KA level, μ M	QA:KA	PA level, μ M
Controls	0.07 (0.05–0.10) ^a	0.07 (0.06–0.08)	1.0 (0.77–1.32) ^a	0.08 (0.07–0.10) ^b
Patients with severe malaria	0.80 (0.66–0.97)	0.06 (0.06–0.07)	13.0 (10.7–15.9)	0.19 (0.17–0.21)

NOTE. Data are geometric mean (95% confidence interval). KA, kynurenine acid; PA, picolinic acid; QA, quinolinic acid.

^a *P* < .0001.

^b *P* < .001.

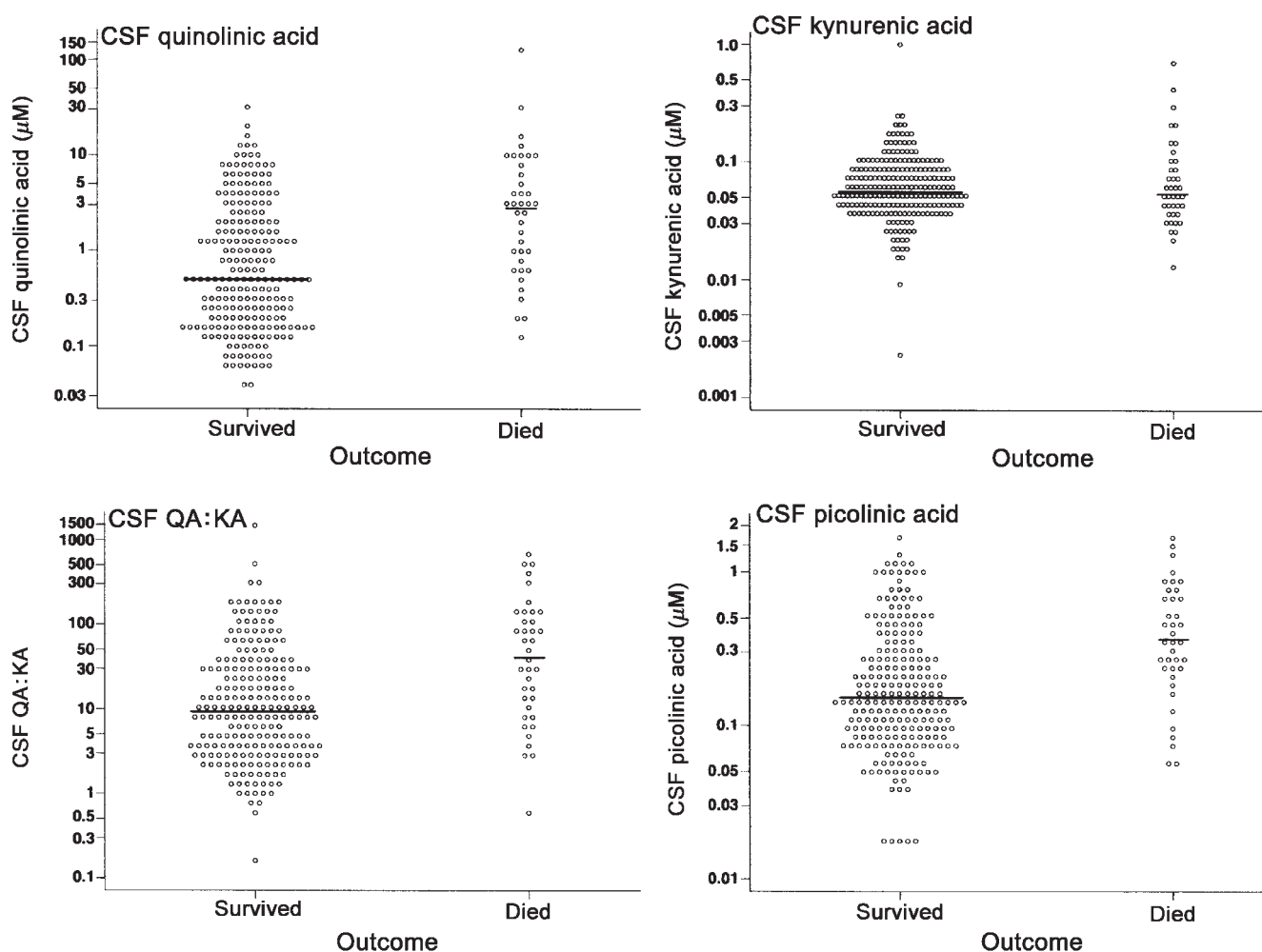


Figure 1. Kynurenine metabolite levels and clinical outcome in patients with severe malaria. CSF, cerebrospinal fluid; QA:KA, quinolinic acid:kynurenic acid ratio.

independently with outcome, but they were related to poor outcome as a consequence of impaired renal function. For example, using logistic regression to adjust for plasma creatinine, the association of QA:KA with mortality is dramatically reduced (unadjusted [$P < .001$] or adjusted [$P < .03$] for creatinine). An alternative explanation to the one we have presented would be that poor outcome was related to the KYN metabolites being increased as a consequence of renal failure. Although we cannot exclude the latter, such a hypothesis would not explain why some patients with renal failure died without any prior impairment in consciousness.

Relationship between lactate and markers of severe malaria infection. CSF lactate levels were significantly higher in patients who died than in survivors (6.0 mM [range, 5.0–7.0 mM] vs. 4.0 mM [3.8–4.3 mM], respectively; $P < .001$). Cerebral lactate accumulation can occur with seizures, but there was not a significant association between convulsions and either CSF lactate ($P = .27$) or lactate:pyruvate ratio ($P = .46$). However,

there was a positive association with CSF lactate levels and jaundice ($P < .001$), shock ($P < .0001$), renal failure ($P < .01$), and pulmonary edema ($P < .01$). There were no associations with hyperparasitemia or anemia. All groups showed an increased CSF lactate:pyruvate ratio, but there was no significant difference between the groups. Using the same multivariate model described previously for the KYN metabolites, it was evident that CSF lactate remains an independent and significant predictor of poor outcome in severe malaria.

Discussion

This study examined levels of metabolites from the KYN pathway in the CSF of adult patients with severe malaria to determine whether changes in potentially neurotoxic or neuroprotective mediators reflected the prognosis of the disease and the incidence of coma or convulsions. A major excitotoxic metabolite of the KYN pathway, QA, has been implicated in a wide

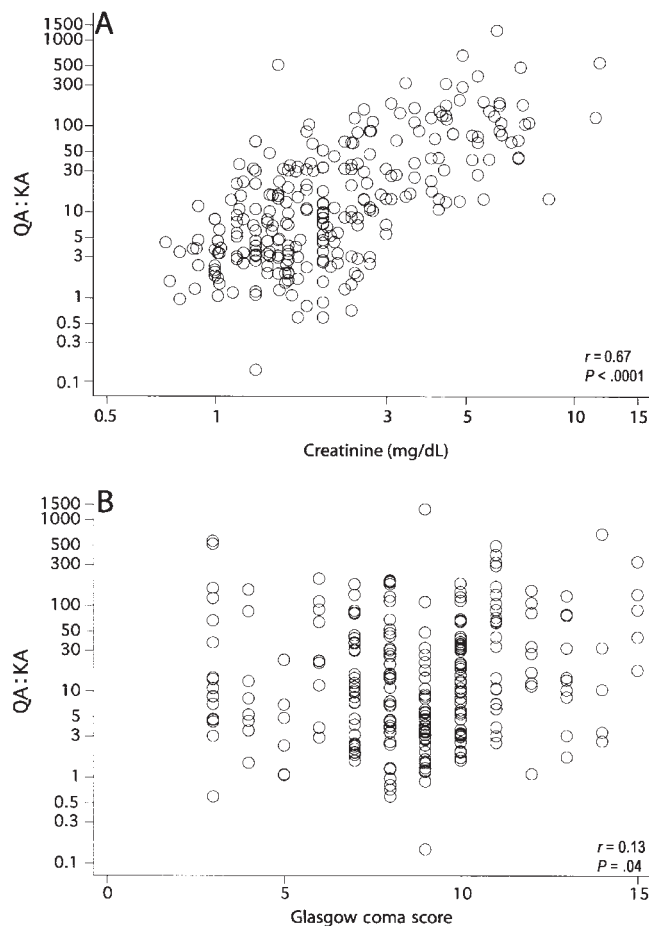


Figure 2. Plots of Glasgow coma score and plasma creatinine levels against the quinolinic acid:kynurenic acid ratio (QA:KA) in patients with severe malaria.

range of neuropathological disorders in humans and recently in children with CM [3]. We found very high levels of QA in our study subjects, compared with levels in subjects in other studies of nervous system disease (table 2). QA has been shown both in vitro and in vivo to cause neurotoxicity via the NMDA receptor by increasing intracellular calcium levels (reviewed in [6]). The suggestion that excitotoxicity could be involved in the pathogenesis of CM in adults from Southeast Asia is based on several lines of evidence: (1) convulsions are common in CM; (2) some pathologic features in the cerebral cortex of patients who died from CM share similarities with excitotoxic-mediated damage; (3) studies have shown findings in a mouse model of CM; and (4) studies have also shown findings in African children with CM.

Convulsions are a feature of CM, although they occur more frequently in African children (40%) than in Vietnamese adults (10%) [16]. The cause of convulsions in severe malaria, similar to the pathogenesis of coma, is not clear and is likely to be multifactorial (reviewed in [17]). The findings of this study of adults

do not suggest that there is an association between convulsions and the levels of QA in the CSF, despite considerably elevated CSF QA levels, compared with levels in controls, and despite these elevations not being associated with a rise in CSF KA levels, the natural antagonist. High concentrations of CSF QA levels in the absence of seizures have been reported in a model of bacterial septicemia in macaque monkeys [18] and in people with human immunodeficiency virus infection [19].

QA is also reported to cause excitotoxic cell death. Previous reports of cortical neuropathology in patients with CM would fit with an excitotoxic etiology [20]. However, other regions of the brain that are highly susceptible to excitotoxin-induced injury, including the striatum and hippocampus, have not been routinely sampled and analyzed in postmortem studies. We found that 114 (43.9%) patients with severe malaria had QA levels $>1 \mu M$, concentrations that would be compatible with neurotoxicity in other settings. However, it should be noted that no survivors of severe malaria in this study developed neurologic sequelae.

Two recent studies concluded that excitotoxic mechanisms may contribute to the pathogenesis of CM [3, 4]. An increase in the QA:KA ratio was seen in the murine model of CM, but not in malaria-infected mice without neurologic involvement, at a time when the mice were exhibiting neurologic symptoms [4]. Large increases in concentrations of CSF QA were found in 97 Kenyan children with CM who were compared with a reference population: there was a graded increment in QA concentration across outcome groups of increasing severity [3]. However, these studies did not take into account possible modulation of QA by other KYN pathway metabolites nor the dependence of QA concentrations on renal function. Impaired renal function

Table 2. Comparison of cerebrospinal fluid (CSF) quinolinic acid (QA) levels in patients with malaria and other neurologic or inflammatory disease.

Patient group, by diagnosis [reference]	No. of patients	CSF QA level, nM ^a
Healthy controls [14]	12	110 (26–360)
Healthy controls ^b	20	70 (50–100)
Congenital hyperammonemia [14]	4	565 (252–990)
Meningeal inflammation [15] ^c	13	643 (265–1561)
Parenchymal inflammation [15] ^d	8	209 (85–515)
Septicemia [15] ^e	6	730 (206–2588)
Other inflammatory disease [15] ^f	9	617 (118–3241)
<i>Plasmodium falciparum</i> malaria		
African children/Kenya [3]	97	229 (188–278)
Vietnamese adults who survived ^b	223	670 (550–810)
Vietnamese adults who died ^b	38	2400 (1520–4070)

^a Data are geometric mean (95% confidence interval).

^b Current study.

^c Included viral, bacterial, listerial, or aseptic and neurocysticercosis.

^d Included cerebral lupus, multiple sclerosis, encephalitis, and central nervous system vasculitis.

^e Included *Candida albicans* vasculitis and *Staphylococcus aureus*.

^f Included headache, syphilis, and head trauma.

occurs commonly in children with severe malaria, although acute renal failure is very unusual.

The concentration of QA under normal and also pathologic conditions in the brain and CSF reflect 3 major sources of production: (1) *de novo* synthesis by microglia or blood-derived monocytes, (2) spillover from the blood, or (3) changes in peripheral metabolism of tryptophan seen in patients with vital organ dysfunction or infection. All 3 sources of increased QA production may operate in concert in malaria infection. Microglial activation has been observed previously in human malaria infection [21–23] and in the murine model [24–27]. However, direct evidence for the induction of indoleamine 2,3-dioxygenase, the first and rate-limiting enzyme in the KYN pathway, would be necessary to confirm these results.

Renal impairment is the most likely mechanism for the increased CSF QA levels in CM. One-quarter of the patients in this study had acute renal failure on admission and required treatment. The mechanisms causing increased CSF KYN metabolite levels during renal insufficiency in malaria are unknown. Marked increases in KYN and QA have been observed in the serum and CSF of rats and humans with renal failure not associated with malaria [28]. In rats, KYN and QA levels in serum, brain, and CSF increased in parallel with the severity of renal insufficiency. It was found that the accumulation of serum KYN and QA in this model was not related to a decrease of renal excretion but to a decrease in degradation combined with an increase in production. Furthermore, it was suggested that increased CSF QA is secondary to QA from plasma and/or QA precursors passing into the brain [28].

Like QA, the increase in PA was associated significantly with outcome but was not independent of other complicating factors, notably renal failure. Also, the PA levels could not predict those patients with or without cerebral complications. These results contrast with our findings for lactate, which was significantly and independently associated with death from severe malaria.

In summary, there was a considerable increase in the level of the KYN metabolites QA and PA in the CSF of patients with severe malaria, compared with levels in controls in the United Kingdom and levels in previous studies. More than 40% of severe malaria patients showed QA CSF concentrations that would be compatible with neurotoxicity in other settings. Although the levels of excitotoxic metabolites of the KYN pathways QA and PA were significantly increased, with no concomitant increase in the neuroprotective metabolite KA, there was no association between QA and PA concentrations and either convulsions or a clinical diagnosis of CM. Increased levels of QA, PA, and the QA:KA ratio were strongly predictive of a fatal outcome, but, using multivariate analysis, we found that these metabolite levels were significantly related to poor outcome as a consequence of impaired renal function in this patient group. Many studies of neurotoxic metabolites in human disease or animal models concentrate solely on CNS levels of metabolites without taking into account the possible contribution of systemic production and

clearance of these factors. This study highlights the need to correlate markers within the CSF with vital organ function, especially in diseases such as malaria, in which multisystem organ failure is common.

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