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MAJOR ARTICLE

Cerebrospinal Fluid Pterins, Pterin-Dependent Neurotransmitters, and Mortality in Pediatric Cerebral Malaria

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Background. Cerebral malaria (CM) pathogenesis remains incompletely understood. Having shown low systemic levels of tetrahydrobiopterin (BH_4), an enzymatic cofactor for neurotransmitter synthesis, we hypothesized that BH_4 and BH_4-dependent neurotransmitters would likewise be low in cerebrospinal fluid (CSF) in CM.

Methods. We prospectively enrolled Tanzanian children with CM and children with nonmalaria central nervous system conditions (NMCs). We measured CSF levels of BH_4, neopterin, and BH_4-dependent neurotransmitter metabolites, 3-*O*-methyldopa, homovanillic acid, and 5-hydroxyindoleacetate, and we derived age-adjusted z-scores using published reference ranges.

Results. Cerebrospinal fluid BH_4 was elevated in CM (n = 49) compared with NMC (n = 51) (z-score 0.75 vs −0.08; $P < .001$). Neopterin was increased in CM (z-score 4.05 vs 0.09; $P < .001$), and a cutoff at the upper limit of normal (60 nmol/L) was 100% sensitive for CM. Neurotransmitter metabolite levels were overall preserved. A higher CSF BH_4/BH_2 ratio was associated with increased odds of survival (odds ratio, 2.94; 95% confidence interval, 1.03–8.33; $P = .043$).

Conclusion. Despite low systemic BH_4, CSF BH_4 was elevated and associated with increased odds of survival in CM. Coma in malaria is not explained by deficiency of BH_4-dependent neurotransmitters. Elevated CSF neopterin was 100% sensitive for CM diagnosis and warrants further assessment of its clinical utility for ruling out CM in malaria-endemic areas.

Keywords. cerebral malaria; neopterin; neurotransmitter; *P falciparum*; tetrahydrobiopterin.

Malaria from *Plasmodium falciparum* remains a major cause of childhood mortality and morbidity, particularly in African children, with an estimated 435 000 malaria-related deaths in 2017 [1]. Cerebral malaria (CM), characterized by coma, seizures, and brain swelling, is one of the most common and most serious complications in pediatric falciparum malaria, carrying a case fatality ratio of 15%–20% despite best available treatments [2, 3]. Earlier reductions in malaria incidence worldwide [4, 5] have not been sustained [1], and in those settings with decreased malaria transmission, there is an increased proportion of malaria cases presenting as CM [6, 7]. The predominant pathophysiology of severe malaria, including CM, implicates cytoadherence of parasitized red blood cells to endothelial cells, endothelial activation, and endothelial dysfunction, which collectively result in microcirculatory congestion and impaired tissue perfusion [8, 9]. The relatively low prevalence of neurologic sequelae among CM survivors—reported as low as 10% in cohort studies, but generally thought to occur in up to one third of survivors [2, 3, 10]—has spurred investigation of metabolic derangements and cytotoxic mechanisms as potential contributors to CM pathogenesis [11, 12].

We have previously demonstrated elevated plasma phenylalanine in children and adults with falciparum malaria [13]. In addition, we showed that systemic levels of urinary tetrahydrobiopterin (BH_4), an essential cofactor required for enzymatic conversion of phenylalanine to tyrosine, are low in children and adults with severe malaria [14, 15]. Tetrahydrobiopterin is also an essential cofactor for the enzymatic production of nitric oxide (NO) by NO synthases (NOS), and low systemic BH_4 may contribute to the low levels of NO

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demonstrated in severe malaria [16, 17]. In addition, BH_4 is the essential cofactor for mono-oxygenase enzymes required for the synthesis of biogenic amine neurotransmitters including catecholamines, via phenylalanine hydroxylase and tyrosine hydroxylase, and serotonin via tryptophan hydroxylase [18]. (See Supplementary Figure 1 for overview of BH_4 metabolism and enzymatic pathways for these BH_4-dependent neurotransmitters.) We hypothesized that levels of BH_4 and metabolites of BH_4-dependent neurotransmitters would be significantly lower in the cerebrospinal fluid (CSF) of children with CM compared with children with nonmalaria central nervous system conditions (NMCs). To test this hypothesis, we conducted a prospective observational study of pediatric CM in Dar es Salaam, Tanzania. Measurements of BH_4 and BH_4-dependent neurotransmitters in children with CM were compared with established, age-stratified reference ranges and to a contemporaneously enrolled clinical group of children with NMCs—a unique comparison group for prospective clinical studies of CM. To further characterize any observed differences in BH_4 and BH_4-dependent neurotransmitters between CM and NMC, we also measured neopterin, a metabolite of biopterin synthesis, a documented surrogate of acute and chronic inflammation [19, 20].

METHODS

Ethics Statement

This study was approved by the institutional review boards of Hubert Kairuki Memorial University, United Republic of Tanzania National Institute of Medical Research, University of Utah, and Duke University. Written informed consent was obtained from parents or guardians of all children enrolled.

Enrollment Criteria

Children ages 6 months to 6 years were enrolled from Amana or Mwanayamala District Hospitals in Dar es Salaam, Tanzania from November 2007 to January 2012, as previously described [14]. The case definition for CM, based on the World Health Organization (WHO) definition at the time of study [21], and the case definition for NMC are provided in the Supplementary Material.

Clinical and Laboratory Evaluations

Demographic and clinical details were documented on standardized case report forms. Details of clinical testing of venous blood and CSF specimens are provided in the Supplementary Material. Children were treated according to national and hospital protocols at the time of the study, including intravenous quinine for CM.

Biochemical Measurements of Cerebrospinal Fluid

Cerebrospinal fluid was also collected into sterile tubes containing dithioerythritol (approximately 1 mg/mL CSF) and diethylene triamine penta-acetic acid (approximately 0.1 mg/mL CSF) to prevent oxidation and preserve biopterins and in their in vivo redox state. For neopterins, reduced (dihydroneopterin [NH_2]) and oxidized (N_0), we reported the total as "neopterin" having oxidized NH_2 to N_0 in vitro for quantification. Quantification of BH_4, dihydrobiopterin (BH_2), biopterin (B_0), and neopterin was performed via high-performance liquid chromatography (HPLC) using sequential electrochemical and fluorescence detection as previously described [22]. Catecholamine metabolites, homovanillic acid (HVA) and 3-*O*-methyldopa (3-OMD), and serotonin metabolite, 5-hydroxy-indoleacetate (5-HIAA) were also measured by HPLC as previously described [23], along with measurements of enzymatic cofactors, pyridoxal-5' phosphate and 5-methyltetrahydrofolate (5-MTHF).

Metagenomics for Pathogen Detection in Cerebrospinal Fluid

To characterize the etiologies of NMC cases, we performed pathogen detection in CSF using metagenomics next-generation sequencing (see Supplementary Material).

Statistical Analysis

We performed statistical analysis using STATA 15.0 (StataCorp, College Station, TX). The significance threshold was set at a 2-sided $P < .05$. Cerebral malaria and NMC values were compared by Student's *t* test or Wilcoxon rank-sum test according to distribution. A χ^2 test was used to compare proportions between CM and NMC. Values of biopterins, neopterin, neurotransmitter metabolites, and cofactor metabolites were assigned as low, high, or within-normal-range by comparing to age-stratified reference ranges previously established by Medical Neurogenetics Laboratories, a North American reference laboratory that measures these analytes for clinical care [24]. Based on age-stratified reference ranges—established among children and adolescents in Britain—for BH_4, BH_2, neopterin, HVA, 3-OMD, 5-HIAA, and 5-MTHF [23, 25], we calculated age-adjusted z-scores for these analytes, using the median and range of values for each reference age strata. This approach adjusted for differences in age between the CM and NMC cohorts. By displaying the magnitude of the difference between the observed levels and the expected levels for children of a given age, this approach also corrected for the variability in the reference values. This approach was extended to the CSF BH_4/BH_2 ratio. Because BH_2 inhibits NOS, this ratio is a critical determinant of NO synthesis by endothelial NOS [26] and is a measure of redox balance.

Spearman's test was used to examine correlations between CSF and urinary levels of BH_4 and total neopterin, respectively. Area under receiver operator characteristic (AUROC) curve with 95% confidence intervals (CIs) was derived for CSF neopterin to examine its diagnostic utility for CM. Among CM, we assessed associations with survival by logistic regression,

controlling for age in months, for each of the CSF mediators found to be altered in CM—neopterin, BH_4, BH_4/BH_2 ratio, HVA, 3-OMD, and 5-HIAA.

In addition to comparisons between CM and NMC, CM was also compared with the NMC subset with unarousable coma (Blantyre coma scale [BCS] ≤2) and to the NMC subset with evidence of meningitis (CSF white blood cell ≥10 cells/μL) [27, 28]; comparisons of CSF analytes between NMC with coma and NMC without coma were also undertaken. Spearman's test was used to examine correlations between CSF pterins and neurotransmitter metabolites and the following: *P falciparum* histidine-rich protein 2; seizures; coma duration; BCS at enrollment; and CSF opening pressure. Given the multiple statistical comparisons for these exploratory correlations and the exploratory analyses comparing CM with NMC subgroups and comparing NMC with coma to NMC without coma, we used the Benjamini-Hochberg adaptive step-up procedure to control the false discovery rate—the expected proportion of rejected null hypotheses that are false positives [29].

RESULTS

During the study period, 270 children were screened for CM or NMC eligibility. Fifty-eight children were enrolled as CM, but 6 of these were excluded due to inadequate specimen collection (urine not obtained [n = 1] or CSF not obtained [n = 1]) or other discrepancies during their enrollment (malaria rapid diagnostic test positive, but blood smear was negative for malaria [n = 2] and failure to report serious adverse events within 24 hours [n = 2]). Fifty-two children with NMC were enrolled. Baseline clinical and laboratory characteristics are shown in Table 1. Compared with NMC, CM had significantly higher age, weight, temperature, and respiratory rate and lower BCS, hemoglobin, and platelet counts. Among children with NMC, none had a positive aerobic CSF culture. For 9 (17.3%) NMC children, a bacterial pathogen was detected by metagenomic next-generation sequencing. Supplementary Table 1 shows the CSF profiles and metagenomics results for these patients. In total, 26 (50.0%) of children with NMC had CSF-evidence of meningo-encephalitis. All of the CM children and 3 (5.8%) of the NMC children received intravenous quinine before lumbar puncture. Median time from quinine therapy to CSF collection was 6 (interquartile range [IQR], 4–8.7) hours for CM children, and the range among NMC children was 2–7.1 hours. Seven (13.5%) CM and 6 (11.5%) NMC children died in-hospital.

Table 2 shows the CSF measurements of biopterins, total neopterin, neurotransmitters, and cofactor metabolites. The BH_4 CSF levels were significantly higher in CM than in NMC in absolute terms and by age-adjusted z-score (0.75 vs −0.08; $P < .001$) (Figure 1A). Among CM, 31 (63.3%) had high BH_4 compared with 8 (15.7%) of NMC children. Only 3 (6.1%) of 49 CM and 5 (9.8%) of 51 NMC had BH_4 levels below the lower limit of the reference range. Neither the absolute values or age-adjusted z-scores for the CSF BH_2 significantly differed between CM and NMC; this was likewise for the BH_4/BH_2 ratio (Table 2, Figure 1B and C). Cerebrospinal fluid neopterin concentration was elevated in CM compared with NMC: CM z-score 4.05 vs NMC z-score 0.09, with 38 (77.6%) of 49 CM having a z-score >2 (Figure 1D). It is notable that all 49 CM children with a neopterin measurement had an elevated CSF neopterin level (>60 nmol/mL), whereas neopterin was elevated in only 20 (39.2%) of 51 NMC children. Cerebrospinal fluid neopterin had an AUROC of 0.78 (95% CI, 0.68–0.89) for discriminating CM from NMC (Supplementary Figure 2). Cerebrospinal fluid neopterin levels correlated with urine neopterin levels among CM (r = 0.45; $P = .002$) and among NMC (r = 0.65; $P < .001$), but CSF BH_4 levels did not correlate with urine BH_4 levels neither among CM (r = −0.18; $P = .23$) nor among NMC (r = 0.13; $P = .39$).

For neurotransmitter measurements (Figure 2A–C), 5-HIAA was lower in CM than in NMC, but only a minority in both groups had 5-HIAA levels below the lower limit of normal [24]: 13 (26.0%) of 50 CM and 8 (15.7%) NMC. The absolute values of HVA and 3-OMD were lower in CM, but the age-adjusted z-scores did not differ significantly between CM and NMC (see Table 2). For HVA, 44 (88.0%) of 50 children in each group had values within a pediatric normal range; and for 3-OMD, all CM children and 50 (98.0%) of 51 NMC children had values within normal range. Among the 44 CM children with a CSF measurement 5-MTHF, only 4 (9.0%) had low levels detected; and among the 9 CM with measured CSF pyridoxyl-phosphate, none had low levels.

With respect to associations with clinical outcome in CM, the CSF BH_4/BH_2 ratio was associated with survival when adjusted for age. For every natural log scale increase in BH_4/BH_2, the odds of survival were increased 2.94-fold (95% CI, 1.03–8.33; $P = .043$). A similar pattern was observed for BH_4 by itself ($P = .08$). Neopterin, 5-HIAA, HVA, and 3-OMD had no significant association with odds of survival among CM.

Because of the heterogeneity of diseases and disease severity in the NMC group, we compared CSF pterin levels and neurotransmitter metabolites between CM and NMC with unarousable coma (BCS ≤2) and between CM and NMC with meningitis (age-adjusted z-scores in Table 3 and absolute values in Supplementary Table 2). Compared with NMC with coma, CM children had a higher z-score for BH_4 (however, $P > .05$ after adjusting for multiple comparisons). Compared with NMC with meningitis, CM children had significantly higher BH_4 z-score (0.75 vs −0.07; $P < .001$, adjusted $P = .009$). Differences in neopterin and neopterin z-scores for CM compared with either NMC subgroup were not significant. Thirteen (50%) of NMC with meningitis and 5 (45.5%) NMC with coma

had a CSF neopterin level above the upper limit of normal. The AUROC for CSF neopterin was 0.75 (95% CI, 0.52–0.97) for distinguishing CM from NMC with coma—the subgroup clinical phenotype most similar to CM.

For neurotransmitter metabolites, there were no significant differences when comparing CM z-scores with either NMC with meningitis or with NMC with coma (Table 3). Likewise, the comparisons of CSF analytes between NMC with coma and NMC without coma did not demonstrate any significant differences (Supplementary Table 3). For CM patients, none of the CSF analytes (pterins, neurotransmitter metabolites, or cofactors) correlated with plasma PfHRP-2 levels, nor did we find any association between CSF analytes and the presence of seizures, duration of coma, depth of coma, or CSF opening pressure.

DISCUSSION

Contrary to our hypothesis and to our findings of low systemic (urine) BH_4 levels in children with CM [14], we found that CSF levels of BH_4 were actually elevated in CM compared with established reference range values and compared with children with NMCs. The importance of maintaining BH_4 in the central nervous system (CNS) despite systemic BH_4 deficiency is highlighted by the association between increasing CSF BH_4/BH_2 and the increased odds of survival in CM. Although BH_4-dependent CSF neurotransmitter levels were lower in CM than in NMC, most differences were not statistically significant, and most CM children had neurotransmitter levels that were within the pediatric reference ranges from Europe and North America [23, 24]. Consistent with our findings of high systemic neopterin levels

Table 1. Baseline Clinical Characteristics of Children With Cerebral Malaria and Children With Nonmalaria Central Nervous System Conditions

Clinical Characteristics	CM (n = 52)	NMC (n = 52)	*P* Value
Age, months	51 (47–55)	28 (22–33)	<.001
Male sex, no. (%)	34 (65.4)	29 (55.8)	.32[a]
Weight, kg	15.4 (14.4–16.4)	11.2 (10.2–12.1)	<.001
Last meal, hours	3.5 [1.3–7.0]	2.3 [1–4]	.10[b]
History of seizure, no. (%)	45 (86.5)	46 (90.1)	.56[a]
Axillary temperature, °C	38.3 (38.0–38.6)	37.8 (37.4–38.1)	.023
Respiratory rate, breaths/minute	43 (40–47)	37 (34–41)	.015
Blantyre coma score	1.7 (1.5–1.8)	3.9 (3.4–4.3)	<.001
Blantyre coma score ≤2	52 (100)	11 (21.1)	<.001[a]
Duration of coma (hours)[C]	14 [8.5–22]	9 [5–24]	.56
In-hospital death	7 (13.4)	6 (11.5)	.09
Clinical Laboratory Characteristics Blood			
White blood cells × 10^3/μL	8.7 [6.4–11.9]	11.9 [8.6–17.8]	.002[b]
Absolute neutrophil count × 10^3/μL	4.8 [3.5–6.4]	7.2 [4.3–11.5]	.006[b]
Hemoglobin, g/dL	7.0 (6.5–7.5)	9.8 (9.3–10.2)	<.001
Platelet × 10^3/μL	70 [55–101]	392 [197–570]	<.001[b]
Blood glucose, mg/dL	119 (105–132)	120 (93–147)	.94
Hypoglycemia, no. (%)	0 (0.0)	2 (4.7)	.13[a]
Plasma sodium, mmol/L	132 (130–133)	133 (130–136)	.42
Plasma bicarbonate, mmol/L	20 (18.8–21.3)	19.0 (17.0–21.0)	.36
Plasma creatinine, mg/dL	0.8 (0.5–1.0)	0.5 (0.4–0.7)	.12
Parasite density, trophozoites/μL [IQR]	45 231 [9396–128 231]	N/A	--
PfHRP-2, ng/dL [IQR]	1086.5 [379.1–2585.5]	N/A	--
Cerebrospinal Fluid			
Opening pressure, cm H_2O	18.4 (16.8–19.9)	18.3 (17.0–19.5)	.88
Opening pressure >20 cm H_2O, no. (%)	16 (30.8)	13 (26.4)	.38[a]
White blood cells/μL	5 [2–10]	9 [0.5–160]	.06[b]
Neutrophils (%), cells/μL	36.1 (28.8–43.5)	47.1 (38.9–55.2)	.048
Neutrophils absolute, cells/μL	1.8 [0.5–4.6]	3.6 [0.2–88]	.036[b]
Red blood cells/μL	0 [0]	2.5 [0–90]	<.001[b]
Protein, mg/dL	17.3 [15–26]	14.7 [11.8–28.2]	.33[b]
Glucose, mg/dL	76 (71–81)	65 (55–76)	.06
Hypoglycorrhachia	20 (43.5)	22 (56.4)	.24[a]

Abbreviations: CM, cerebral malaria; IQR, interquartile range; N/A, not applicable; NMC, nonmalaria central nervous system conditions.

NOTES: Mean (95% confidence interval); median [IQR]. Student's *t* test unless otherwise indicated. Hypoglycemia defined as plasma glucose <40 mg/dL. Hypoglycorrhachia defined as cerebrospinal fluid glucose <2/3 of plasma glucose value.

[a]χ^2 test of proportions.

[b]Wilcoxon rank-sum.

[c]Duration of coma at the time of enrollment.

[14], CSF neopterin was elevated in CM, and a CSF neopterin level cutoff at the upper limit of normal (60 nmol/L) had 100% sensitivity for excluding CM as the diagnosis. We discuss each of these key findings in the subsections below.

Significance of Central Nervous System Pterins: Our Findings and Findings From Previous Studies

The chief purpose of our prospective observational study was to determine BH_4 status in the CNS of children with CM. We hypothesized that CNS deficiency of BH_4 would lead to insufficient levels of BH_4-dependent neurotransmitters. Such a deficiency would implicate a metabolic mechanism that contributes to the other pathophysiologic mechanisms established in malarial coma—diffuse sequestration of infected red blood cells throughout the microvasculature [9] and brain swelling [30]. A metabolic contribution to coma might also point toward potential adjunctive therapies to reduce the morbidity and mortality of CM [31, 32]. In addition, a metabolic mechanism contributing to CM pathophysiology would be consistent with the rapid recovery of consciousness that may be observed in CM. Our hypothesis of deficiency in CNS BH_4 was also plausible given previous findings of low urinary BH_4 and elevated plasma phenylalanine in CM. Phenylalanine is an amino acid whose conversion to tyrosine in the liver requires BH_4 as an enzymatic cofactor. We unexpectedly found that CSF BH_4 levels in CM are actually elevated compared with levels in NMC, a group that included 11 children with unarousable coma, 26 with meningitis defined by CSF pleocytosis, and 9 with pathogens detected by metagenomic next-generation sequencing (see Supplementary Table 1). We noted that CSF metabolites of BH_4-dependent neurotransmitters (serotonin, dopamine, epinephrine, and norepinephrine, and L-dopa) are generally within normal limits. To our knowledge, only 1 prior study reports neurotransmitter metabolite levels in CM, and 5-HIAA was the only neurotransmitter metabolite measured in that [11]. Our study provides important evidence that coma in malaria is not due to insufficient levels of serotonin, dopamine, or the other catecholamines, epinephrine, and norepinephrine. Measurements of CSF 5-MTHF, a vitamin required for the BH_4 salvage pathway, and pyridoxyl-5-phosphate, a vitamin required for dopamine and serotonin synthesis, were likewise generally normal.

Our findings help clarify 2 previous studies of pterins in CM [11, 33] that had conflicting results. (1) The study by Weiss et al [33] did not measure biopterins with different oxidation states. They measured only CSF total biopterin (the sum of BH_4, BH_2, and B_0) in 130 Zambian children with CM and found total biopterin to be low [33]. (2) The study by Dobbie et al [11] used methods very similar to ours to measure BH_4 and BH_2 in 97 Kenyan children in the recovery phase of CM and found that BH_4 was increased compared with the reference range established in British children [23]. From our findings and those

Table 2. Cerebrospinal Fluid Concentrations (nmol/L) of Pterins and BH_4-Dependent Neurotransmitter Metabolites in Children With Cerebral Malaria and Children With Nonmalaria Central Nervous System Conditions

Analyte Tested (Number of CM/NMC)	CM	NMC	*P* Value	CM z-Score	NMC z-Score	*P* Value
BH_4 (49/51)	57 [37–69]	35 [23–53]	.001	0.75 [0.13–1.16]	−0.08 [−0.31 to 0.38]	<.001
BH_4 high (%)	31 (63.3)	8 (15.7)	<.001[a]			
BH_2 (49/51)	16 [10.4–20.0]	13 [7.6–30.0]	.60	0.65 [0.24–0.94]	0.43 [0.04–1.68]	.27
BH_2 high, n (%)	27 (55.1)	22 (43.1)	.23			
B_0 (49/50)	3.4 [2.2–5.5]	3.6 [2.5–7.7]	.25			
Total biopterin (49/50)	76.4 [56.7–92.6]	56.8 [42.6–92.7]	.06			
BH_4/BH_2 (49/50)	3.8 [2.9–4.7]	3.0 [1.2–5.0]	.13	1.18 [0.65–2.00]	0.18 [−0.37 to 1.56]	.70
BH_4/total biopterin (49/50)	0.8 [0.7–0.8]	0.7 [0.5–0.8]	.06			
Neopterin (49/51)	249 [152–367]	41 [27–148]	<.001	4.05 [2.07–5.98]	0.09 [−0.16 to 2.17]	<.001
Neopterin high, n (%)	49 (100)	20 (39.2)	<.001			
5-HIAA (50/51)	120 [72–161]	188 [114–319]	<.001	−0.32 [−0.53 to −0.15]	−0.25 [−0.44 to 0.00]	.044
5-HIAA low, n (%)	13 (26.0)	8 (15.7)	.09			
HVA (50/50)	393 [266–542]	510 [342–691]	.009	−0.25 [−0.45 to −0.06]	−0.18 [−0.35 to −0.04]	.18
HVA low, n (%)	6 (12.0)	6 (12.0)	.90			
3-OMD (50/51)	20 [15–32]	39 [22–60]	<.001	−0.86 [−0.88 to −0.78]	−0.82 [−0.87 to −0.68]	.26
3-OMD Low, n (%)	0 (0)	0 (0)	-	-	-	-
5-MTHF (45/50)	64 [54–86]	77 [53–109]	.32	−0.24 [−0.35 to −0.08]	−0.18 [−0.4 to −0.02]	.84
5-MTHF low, n (%)	4 (8.9)	5 (10.0)	.98			
Pyridoxine (9/5)	27 [20–31]	14 [11–19]	.06			
Pyridoxine low, n (%)	0 (0)	2 (40.0)	.040[a]			

Abbreviations: B_0, biopterin; BH_2, dihydrobiopterin; BH_4, tetrahydrobiopterin; CM, cerebral malaria; HVA, homovanillic acid; NMC, nonmalaria central nervous system conditions; 3-OMD, 3-*O*-methyldopa; 5-HIAA, 5-hydroxy-indoleacetate; 5-MTHF, 5-methyl-tetrahydrofolate.

[a] χ^2 test of proportions.

NOTES: Median [interquartile range], Wilcoxon rank-sum test unless otherwise indicated. Age-adjusted z-scores are provided for BH_4, BH_2, neopterin, 5-HIAA, HVA, 3-OMD, and 5-MTHF as well as the ratio of BH_4/BH_2 z-scores. See Methods for derivation of age-adjusted z-scores. Designations of "high" or "low" are based upon pediatric reference range values [24].

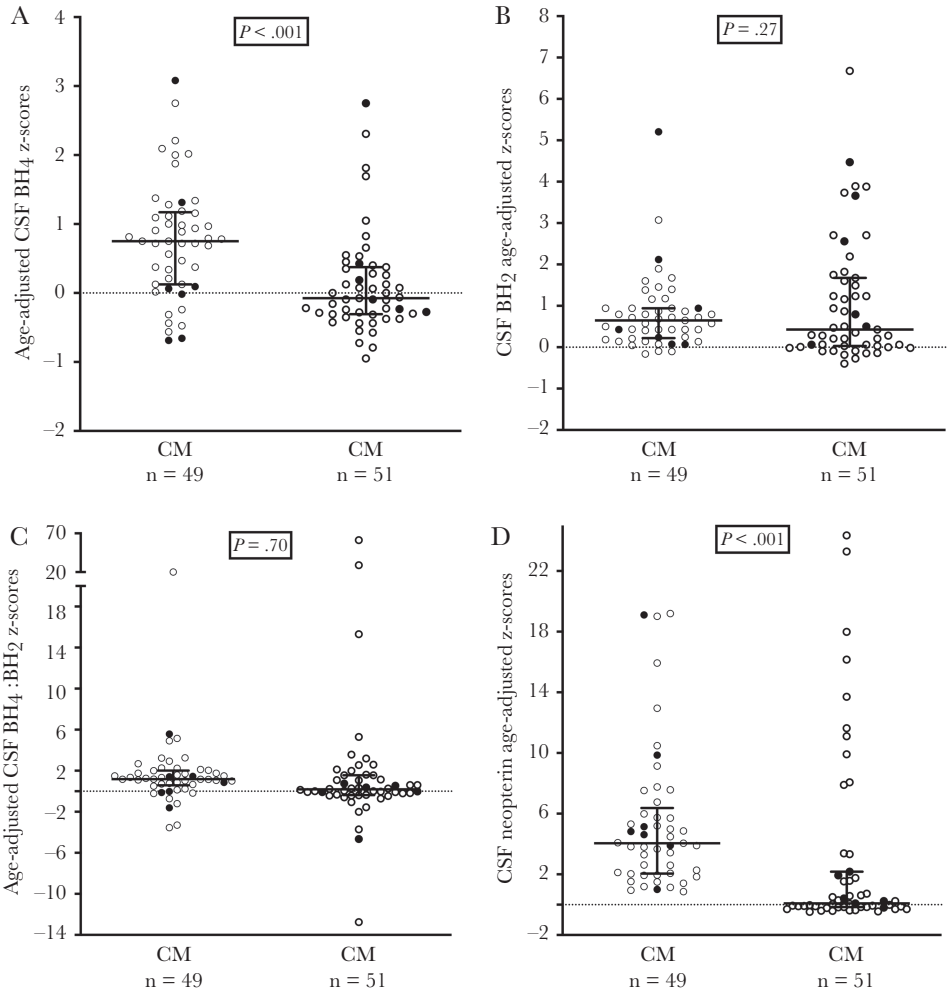

Figure 1. Cerebrospinal fluid (CSF) tetrahydrobiopterin, dihydrobiopterin, tetrahydrobiopterin/dihydrobiopterin, and neopterin measurements in children with cerebral malaria (CM) and children with nonmalaria central nervous system conditions (NMCs). Nanomolar measurements for each individual CM and NMC participant are presented as age-adjusted z-score to normalize comparisons of the 2 groups. Median is shown by horizontal mid-line and interquartile range is shown by upper and lower whiskers. Fatal cases are denoted by filled circles, whereas nonfatal cases are circles with no fill. (A) Cerebrospinal fluid tetrahydrobiopterin (BH_4) measurements. (B) Cerebrospinal fluid dihydrobiopterin (BH_2) measurements. (C) The ratio of CSF BH_4 to its oxidized metabolite, CSF BH_2. (D) Cerebrospinal fluid total neopterin measurements.

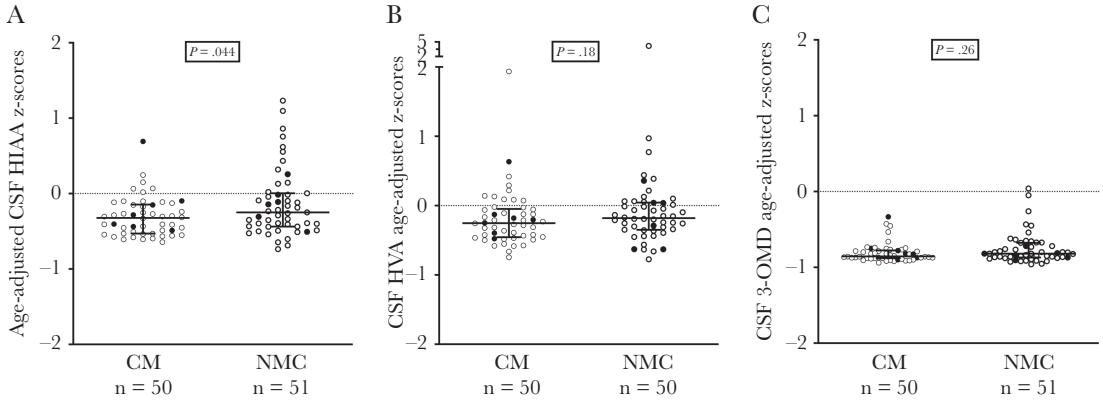

Figure 2. Cerebrospinal fluid measurements (CSF) of BH_4-dependent neurotransmitter metabolites in children with cerebral malaria (CM) and children with nonmalaria central nervous system conditions (NMCs). Nanomolar measurements for each individual CM and NMC participant are presented as age-adjusted z-score to normalize comparisons of the 2 groups. Median is shown by horizontal mid-line and interquartile range is shown by upper and lower whiskers. Fatal cases are denoted by filled circles, whereas nonfatal cases are circles with no fill. Cerebrospinal fluid 5-hydroxyindoleacetate (5-HIAA) measurements. (B) Cerebrospinal fluid homovanillic acid (HVA) measurements. (C) Cerebrospinal fluid 3-*O*-methyldopa (3-OMD) measurements.

of Dobbie et al [11], we conclude that coma in malaria is not a result of CNS BH_4 deficiency. Although we did not find an actionable CSF BH_4 deficiency, we found that pterin metabolism might have important prognostic significance. Despite our small sample size, we detected that an increased CSF BH_4/BH_2 ratio is associated with increased odds of survival. This ratio reflects the balance between reduced biopterin, BH_4, and the most abundant oxidized form of biopterin, BH_2; so its prognostic relevance might indicate the importance of maintaining CNS redox metabolism amid oxidative stress in CM [34, 35].

Our finding of elevated CSF BH_4 in CM stands in contrast to the concurrent decrease in systemic levels of BH_4, as measured by urinary excretion, in these same children [14]. In our view, the underlying mechanism for this difference is an open question. Given that BH_4 is unstable and readily oxidized, we speculate that the CNS has developed adaptive mechanisms to counteract oxidative stress so as to ensure adequate BH_4 for neurotransmitter synthesis, including NO synthesis. In comparing the CNS and systemic compartments, the levels of total biopterins ($BH_4 + BH_2 + B_0$) are elevated in both, but the ratios of reduced (BH_4) to oxidized BH_2 are high in the CSF (median 3.8; IQR, 2.9–4.7) and low in the urine (median 0.5; IQR, 0.2–1.1). Maintaining replete BH_4 is physiologically significant given that BH_4 deficiency leads to superoxide formation catalyzed by NOS, a process known as NOS uncoupling [36], and given that deficiency in BH_4 can lead to mitochondrial generation of reactive oxygen species, independent of NOS [37]. This suggests that BH_4 might be both an indicator of and a factor involved in CNS adaptive mechanisms to mitigate oxidative stress—mechanisms that are not present in the systemic compartment. To our knowledge, there are no prior published reports of elevated CSF BH_4 in CNS infections other than CM; but high CSF measurements of total biopterins is observed in inflammatory conditions such as Aicardi-Goutières and bacterial meningitis [20, 38], and clinical experience indicates that in those conditions, the elevated biopterins are predominantly BH_4 and not the oxidized forms, BH_2 or B_0 (K.H., unpublished data, 2020). Given the potential importance of elevated CSF BH_4 as a characteristic of CM and other serious inflammatory diseases of the brain, and because BH_4 is an obligate cofactor for NO synthesis, neurotransmitter synthesis, and redox metabolism, further studies are warranted to determine the biological significance of elevated CSF BH_4 in CM.

Cerebrospinal Fluid Neopterin Elevation in All Children With Cerebral Malaria

A potentially important finding from our study is the marked elevation of neopterin in the CSF of all children with CM. Cerebrospinal fluid neopterin is derived from cells of the mononuclear phagocyte lineage—brain monocytes/macrophages and microglial cells [39, 40]. It should be noted that intravascular monocyte infiltrates [41] and perivascular monocyte infiltrates [42] are significant brain pathology findings in retinopathy-positive CM. These mononuclear phagocytes and microglial cells lack the enzyme 6-pyruvoyltetrahydropterin synthase ([PTPS]; see Supplementary Figure 1), leading to the accumulation of dihydroneopterin triphosphate. When cells lacking PTPS are activated with interferon-γ or tumor necrosis factor-α, GTP cyclohydrolase ([GTPCH]; see Supplementary Figure 1) gene transcription is upregulated, augmenting the pathway to neopterins—NH_2 and the oxidized metabolite, N_0, measured collectively by our assay as total neopterin. Neopterin is a recognized biomarker of cell-mediated immune responses [19]. Elevated CSF neopterin has been observed in various CNS infections [20], including CM [11, 33], and in other inflammatory conditions, such as inherited type I interferonopathies [43, 44]. Although high CSF neopterin is not specific to malaria, in our study all CM children had CSF neopterin levels above an established reference range, and more than 75% had age-adjusted z-scores greater than 2. Our results suggest the potential clinical utility of measuring CSF neopterin in children presenting with coma—an absence of CSF neopterin elevation may be a

Table 3. Age-Adjusted z-Scores and *P* Values for Cerebrospinal Fluid Pterin and BH_4-Dependent Neurotransmitter Metabolites in Children With Cerebral Malaria and Nonmalaria Central Nervous System Conditions Who Had Unarousable Coma or Meningitis

Analyte	CM z-Score	NMC Coma z-Score (n = 11)	*P* Value/Adjusted *P* Value[a]	NMC Meningitis z-Score (n = 26)	*P* Value/Adjusted *P* Value[a]
BH_4	0.75 [0.13–1.16]	0.00 [−0.27 to 0.40]	.013/.07	−0.07 [−0.31 to 0.26]	<.001/.009
BH_2	0.65 [0.24–0.94]	1.24 [0.06–2.71]	.43/.84	1.24 [0.28–2.56]	.023/.36
BH_4/BH_2	1.18 [0.65–2.00]	0.23 [−0.02 to 1.13]	.08/.35	−0.03 [−0.34 to 0.54]	.001/.046
Neopterin	4.05 [2.07–5.98]	0.24 [−0.16 to 7.89]	.017/.08	0.52 [−0.07 to 9.91]	.029/.12
5-HIAA	−0.32 [−0.53 to −0.15]	−0.01 [−0.49 to 0.15]	.07/.18	−0.19 [−0.44 to 0.15]	.049/.17
HVA	−0.25 [−0.45 to −0.06]	−0.07 [−0.44 to 0.36]	.28/.42	−0.16 [−0.30 to 0.04]	.12/.27
3-OMD	−0.86 [−0.88 to −0.78]	−0.80 [−0.88 to −0.65]	.23//.40	−0.80 [−0.86 to −0.63]	.05/ .17
5-MTHF	−0.24 [−0.35 to −0.08]	−0.16 [−0.40 to 0.03]	.99//.99	−0.25 [−0.49 to −0.03]	.80/.80

Abbreviations: B_0, biopterin; BH_2, dihydrobiopterin; BH_4, tetrahydrobiopterin; CM, cerebral malaria; HVA, homovanillic acid; NMC, nonmalaria central nervous system conditions; 3-OMD, 3-*O*-methyldopa; 5-HIAA, 5-hydroxy-indoleacetate; 5-MTHF, 5-methyl-tetrahydrofolate.

[a] *P* value adjusted for multiple statistical comparisons using the Benjamini-Hochberg adaptive step-up procedure.

NOTES: Values presented are median [interquartile range]. See Methods for derivation of age-adjusted z-scores. Wilcoxon rank-sum test of significance. Cerebral malaria number tested 49 for BH4 and neopterin; 50 for 5-HIAA, HVA, 3-OMD; and 45 for 5-MTHF.

sensitive test to rule out CM. Validation of CSF neopterin with larger sample sizes from diverse malaria endemic-settings and in retinopathy-positive CM is warranted.

Study Limitations

We note several limitations. Our NMC cases were not age-matched to each CM case, resulting in a comparator group that was younger than our CM group. Nonmalaria central nervous system condition was uncommon in our enrollment sites rendering age-matching unfeasible for study timelines. Nevertheless, we expressed concentrations of pterins and neurotransmitter metabolites as age-adjusted z-scores, which normalize the data for statistical comparison. Reference ranges for CSF pterins and neurotransmitter metabolites used in this analysis were derived from European and North American populations, because no such reference ranges among African children are available. Our NMC group was etiologically heterogeneous, and only 11 had unarousable coma on par with CM case definitions. The WHO CM case definition that we used [21] did not require presence of retinopathy, a finding that improves the specificity for identifying CM. We did not include any direct measurements of NO.

CONCLUSIONS

We conclude that even though low systemic concentrations of BH_4 are characteristic of CM, there is not deficiency of CSF BH_4. Cerebrospinal fluid BH_4 concentrations are actually significantly elevated in CM, and levels of BH_4-dependent neurotransmitters are largely preserved. An elevated CSF BH_4/BH_2 ratio is associated with survival. Cerebrospinal fluid neopterin is significantly elevated in CM, and this finding may be useful in excluding the diagnosis of CM among children presenting with unarousable coma in malaria-endemic areas.

Supplementary Data

Supplementary materials are available at The *Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Supplementary 1. Methods.

Supplementary Figure 1. Tetrahydrobiopterin-dependent pathways for neurotransmitter synthesis.

Supplementary Figure 2. Receiver operator characteristic curve analysis of the diagnostic accuracy of CSF neopterin to distinguish cerebral malaria (CM) from nonmalaria CNS conditions (NMC).

Supplementary Table 1. Clinical characteristics among 9 children with a nonmalaria central nervous system condition for whom a pathogen was detected by metagenomic next-generation sequencing.

Supplementary Table 2. Cerebrospinal fluid concentrations (nmol/L) of pterins and BH_4-dependent neurotransmitter metabolites in children with cerebral malaria (CM) and children with nonmalaria central nervous system conditions who had unarousable coma (NMC Coma) or meningitis (NMC Meningitis).

Supplementary Table 3. Cerebrospinal fluid concentration of pterins and BH_4-dependent neurotransmitter metabolites in nonmalaria CNS conditions causing unarousable coma (NMC coma) and nonmalaria CNS conditions without coma (NMC without coma) causing unarousable (NMC coma) and children with nonmalaria CNS conditions NMC without coma.

Notes

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Author contributions. D. L. G., B. K. L., J. B. W., N. M. A., T. W. Y., and E. D. M. conceived and designed the study. S. M. F., J. P. M., E. D. M., D. L. G., J. B. W., and M. P. R. performed the study. K. H. performed the laboratory measurements of pterins and pterin-dependent neurotransmitters. C. L., S. N., and J. L. D. performed the metagenomics pathogen detection laboratory analyses. M. P. R., R. A. S., T. W. Y., N. M. A., D. L. G., and J. B. W. analyzed the data. M. P. R., S. M. F., J. P. M., E. D. M., N. M. A., T. W. Y., B. K. L., R. A. S., K. H., C. L., S. N., J. L. D., D. L. G., and J. B. W. wrote and edited the manuscript.

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