Journal of the Pediatric Infectious Diseases Society

ORIGINAL ARTICLE

Retinopathy-Positive Cerebral Malaria Is Associated With Greater Inflammation, Blood-Brain Barrier Breakdown, and Neuronal Damage Than Retinopathy-Negative Cerebral Malaria

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Background. Our prior study findings suggest that *Plasmodium falciparum* is the cause of disease in both malaria retinopathy-positive (RP) and most retinopathy-negative (RN) cerebral malaria (CM), and that absence of retinopathy and decreased disease severity in RN CM may be due to shorter duration of illness, lower parasite biomass, and decreased *var* gene expression in RN compared to RP CM. In the present study, we assessed the pathophysiology of RP and RN CM.

Methods. We compared markers of systemic and central nervous system inflammation, oxidative stress, neuronal injury, systemic endothelial activation, angiogenesis, and platelet activation in Ugandan children with RP (n = 167) or RN (n = 87) CM.

Results. RP children had higher plasma C-reactive protein ($P = .013$), ferritin and erythropoietin (both $P < .001$) levels, an elevated cerebrospinal fluid (CSF):plasma albumin ratio ($P < .001$), and higher CSF tau protein levels ($P = .049$) than RN children. Levels of plasma and CSF proinflammatory and anti-inflammatory cytokines and oxidative stress markers did not differ between RP and RN children. RN children had higher plasma levels of endothelin 1 ($P = .003$), platelet-derived growth factor ($P = .012$), and platelet factor 4 ($P = .034$).

Conclusions. RP and RN CM may represent different phases of CM. RN CM may be driven by early vasospasm and platelet activation, whereas the more advanced RP CM is associated with greater inflammation, increased erythropoietic drive, blood-brain barrier breakdown, and neuronal injury, each of which may contribute to greater disease severity.

Key words. blood-brain barrier; cerebral malaria; falciparum; inflammation; retinopathy; tau.

Malaria remains one of the world's deadliest diseases, and cerebral malaria (CM) is the most severe complication of malaria, with a case fatality rate varying from 15% to 40% and long-term neurocognitive sequelae detected in 24% of survivors [1–3]. Despite insights gleaned from a broad range of clinical, histopathological, and laboratory studies, the pathogenesis of CM remains incompletely understood. Several mechanisms have been proposed, including cytoadherence and sequestration of infected erythrocytes, platelet activation, excessive inflammatory cytokine production, endothelial injury, blood-brain barrier (BBB) dysfunction, and neuronal damage [4–7].

Among children with CM, a subset has a characteristic retinopathy that has diagnostic and prognostic significance [8, 9]. The presence and severity of malaria retinopathy (MR) correlates with parasite sequestration, markers of parasite biomass and virulence, and clinical severity of illness including coma duration and death [9–14]. It has been proposed that retinopathy-positive (RP) CM is true cerebral malaria, and that retinopathy-negative (RN) children may have alternative etiologies of coma with incidental parasitemia [8]. However, our recent studies show that *Plasmodium falciparum var* genes associated with severe malaria are expressed in children with RP and RN CM but not in children with asymptomatic ("incidental") parasitemia, though the *var* genes are expressed at lower levels in RN than RP CM; that parasite biomass in RN CM is lower than in RP CM but still higher than in another form of severe malaria, severe malarial anemia; and that children with RN CM typically present with shorter coma duration and typically have milder clinical illness than children with RP CM [11, 12, 14]. Together, these findings suggest that most cases of RN CM are caused by *P falciparum* and not alternative causes of coma, and that RN CM represents a milder form of

Received 25 June 2019; editorial decision 12 November 2019; accepted 15 November 2019; Published online December 6, 2019.

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Journal of the Pediatric Infectious Diseases Society 2020;9(5):580–6

DOI: 10.1093/jpids/piz082

CM than RP CM, possibly because of the earlier presentation of children with RN CM. In addition, a recent study using genetic traits to assess risk for severe malaria in Malawian children also concluded that many cases of RN CM are likely due to disease caused by *P falciparum* [15].

The studies to date provide strong evidence that *P falciparum* is the likely cause of most RN CM, and that *P falciparum var* gene expression and parasite biomass are contributors to disease severity, but few studies to date have assessed how these factors may affect pathophysiology in RN and RP CM. To explore how pathophysiologic mechanisms differ in RP and RN CM, we compared markers of endothelial activation, angiogenesis, platelet activation, oxidative stress, inflammation, and neuronal injury in patients with RP and RN CM.

MATERIALS AND METHODS

Study Population

The study was conducted at Mulago Hospital (Kampala, Uganda) from 2008 to 2013. Children with CM were enrolled if they were between 18 months and 12 years of age. CM was defined as coma (Blantyre Coma Scale score ≤2), *P falciparum* on blood smear, and no other known cause of coma (eg, meningitis, a prolonged postictal state, or hypoglycemia). Exclusion criteria included known chronic illness requiring medical care, known developmental delay, history of coma, head trauma, hospitalization for malnutrition, or cerebral palsy. Children with CM were managed according to the Ugandan Ministry of Health treatment guidelines current at the time of the study, including quinine for treatment of CM through December 2012 and artesunate during January–December 2013. This study population has recently been described in an article examining the clinical features of RP and RN CM [14].

Diagnosis of Retinopathy

Children were assessed for MR by means of indirect ophthalmoscopy. An ophthalmologist experienced in the evaluation of malarial retinopathy (Dr Susan Lewallen) trained the 2 senior study investigators (C. C. J. and R. O. O.), a study ophthalmologist, and study medical officers in identifying and recording findings of malarial retinopathy. The study investigators and ophthalmologist then continued training and assessing the study medical officers for accuracy in this assessment and recording of ophthalmoscopic findings, and interim retraining and assessment was done by Dr Simon Harding, a second expert in malaria retinopathy. Ophthalmology evaluation was done by medical officers in all CM patients on admission, and repeated every 24 hours while they remained comatose. Before each examination, pupils were dilated with sequential instillation of cyclopentolate 1% and tropicamide 1%. Using a binocular indirect ophthalmoscope, an eye examination was performed 30–60 minutes later. The findings were noted on case report forms with record made of the presence and severity of retinal hemorrhages, vessel changes, and retinal whitening. Children were considered to have malarial retinopathy if at least 1 of these findings was present.

Laboratory Testing

Peripheral blood smears were assessed for *Plasmodium* species by microscopy with Giemsa staining according to standard protocols. Two independent readings were conducted and, if not consistent, were resolved by a third reading. Plasma *P falciparum* histidine-rich protein 2 (*Pf*HRP-2) levels were quantified using the commercially available Malaria Ag complement enzyme-linked immunosorbent assay (ELISA) kit (Cellabs, Brookvale, Australia), and were used to calculate parasite biomass as described previously [11]. Plasma and cerebrospinal fluid (CSF) cytokine and chemokine levels were measured in a magnetic cytometric bead assay using a Bio-Plex Pro Human Cytokine Multiplex Assay (Bio-Rad, Hercules, California) for the following biomarkers: interleukin (IL) 1β, IL-1 receptor antagonist, IL-10, vascular endothelial growth factor, platelet-derived growth factor (PDGF-bb), interferon-γ, and granulocyte colony-stimulating factor; in a 1:4 dilution. Tumor necrosis factor alpha (TNF-α) and IL-6 levels were tested per the manufacturer's instructions using the Millipore Human Cytokine and Chemokine Kit (EMD-Millipore, Billerica, Massachusetts). Plasma soluble intracellular adhesion molecule 1 (sICAM-1), soluble vascular cellular adhesion molecule 1 (sVCAM-1), soluble E-selectin, and soluble P-selectin were also measured by magnetic cytometric bead assay (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions. Plasma concentrations of C-reactive protein (CRP) and ferritin were measured with the use of a Luminex immunoassay (Milliplex MAP kit; EMD Millipore, Billerica, Massachusetts) and a standard ELISA kit (Ramco Laboratories Inc, Stafford, Texas), respectively. Human endothelin-1 (ET-1) concentrations in plasma were measured using the QuantiGlo Human Endothelin-1 Immunoassay (R&D Systems, Minneapolis, Minnesota). Platelet factor 4 (PF-4) was measured using the IMUCLONE Platelet Factor 4 ELISA (American Diagnostica Inc, Stamford, Connecticut). CSF tau was measured using the Luminex-based Human Tau (total) Singleplex Bead Kit (Invitrogen, Carlsbad, California) and the Human Neuroscience Buffer Reagent Kit (Invitrogen). All cytometric bead assay testing was conducted using the BioPlex-200 system (Bio-Rad).

Plasma angiopoietin 1 and 2 (Ang-1 and -2) and von Willebrand factor (vWF) levels were quantified using the human Ang-1 and 2 DUO ELISA kit (R&D Systems) and REAADS vWF activity ELISA kit (Corgenix, Broomfield, Colorado), respectively. Plasma and CSF malondialdehyde (MDA) was measured by the measurement of thiobarbituric acid reactive substances using high-pressure liquid chromatography (Eagle Biosciences,

Amherst, New Hampshire). Cytosolic Cu/Zn superoxide dismutase 1 (SOD-1) expression was measured in the plasma and CSF by commercial ELISA (Calbiochem, San Diego, California). Plasma and CSF erythropoietin (EPO) levels were tested via a high-sensitivity radioimmunoassay, as previously described [16]. Plasma protein carbonyls were measured by Protein Carbonyl ELISA (OxiSelect Protein Carbonyl ELISA Kit, Cell BioLabs, San Diego, California). Plasma heme oxygenase 1 (HO-1) levels were measured using the Human HO-1 ELISA Kit (Stressgen, Bioreagents Corporation, Ann Arbor, Michigan). CSF neuron-specific enolase (NSE) levels in plasma were measured per the manufacturer's instructions using the NSE Enzyme Immunoassay Kit (ALPCO Diagnostics, Salem, New Hampshire).

Statistical Analysis

Data were analyzed using Stata/SE version 13.1 software (StataCorp, College Station, Texas). The Student *t* test was used to compare means of normally distributed data. For nonnormally distributed continuous variables, we used the Kruskal-Wallis test. $P < .05$ was considered significant. For each table, which represents potential different mechanisms associated with RP vs RN CM, the Benjamini-Hochberg correction was used to adjust for multiple comparisons.

Ethics Statement

Ethical approval was granted by the institutional review boards for human studies at the Makerere University School of Medicine and the University of Minnesota. Written consent was obtained from parents or guardians of study participants.

RESULTS

During the period of study, a total of 261 children with CM were enrolled. Indirect ophthalmoscopy was performed on 254 children, who were classified into 2 groups based on fundoscopic findings: RN (n = 87) and RP (n = 167). Seven children did not have a documented fundoscopic examination due to equipment or staffing issues. As previously described, there was no significant difference in time to first ophthalmologic assessment between the 2 groups, and in 54 eye examinations done by both an ophthalmologist (within 6–12 hours of the initial medical officer and examination) and a medical officer, 18.5% had discordant findings. Thirteen percent were findings of retinopathy by the ophthalmologist with a negative examination by the medical officer, whereas 5.5% were findings of no retinopathy by the ophthalmologist in a child previously noted to have retinopathy by the medical officer [14].

Demographic and Clinical Characteristics

The age range was from 1.5 to 11.7 years, with a mean age of 3.89 (standard deviation, 1.93) years. Of the 261 patients, 154 (59%) were males. There were no significant differences in age or sex between groups (Table 1). As reported earlier, duration of fever did not differ significantly between groups, but duration of coma was longer in children with RP CM and mortality was also higher in RP CM, though this difference did not reach statistical significance (Table 1). Comprehensive demographic and clinical characteristics, including measures of *Pf*HRP-2, a marker of parasite biomass, have been described previously [11, 14].

Endothelial Activation, Endothelin-1 Concentrations, and Angiogenesis

There were no differences in plasma concentrations of standard endothelial activation markers (vWF, E-selectin, P-selectin, sICAM-1, or sVCAM-1) between children with RP and RN CM (Table 2). However, plasma concentrations of ET-1, a marker of endothelial vasospasm, were higher in children with RN CM ($P = .003$). Among markers of angiogenesis, a trend toward a higher Ang-2:Ang-1 ratio was seen in children with RP CM ($P = .061$, Table 3).

Platelet Activation

Children with RN CM had higher platelet-derived growth factor ($P = .012$) and PF-4 ($P = .034$), 2 factors released during platelet activation, than children with RP CM (Table 4).

Oxidative Stress and Ischemia

There were no significant differences in plasma or CSF markers of oxidative stress (protein carbonyls, MDA, SOD-1, or HO-1 concentrations) in children with RP compared to RN CM (Table 5).

In contrast, both plasma and CSF EPO levels were significantly higher in children with RP compared to RN CM (both $P < .001$, Table 6), and this difference remained statistically significant after controlling for hemoglobin levels ($P = .01$ and $P = .03$, respectively).

Table 1. Demographic Characteristics and Mortality in Children With Retinopathy-Positive Versus Retinopathy-Negative Cerebral Malaria

Characteristic	RP (n = 167)	RN (n = 87)	*P* Value[a]
Age, y, mean (SD)	3.80 (1.97)	4.12 (1.88)	.217
Male sex, % (95% CI)	57.5 (49.6–65.1)	60.9 (49.9–71.2)	.598
Reported duration of coma before presentation, h, mean (SD)	17.9 (14.3)	13.8 (11.3)	.023
Reported duration of fever before presentation, d, mean (SD)	3.33 (1.46)	3.10 (1.45)	.242
Mortality rate, % (95% CI)	14.4 (9.4–20.6)	8.0 (3.3–15.9)	.144
Coma duration in hospital, h, median (IQR)	58.0 (32.0–88.0)	38.8 (20.0–56.3)	**< .001**

Bold values indicate statistical significance at $P < .016$.

Abbreviations: CI, confidence interval; IQR, interquartile range; RN, retinopathy negative; RP, retinopathy positive; SD, standard deviation.

[a]For categorical data, the χ^2 test was used to compare the 2 groups. For data represented with mean and SD, the *t* test was used. For data represented with median and IQR, the Kruskal-Wallis test was used. *P* values < .016 were considered statistically significant after correction for multiple comparisons.

Table 2. Plasma Markers of Endothelial Activation and Vasospasm in Children With Retinopathy-Positive Versus Retinopathy-Negative Cerebral Malaria

Marker	RP (n = 167)	RN (n = 87)	*P* Value[a]
vWF, % of normal	177 (101–282) n = 133	160 (97–277) n = 63	.561
E-selectin, ng/mL	180 (144–239) n = 143	192 (136–254) n = 68	.397
P-selectin, ng/mL	55 (37–79) n = 138	51 (39–78) n = 63	.893
sICAM-1, ng/mL	674 (242–1541) n = 143	796 (261–1523) n = 68	.770
sVCAM-1, ng/mL	3826 (2715–5985) n = 143	3847 (2727–7185) n = 68	.502
Endothelin-1, pg/mL	0.62 (0.41–0.93) n = 160	0.78 (0.54–1.15) n = 80	**.003**

Bold values indicate statistical significance at $P < .017$.
Data are presented as median (interquartile range) unless otherwise indicated.
Abbreviations: RN, retinopathy negative; RP, retinopathy positive; sICAM-1, soluble intracellular adhesion molecule 1; sVCAM-1, soluble vascular cellular adhesion molecule 1; vWF, von Willebrand factor.
[a]The Kruskal-Wallis test was used to compare groups. *P* values of < .017 were considered statistically significant after correction for multiple comparisons.

Inflammation

Children with RP CM had higher levels of CRP ($P = .013$) and ferritin ($P < .001$), acute-phase reactants that serve as markers of inflammation, than children with RN CM (Table 6). However, there were no significant differences in proinflammatory or anti-inflammatory cytokines between the 2 groups (Table 7).

BBB and Neuronal Injury

Children with RP CM had a significantly higher CSF:plasma albumin ratios than children with RN CM ($P < .001$), indicative of increased BBB breakdown in children with RP CM (Table 7). Children with RP CM also had increased concentrations of CSF tau, a marker of neuronal damage, than children with RN CM ($P = .049$), suggesting greater CNS neuronal damage in the children with RP CM (Table 8).

Table 3. Plasma Markers of Angiogenesis in Children With Retinopathy-Positive Versus Retinopathy-Negative Cerebral Malaria

Marker	RP (n = 167)	RN (n = 87)	*P* Value[a]
Ang-1, platelet-free plasma, ng/mL	2.67 (1.44–4.98) n = 151	3.39 (1.51–6.89) n = 74	.181
Ang-2, platelet-free plasma, ng/mL	1.91 (1.02–3.28) n = 151	1.80 (0.75–3.14) n = 74	.377
Ang-2:Ang-1 ratio, platelet-free plasma	0.73 (0.33–1.77) n = 151	0.53 (0.25–0.99) n = 74	.061
VEGF, pg/mL	51 (30–98) n = 156	48 (38–95) n = 76	.856
FGF, pg/mL	30 (13–45) n = 156	35 (11–55) n = 76	.335

Data are presented as median (interquartile range) unless otherwise indicated.
Abbreviations: Ang-1, angiopoietin 1; Ang-2, angiopoietin 2; FGF, fibroblast growth factor; RN, retinopathy negative; RP, retinopathy positive; VEGF, vascular endothelial growth factor.
[a]The Kruskal-Wallis test was used to compare groups. *P* values of < .01 were considered statistically significant after correction for multiple comparisons.

Table 4. Plasma Markers of Platelet Activation in Children With Retinopathy-Positive Versus Retinopathy-Negative Cerebral Malaria

Marker	RP (n = 167)	RN (n = 87)	*P* Value[a]
Platelet count, $\times 10^3/\mu L$	61 (37–108) n = 163	57 (30–111) n = 87	.896
PDGF-bb, pg/mL	483 (232–1134) n = 156	762 (313–1528) n = 76	**.012**
PF-4, platelet-free plasma, ng/mL	370 (194–690) n = 149	467 (269–1241) n = 70	.034

Bold values indicate statistical significance at $P < .017$.
Data are presented as median (interquartile range) unless otherwise indicated.
Abbreviations: PDGF-bb, platelet-derived growth factor; PF-4, platelet factor 4; RN, retinopathy negative; RP, retinopathy positive.
[a]The Kruskal-Wallis test was used to compare groups. *P* values of < .017 were considered statistically significant after correction for multiple comparisons.

DISCUSSION

The present study adds to our knowledge about what contributes to RP or RN CM by providing novel data showing that RP CM is characterized by increased systemic inflammation, erythropoietic drive and oxygen demand, BBB breakdown, and CNS neuronal damage than RN CM, whereas RN CM is characterized by greater platelet activation and ET-1 concentrations than RP CM. Together with our prior study findings of prolonged coma duration, higher *P falciparum* parasite biomass, and greater *var* gene expression in RP than RN CM, the current study findings suggest that children with RN CM may present earlier in the disease process, at a stage where

Table 5. Plasma and Cerebrospinal Fluid Markers of Oxidative Stress in Children With Retinopathy-Positive Versus Retinopathy-Negative Cerebral Malaria

Marker	RP (n = 167)	RN (n = 87)	*P* Value[a]
Plasma			
Protein carbonyl, nmol/mg	1225 (897–1885) n = 121	1264 (894–1812) n = 50	.938
MDA, µmol/L	1.35 (1.01–1.62) n = 46	1.37 (0.98–1.62) n = 22	.849
SOD-1, ng/mL	340 (208–508) n = 132	291 (216–478) n = 59	.838
HO-1, ng/mL	17 (10–28) n = 137	18 (8–26) n = 65	.662
Erythropoietin, mU/mL	1379 (534–3240) n = 138	388 (89–1154) n = 63	**< .001**
Cerebrospinal fluid			
MDA, µmol/L	0.35 (0.28–0.48) n = 87	0.35 (0.28–0.47) n = 43	.919
SOD-1, ng/mL	88 (59–125) n = 101	78 (49–119) n = 44	.430
Erythropoietin, mU/mL	12 (7–22) n = 100	4 (3–7) n = 52	**< .001**

Bold values indicate statistical significance at $P < .01$ for plasma and $P < .017$ for cerebrospinal fluid.
Data are presented as median (interquartile range) unless otherwise indicated.
Abbreviations: HO-1, heme oxygenase 1; MDA, malondialdehyde; RN, retinopathy negative; RP, retinopathy positive; SOD-1, superoxide dismutase 1.
[a]The Kruskal-Wallis test was used to compare groups. *P* values of < .01 for plasma and < .017 for cerebrospinal fluid were considered statistically significant after correction for multiple comparisons.

Table 6. Plasma Markers of Inflammation in Children With Retinopathy-Positive Versus Retinopathy-Negative Cerebral Malaria

Marker	RP (n = 167)	RN (n = 87)	*P* Value[a]
CRP, μg/mL	642 (467–863) n = 157	522 (313–736) n = 77	**.013**
Ferritin, ng/mL	1241 (795–1945) n = 159	821 (480–1399) n = 81	**< .001**

Bold values indicate statistical significance at $P < .05$.
Data are presented as median (interquartile range) unless otherwise indicated.
Abbreviations: CRP, C-reactive protein; RN, retinopathy negative; RP, retinopathy positive.
[a]The Kruskal-Wallis test was used to compare groups.

Table 8. Cerebrospinal Fluid Markers of Neuronal Damage in Children With Retinopathy-Positive Versus Retinopathy-Negative Cerebral Malaria

Marker	RP (n = 167)	RN (n = 87)	*P* Value[a]
Tau, pg/mL	449 (266–1020) n = 100	276 (196–862) n = 44	**.049**
Neuron-specific enolase, ng/mL	2.80 (1.58–5.03) n = 99	2.35 (1.43–4.78) n = 44	.696

Bold values indicate statistical significance at $P < .05$.
Data are presented as median (interquartile range) unless otherwise indicated.
Abbreviations: RN, retinopathy negative; RP, retinopathy positive.
[a]The Kruskal-Wallis test was used to compare groups.

platelet activation and a degree of endothelial vasospasm may predominate, and in which inflammation, BBB breakdown, and neuronal damage are less advanced and less severe than in RP CM [11, 12, 14]. Children with RP CM also had significantly higher plasma and CSF levels of EPO, a marker of oxygen demand and erythropoietic drive that has been shown to be associated with prolonged coma duration and mortality in CM [16]. These are systemic markers of illness severity, which correlate with the more severe illness that has been reported for RP CM children and that have been described in this cohort of patients [13, 14, 17].

Table 7. Plasma and Cerebrospinal Fluid Proinflammatory and Anti-inflammatory Cytokines in Children With Retinopathy-Positive Versus Retinopathy-Negative Cerebral Malaria

Cytokine	RP (n = 167)	RN (n = 87)	*P* Value[a]
Plasma			
TNF-α, pg/mL	104 (46–190) n = 158	123 (54–211) n = 80	.618
G-CSF, pg/mL	71 (36–169) n = 156	86 (48–214) n = 76	.257
IFN-γ, pg/mL	92 (60–157) n = 156	116 (60–233) n = 76	.069
IL-1β, pg/mL	3.03 (2.02–5.00) n = 156	3.67 (2.08–5.78) n = 76	.297
IL-1 receptor antagonist, pg/mL	1493 (506–4474) n = 156	1308 (469–3736) n = 76	.891
IL-10, pg/mL	230 (82–818) n = 156	296 (80–663) n = 76	.982
Cerebrospinal fluid			
CSF:plasma albumin ratio	7.2 (3.8–12.6) n = 104	3.4 (2.5–4.8) n = 45	**< .001**
TNF-α, pg/mL	1.46 (0.55–3.71) n = 117	1.20 (0.55–2.25) n = 57	.131
G-CSF, pg/mL	50 (22–134) n = 101	32 (23–85) n = 44	.222
IFN-γ, pg/mL	22 (12–32) n = 101	22 (15–38) n = 44	.452
IL-1β, pg/mL	0.48 (0.48–0.48) n = 101	0.48 (0.48–0.48) n = 44	.341
IL-1 receptor antagonist, pg/mL	51 (30–100) n = 101	43 (28–70) n = 44	.491
IL-10, pg/mL	5.13 (3.45–11.57) n = 101	4.66 (3.17–7.77) n = 44	.315

Bold values indicate statistical significance at $P < .014$.
Data are presented as median (interquartile range) unless otherwise indicated.
Abbreviations: CSF, cerebrospinal fluid; G-CSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; RN, retinopathy negative; RP, retinopathy positive; TNF, tumor necrosis factor.
[a]The Kruskal-Wallis test was used to compare groups. *P* values of < .014 were considered statistically significant after correction for multiple comparisons.

Prior studies have shown higher Ang-2 concentrations and Ang-2:Ang-1 ratios in RP compared to RN CM [18], and a lack of difference in vWF levels in RP and RN CM [19], both similar to our findings. The findings regarding other markers of endothelial activation, endothelial vasospasm, platelet activation, inflammation, oxidative stress, and erythropoietic drive and oxygen demand, as well as central nervous system inflammation and neuronal injury, are new, and may provide valuable insights into the pathophysiology of the 2 conditions.

The primary biomarkers seen predominantly in children with RN CM were higher plasma ET-1 concentrations and higher levels of PF-4 and PDGF-bb, which are both markers of platelet activation. ET-1 has been shown to be a key mediator of microvascular dysfunction in murine models of CM [20, 21]. Interestingly, these findings have been recently corroborated by a study from the Democratic Republic of Congo in which transcranial Doppler (TCD) ultrasound showed cerebral vasospasm in 13% of children with RP CM. Children with vasospasm were more likely to survive, albeit with more neurologic sequelae than children with microvascular obstruction, hyperemia, or low flow [22]. Unfortunately, this study did not assess TCD findings in RN CM, but future studies should evaluate the correlation between TCD findings and plasma ET-1 levels. Studies assessing ET-1 in children have reported a wide range of normal levels, so the difference in levels that would lead to a physiologic change such as vasospasm is unclear and requires further study. Increased vasospasm could explain, in part, the onset of coma prior to the development of retinopathy, though vasospasm may also occur in RP CM, as shown in the Democratic Republic of Congo study. Indeed, though ET-1 levels differed in RP and RN CM, outliers were seen in both groups. Platelet activation has been shown to be important in the pathogenesis of CM but may be pathogenic or protective, depending on the timing of activation [23, 24]. It is possible that increased platelet activation early in disease may limit parasite growth early in infection, leading to less severe disease in RN CM children.

In contrast, children with RP CM had higher plasma CRP and ferritin concentrations, a trend toward higher Ang-2:Ang-1 ratios, higher plasma and CSF EPO concentrations, and a higher CSF:plasma albumin ratio and CSF tau concentrations than children with RN CM. However, there were no differences in other key disease mediators, including plasma and CSF cytokines and chemokines, plasma and CSF oxidative stress markers, and plasma markers of endothelial activation. Thus, the higher parasite biomass and *var* gene expression in RP CM lead to differences in systemic inflammation but not in specific proinflammatory cytokines or oxidative stress; in endothelial dysfunction but not activation; and in evidence of oxygen demand (EPO). One prior study showed increased TNF-a and IL-10 levels in children with RP compared to RN CM [25], findings that differ from the present results and require further study. Together, the findings from the present study suggest that higher levels of systemic inflammation, endothelial dysregulation, and oxygen demand, presumably from greater ischemia with increased sequestration, may lead to greater BBB breakdown and CNS neuronal damage in RP as compared to RN CM.

That markers of vasospasm and platelet activation are elevated in RN CM supports that this condition likely represents the same pathophysiologic mechanism as is proposed for RP CM. Of note, endothelial damage and platelet activation are early components in the proposed series of events leading to pathology in cerebral malaria [5], with inflammation, BBB breakdown, and neuronal injury, which are elevated in patients with RP CM in our study, occurring later in the cascade. This supports the idea that RN CM may be an earlier, less severe form of the same disease process.

A limitation of our study was the lack of examinations by 2 examiners on every child, as previously described [14]. In the subgroup of children who were examined by an ophthalmologist within 12 hours of initial examination, there was discordance of 18.5% on examinations. Changes in malaria retinopathy status can occur over time, so these results may well reflect true variability in examinations [9]. Alternatively, it is possible that there may have been misclassification of retinopathy findings in a small number of children. Still, the percentage of RN children in our cohort was similar to that in studies in Malawi that pioneered this evaluation [13], our group was trained by experts from the Malawi group, and we found a significant difference in *Pf*HRP-2 levels between RP and RN children, as did the Malawi group, all of which would suggest that the eye examinations done in this study were as accurate as can be obtained in this population. One implication is that our category of RN CM children is likely to include true cases of retinopathy below our level of detection. This may be addressed in the future by the ongoing development of digital fundoscopy combined with automated detection of malaria retinopathy [26]. There may be heterogeneity among children with RN CM, and some may still have incidental parasitemia with another cause of coma, but the findings of this study combined with those of our prior study and a recent study from Malawi suggest that *P falciparum* may be responsible for clinical disease in many children with RN CM [14, 15].

We conclude that children with RP and RN cerebral malaria appear to be on a spectrum of illness, and further that RN CM appears to be a pathophysiologically earlier presentation, with greater contributions of vasospasm and platelet activation to the clinical presentation. In our study, children with RP CM presented with greater markers of inflammation, BBB breakdown, and neuronal injury, late findings in the pathophysiology cascade, and markers consistent with the greater severity of illness on presentation. Based on these findings, we hypothesize that *P falciparum* is responsible for illness in most cases of RN CM, and that RN often represents an earlier presentation of CM than RP CM. Further studies are required to determine whether children with RP CM develop more severe illness than children with RN CM because of prolonged duration of illness prior to seeking care, as suggested by the longer duration of coma in children with RP CM; because children with RN CM are better able to control parasitemia and sequestration due to factors such as increased early platelet activation; or because of some combination of these factors.

Notes

Acknowledgments. The authors thank the children and families who participated in the study; the clinical staff at Mulago Hospital; and Dr Susan Lewallen for training the clinical staff.

Financial support. This work was supported by the US National Institutes of Health National Institute of Neurological Disorders and Stroke and the Fogarty International Center (grant numbers R01 NS055349 and D43 NS078280) and the Doris Duke Charitable Foundation through a grant supporting the Doris Duke International Clinical Research Fellows Program at the University of Minnesota. C. V. was a Doris Duke International Clinical Research Fellow.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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