

High Plasma Levels of Soluble Endothelial Protein C Receptor Are Associated With Increased Mortality Among Children With Cerebral Malaria in Benin

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Loss of endothelial protein C receptor (EPCR) occurs at the sites of *Plasmodium falciparum*-infected erythrocyte sequestration in patients with or who died from cerebral malaria. In children presenting with different clinical syndromes of malaria, we assessed the relationships between endogenous plasma soluble EPCR (sEPCR) levels and clinical presentation or mortality. After adjustment for age, for treatment before admission, and for a known genetic factor, sEPCR level at admission was positively associated with cerebral malaria ($P = .011$) and with malaria-related mortality ($P = .0003$). Measuring sEPCR levels at admission could provide an early biological marker of the outcome of cerebral malaria.

Keywords. plasmodium; cerebral malaria; EPCR; sEPCR; protein C.

Cerebral malaria is one of the most lethal complications of *Plasmodium falciparum* malaria. Over 500 000 children in Africa develop this syndrome, with a mortality rate of approximately 20% [1]. Children surviving cerebral malaria have neurological sequelae that manifest as long-term neurocognitive impairments [2]. The cytoadhesion of *P. falciparum*-infected

erythrocytes to endothelial cells in microvessels, via parasite proteins belonging to the *P. falciparum* erythrocyte membrane protein 1 family is considered to be involved in the pathogenesis of complicated malaria. More recently, it has been demonstrated that the endothelial protein C (PC) receptor (EPCR) acts as an endothelial receptor for infected erythrocytes in cerebral malaria [3]. Of note, significantly higher binding in vitro to human brain microvascular endothelial cells via EPCR is observed with parasite isolates from patients with severe malaria, compared with observations involving isolates from children with uncomplicated or mild malaria [3].

EPCR is a 46-kDa type 1 transmembrane glycoprotein, homologous to major histocompatibility complex class I/CD1 family proteins, that is expressed mainly on the luminal surface of endothelial cells. It is encoded by the endothelial PC receptor gene (PROCR), located on chromosome 20 [4]. EPCR is one of the most important components of the PC pathway, classically known as the anticoagulant system. Endothelial cell-bound EPCR is a receptor for PC, and binding to EPCR increases the rate of PC activation by thrombin-thrombomodulin complexes. EPCR also exists in a plasma-soluble form (sEPCR) that binds PC and activated PC with similar affinity [5, 6]. sEPCR levels in plasma at steady state are mostly determined by the genotype of rs867186 (Ser219Gly), a nonsynonymous single-nucleotide polymorphism (SNP) of the PROCR gene [7, 8]. The rs867186-G allele is strongly associated with increased sEPCR levels and with protection from severe malaria [9]. In a recent study of Malawian children with cerebral malaria, loss of endothelial cell-bound EPCR was demonstrated at the sites of infected erythrocyte cytoadherence [10]. In light of these studies, we designed and conducted a study to investigate the relationship between sEPCR plasma levels with clinical manifestations of malaria and associated mortality.

METHODS

Study Design and Malarial Patients

This study was conducted in 3 hospital centers in Cotonou, southern Benin, during the 2013 and 2014 malaria transmission seasons (June–September and May–July, respectively). Children <6 years of age presenting at Hôpital Mère-Enfant de la Lagune, Centre National Hospitalier Universitaire Hubert Koutoukou Maga, and Hôpital Suru-Léré were screened by a rapid diagnostic test for malaria (DiaQuick Malaria *P. falciparum* Cassette, Dialab; Hondastrasse, Austria). Children were included if they presented with a diagnosis either of cerebral malarial, severe but noncerebral malaria (hereafter, “severe malaria”), or

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uncomplicated malaria. Cerebral malaria was defined as *P. falciparum* infection and a Blantyre coma score of ≤ 2 , with no other known cause of coma. Lumbar puncture examination was performed to exclude bacterial infections, such as meningitis. No other obvious respiratory infections were recorded. Severe malaria was defined as the presence of one of the following signs: high *P. falciparum* parasitemia ($>250\,000$ parasites/ μL), *P. falciparum* parasitemia, and severe anemia (hemoglobin level, <5 g/dL). Uncomplicated malaria was defined as *P. falciparum* parasitemia accompanied by fever, headache, or myalgia without signs of severity and/or evidence of vital organ dysfunction, as defined by the World Health Organization (WHO) [11].

After obtaining informed and written consent from parents or guardians, 2–4-mL venous blood samples were collected into tubes containing citrate phosphate dextrose adenine. Fifty microliters of blood was transferred to Whatman 3MM filter paper and stored at room temperature for DNA extraction. Blood samples were centrifuged at 1000g for 15 minutes to obtain plasma that was immediately stored at -80°C until use. Children were followed until hospital discharge. Control visits occurred on day 30 after admission, and repeat blood samples were collected and processed as described above.

Plasmodium falciparum infections were confirmed by microscopy of Giemsa-stained thick blood smears, and parasitemia was recorded as the number of asexual parasites per microliter of blood. Children were treated according to Benin Ministry of Health guidelines. All patients with cerebral malaria or severe noncerebral malaria received parenteral quinine, while patients with uncomplicated malaria received either quinine or oral artemisinin-based combination therapy. Blood transfusion was performed for patients with severe anemia. Diazepam was administered in cases of convulsions.

Ethics Statement

This study was reviewed and approved by the ethics committee of the Research Institute of Applied Biomedical Sciences, Cotonou, Benin (protocols 006/CER/ISBA/12 and 21/CER/ISBA/13).

sEPCR Measurement in Plasma

Plasma sEPCR levels were measured using a commercially available quantitative sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (human soluble endothelial PC receptor sEPCR ELISA kits, Aviscera Bioscience, Santa Clara). Samples were diluted at a ratio of 1:20 and assayed in duplicate. Mean values of duplicates were used for quantification on a standard curve, in accordance with the manufacturer's instructions.

DNA Extraction and PROCR rs867186 SNP Genotyping

Genomic DNA was extracted from filter paper, using the Chelex 100 resin method [12], and genomic DNA were stored at -20°C

for genotyping of the rs867186 SNP by sequencing. The following primers were used to amplify a 660-bp polymerase chain reaction product: forward: CACACGCAGCTTCAGTCAGT; and reverse: TCCCATCCCAAGTCTGACAC [9]. PCR amplification was performed under the following conditions, using a Biometra Thermocycler 3000: 95°C for 15 minutes, and then 35 cycles of 95°C for 30 seconds, 60°C for 40 seconds, and 72°C for 1 minute. PCR products were purified and DNA sequence was determined by Sanger sequencing (GATC Biotech).

Statistical Analysis

Groups were compared by nonparametric tests (the Kruskal–Wallis test was used for continuous variables, and the Fisher exact test was used for categorical variables). Associations between log-transformed sEPCR levels and clinical expression of malaria were tested by linear regression, with adjustment for potential confounding factors with a *P* value of $< .2$ in univariate analyses: age of the patients (continuous), rs867186G mutation in the PROCR gene, or treatment before admission.

RESULTS

Baseline Characteristics of the Study Population, Sample Collection, and Follow-up

A total of 188 children <6 years of age were included: 58 had cerebral malaria, 77 had severe malaria, and 53 had uncomplicated malaria. Patients' characteristics at admission are shown in [Supplementary Table 1](#). The genotype frequencies of the AA and AG alleles of rs867186 (0.84 and 0.16, respectively) showed no difference between clinical groups ($P = .9$). No patient was homozygous recessive rs867186GG.

Seven patients with cerebral malaria and 1 patient with severe malaria were lost to follow-up. Three patients with severe malaria were referred to the University Hospital in Cotonou for special care. Twenty-four deaths were recorded among patients with cerebral malaria (fatality rate, 47%), and 6 deaths occurred among patients with severe malaria (fatality rate, 8%). No patient with uncomplicated malaria died.

Twenty-seven survivors of cerebral malaria were seen at day 30, of whom only 14 permitted collection of blood samples. Similarly, 67 patients with severe malaria were seen at day 30, of whom only 25 permitted blood sample collection. All patients with uncomplicated malaria were seen at day 30, but only 27 permitted collection of blood samples.

Plasma sEPCR Levels and Malaria Status

The level of sEPCR was quantified in plasma specimens obtained at admission from all patients and in plasma specimens obtained 30 days after admission. At admission, median sEPCR levels in patients with cerebral malaria were 2-fold higher than those in patients with severe malaria and patients with uncomplicated malaria (523 ng/mL vs 298 ng/mL and 250 ng/mL,

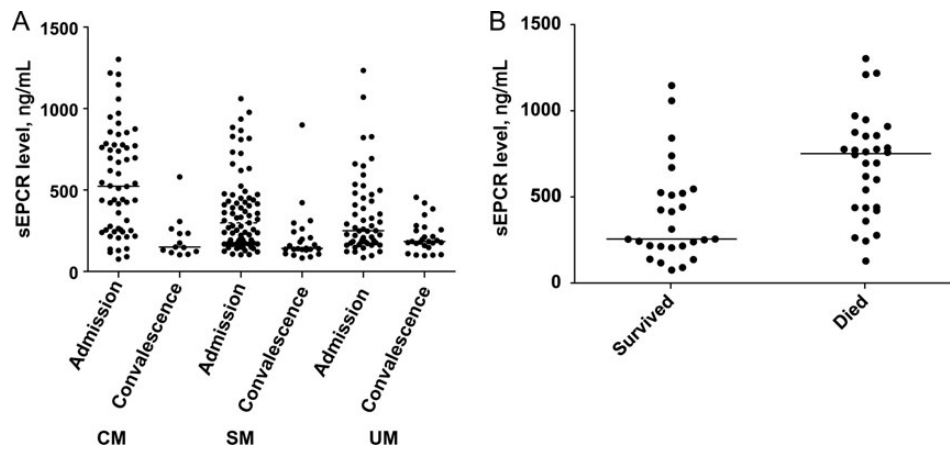


Figure 1. Plasma levels of soluble endothelial protein C receptor (sEPCR), according to malaria clinical form and outcome. Bars represent median values. *A*, sEPCR levels in plasma specimens obtained at admission and at convalescence in patients with malaria, including 58 and 14, respectively, with cerebral malaria (CM); 77 and 25, respectively severe noncerebral malaria (SM); and 53 and 27, respectively, with uncomplicated malaria (UM). At admission, patients with CM had higher sEPCR levels than patients with SM or UM ($P = .0001$). The SM and UM groups had similar sEPCR levels ($P = .5$). *B*, Plasma sEPCR levels at admission in children with CM who survived versus those who died. For children who died, the mean sEPCR level was higher than that for survivors ($P = .0003$).

respectively; $P = .0001$), but levels were similar in the 2 latter groups ($P = .5$; Figure 1*A*). Thirty days later, no such difference was seen among patients who survived ($P = .4$).

Considering the PROC2R gene genotype, the overall analysis at admission showed a trend toward higher plasma sEPCR levels among patients carrying the rs867186AG genotype, compared with those carrying rs867186AA ($P = .17$). However, at convalescence, a clear difference was observed, with children carrying the rs867186AG genotype having higher plasma levels of sEPCR (median, 297 ng/mL; interquartile range [IQR], 174–383 ng/mL) than rs867186AA carriers (median, 158 ng/mL; IQR, 131–211 ng/mL; $P = .03$).

Univariate linear regression analysis also revealed an age-related trend, with younger children having higher sEPCR levels than older children ($P = .07$). Higher sEPCR levels were also associated with receipt of antimalarial treatment before admission (median, 510 ng/mL [IQR, 239–852] in patients with antimalarial treatment vs 313 ng/mL [IQR, 190–510 ng/mL] in patients without antimalarial treatment; $P = .046$). Multivariate analysis that adjusted for all these factors clearly demonstrated a strong positive relationship between high levels of sEPCR on admission and cerebral malaria ($P = .01$), while an age effect persisted ($P = .006$; Table 1).

Plasma sEPCR Levels and Mortality

Comparison of plasma sEPCR levels at admission between patient groups showed significantly higher levels of sEPCR in patients who subsequently died, compared with levels in survivors ($P = .0001$). This difference was particularly pronounced in the cerebral malaria group ($P = .0003$; Figure 1*B*).

DISCUSSION

This study clearly shows a relationship between high plasma levels of sEPCR and cerebral malaria at admission to the hospital. This feature appeared to be specific to cerebral malaria, as no difference in plasma sEPCR levels was observed between patients with severe malaria and those with uncomplicated malaria. This strongly suggests that the increase in plasma sEPCR level results from a pathophysiological mechanism specific to cerebral malaria. This is further supported by the lack of such differences between the clinical groups in samples collected at convalescence.

The present observational study cannot determine causality because of the multiple factors that can lead to an sEPCR increase, including genetic factors. It is striking to note that the influence of factors such as age and rs867186 genotype on the plasma level of sEPCR differs distinctly between samples obtained at admission and those collected at convalescence. Younger children with cerebral malaria had higher sEPCR levels than older children at admission. This age effect could suggest a difference in the development of pathology in younger children. The expected relationship between the rs867186G mutation and elevated sEPCR plasma levels, well described in healthy individuals [13], was not present at admission in our patients but was observed in convalescent samples, further suggesting that the release of sEPCR is altered during acute malarial parasite infection, particularly in those with cerebral malaria.

Previous studies of the pathogenesis of cerebral malaria have suggested various mechanisms of onset, including sequestration of infected erythrocytes, as well as adherence of leukocytes and platelets to the blood-brain barrier endothelium, combined with

Table 1. Factors Associated With Log-Transformed Levels of Soluble Endothelial Protein C Receptor (sEPCR) on Univariate and Multivariate Linear Regression Analyses

Covariate	Univariate Analysis (n = 188)		Multivariate Analysis (n = 170)	
	Crude Coefficient (95% CI)	P Value ^a	Adjusted Coefficient (95% CI)	P Value ^a
Clinical form of malaria				
Uncomplicated	0 (Reference)		0 (Reference)	
Severe	0.06 (–.16 to .29)	.52	0.06 (–.18 to .29)	.63
Cerebral	0.47 (.23 to .71)	.0002	0.34 (.08 to .61)	.01
Age (per 10 mo increase; n = 185)	–0.06 (–.12 to .004)	.07	–0.09 (–.15 to –.02)	.006
rs867186 genotype (n = 172)		.18		.11
AA	0 (Reference)		0 (Reference)	
AG	0.19 (–.08 to .46)		0.21 (–.05 to 0.47)	
Antimalarials before admission	0.28 (.005 to .56)	.046	0.25 (–.06 to .56)	.12

No significant associations were found in univariate analysis between sEPCR levels and the following characteristics on admission: sex, duration of fever before admission, hemoglobin level, and log parasitemia. Blantyre coma score showed a significant association but was highly collinear with the clinical form of malaria. Abbreviation: CI, confidence interval.

^a By the Wald test.

an imbalance of systemic and local proinflammatory and anti-inflammatory cytokines, leading to blood-brain barrier dysfunction and adverse clinical outcomes during cerebral malaria [2]. The observation of petechial lesions in fatal cases of cerebral malaria has led several authors to propose coagulation impairments in the pathogenesis of cerebral malaria, with PC pathway alterations connecting all these mechanisms. Recent work clearly showed cerebral fibrin clots and loss of EPCR and thrombomodulin at the site of infected erythrocyte cytoadherence in postmortem studies of Malawian children who died of cerebral malaria, supporting the concept that low constitutive expression of EPCR and thrombomodulin is most likely causally related to sequestration of infected erythrocytes in the vascular endothelium [10]. Those authors reported a localized increase in sEPCR levels during cerebral malaria, whereas here we highlight significant and specific increased levels of circulating systemic sEPCR in plasma specimens from patients with cerebral malaria, compared to children with other clinical syndromes. Moxon et al also observed a loss of EPCR and thrombomodulin at sites of infected erythrocyte cytoadherence in subcutaneous microvessels in cases of nonfatal cerebral malaria.

In our study, the case-fatality rate among patients with cerebral malaria was particularly high. While it is generally around 20% [1], we here report a case-fatality rate of 45% among our patients with cerebral malaria. The reasons for this high mortality in Benin remain to be clarified, but a delay in accessing hospital care might play an important role. Patients generally arrived for hospital consultation 4 days after the onset of fever (Supplementary Table). Parenteral quinine was used for treatment of cerebral malaria, as it was the recommended treatment in Benin during the period of the study, but artesunate IV

is known to improve outcomes and is now recommended by the WHO [14]. Our observation that high plasma sEPCR levels at admission were strongly associated with subsequent death is both striking and novel because it suggests that systemic sEPCR levels at admission may be an early prognostic marker of the worst outcome of cerebral malaria.

Our findings support the concept of potential therapeutic targets at the coagulation-inflammation interface for African children with cerebral malaria. Augmenting the PC pathway with recombinant activated PC or thrombomodulin was strongly advocated as adjunctive treatment for severe sepsis but was recently withdrawn because of a lack of benefit in follow-up trials. This approach to treatment might be considered in the management of cerebral malaria. Our findings also suggest that in pathological conditions such as cerebral malaria, an early (at admission), significant increase in the level of circulating sEPCR is detrimental. Measuring the sEPCR level at admission could provide a useful early marker of the outcome of cerebral malaria.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interests. All authors: No reported conflicts.

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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