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Angiopoietin-2 levels are associated with retinopathy and predict mortality in Malawian children with cerebral malaria: a retrospective case-control study

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Abstract

Objective—To investigate the relationship between the angiopoietin-Tie-2 system, retinopathy and mortality in children with cerebral malaria (CM).

Design—Case-control study of retinopathy positive versus retinopathy negative children with clinically defined CM.

Setting—Queen Elizabeth Central Hospital in Blantyre, Malawi.

Subjects—155 children presenting with severe malaria and meeting a strict definition of clinical cerebral malaria (Blantyre Coma Score ≥ 2 , *Plasmodium falciparum* parasitemia, no other identifiable cause for coma) were included in the study.

Measurements—Clinical and laboratory parameters were recorded at admission and fundoscopic exams were performed. Admission levels of angiopoietin-1, angiopoietin-2 and a soluble version of their cognate receptor (sTie-2) were measured by ELISA.

Main results—We show that angiopoietin-1 levels are decreased and angiopoietin-2 and sTie-2 levels are increased in children with CM who had retinopathy compared to those who did not. Angiopoietin-2 and sTie-2 were independent predictors of retinopathy (adjusted Odds ratio (95% CI): angiopoietin-2, 4.3 (1.3-14.6), $p=0.019$; sTie-2, 9.7 (2.1-45.8), $p=0.004$). Angiopoietin-2 and sTie-2 were positively correlated with the number of hemorrhages, the severity of retinal whitening and the extent of capillary whitening observed on fundoscopic exam ($p<0.05$ following adjustment for multiple comparisons). Angiopoietin-2 and sTie-2 levels were elevated in children with CM who subsequently died and Angiopoietin-2 was an independent predictor of death (adjusted Odds ratio: 3.9 (1.2-12.7), $p=0.024$). When combined with clinical parameters, Angiopoietin-2 improved prediction of mortality using logistic regression models and classification trees.

Conclusions—These results provide insights into mechanisms of endothelial activation in CM and indicate that the angiopoietin-Tie-2 axis is associated with retinopathy and mortality in pediatric CM.

Keywords

cerebral malaria; retinopathy; mortality; biomarker; angiopoietin; sTie-2

INTRODUCTION

Plasmodium falciparum malaria causes approximately 250 million infections and 1 million deaths annually, with 85% of the fatalities occurring in children [1]. The case fatality rate in cerebral malaria (CM) remains high ($>15\%$) despite the initiation of appropriate anti-malarial therapy and supportive care [2, 3]. Sequestration of parasites in major organs is thought to be central to the pathogenesis of severe malaria syndromes, including CM. Mature stage parasitized erythrocytes bind to cell adhesion molecules expressed on the endothelium (e.g. ICAM-1), where they are believed to contribute to endothelial activation, microvascular obstruction, localised hypoxia and associated tissue injury. Malarial retinopathy has emerged as a powerful tool to better define the pathobiology of severe disease. Specific retinal changes – hemorrhages, retinal whitening, or vessel abnormalities – have been found to be associated with CM. As the retina and the brain are formed from the same embryonic tissue and have analogous blood-tissue-barriers, analysis of retinopathy can provide insights into microvascular and tissue disturbances in the brain that are otherwise

difficult to study. Ophthalmoscopy combined with retinal angiography has demonstrated that retinal whitening is associated with areas of non-perfusion, vessel abnormalities are indicative of non-perfusion of vessels (white colouration) or areas of reduced vascular lumen (orange colouration) [5], and hemorrhage corresponds to focal loss of blood-retinal-barrier integrity. Consistent with these observations and their associated brain pathology, retinopathy is associated with worse outcome in cerebral malaria [6-8].

The vascular pathology observed on retinal examination and at autopsy point to a key role for endothelial activation and dysfunction during the development of CM. The endothelium represents the critical interface between the brain parenchyma and the intravascular compartment containing sequestered parasites. In 75% of fatal CM cases, sequestration was associated with intravascular and perivascular pathology including microthrombus formation, perivascular oedema, and ring hemorrhages in the brain parenchyma surrounding ruptured vessels [9, 10]. Markers of endothelial activation are increased in the plasma of malaria patients and have been positively correlated with disease severity [11-16]. Collectively these observations suggest a role for endothelial activation and loss of barrier integrity in the pathophysiology of CM.

The angiopoietin-Tie-2 system has been shown to regulate endothelial cell function and vascular integrity [17, 18]. Angiopoietin-1 (Ang-1) engages endothelial receptor Tie-2, promoting endothelial cell quiescence and survival, whereas these effects are generally inhibited by Angiopoietin-2 (Ang-2) [19]. Previous reports have demonstrated low Ang-1 and elevated Ang-2 levels in severe compared to uncomplicated malaria [20-22]. In this study, we investigated markers of the angiopoietin-Tie-2 system in CM in relation to retinal findings and clinical outcome. We hypothesized that altered levels of Ang-1, Ang-2, and the soluble form of Tie-2 (sTie-2) would be associated with retinopathy and would correlate with the severity of retinal changes. Since endothelium may have a crucial role in mediating malaria pathogenesis, we further postulated that these markers would have prognostic value in CM. We show that decreases in Ang-1 and increases in Ang-2 and sTie-2 are associated with retinopathy and mortality. We also show that Ang-2 is a clinically informative prognostic biomarker alone or in combination with clinical findings.

MATERIALS AND METHODS

Ethics

Ethical approval for this study was granted from The College of Medicine Research Ethics Committee in Blantyre, Malawi (COMREC) and all parents or guardians gave written informed consent for children to enter the study. This was a retrospective case-control study of plasma samples collected as part of ongoing prospective studies of the pathogenesis and management of CM.

Study Population

Children between 6 months and 14 years of age presenting with fever to the Queen Elizabeth Central hospital in Blantyre, Malawi were recruited between 1997 and 2009. Admission EDTA plasma samples were obtained from children after their parents or guardians had given their informed consent. Samples collected as part of ongoing prospective studies of the pathogenesis and management of CM were used to assess the relationship between endothelial regulatory proteins (Ang-1, Ang-2, sTie-2) and retinopathy and mortality. This retrospective case-control study examined children with clinically defined CM with retinopathy-positive children as cases and retinopathy-negative children as controls. The sample size was calculated based on a case:control ratio of 2:1 with 80% power to detect a clinically significant difference in Ang-2 levels based on data from a previous study of

African children with CM at an alpha of 0.05 [21]. Inclusion criteria included a clinical definition of CM: (*P. falciparum* asexual parasitemia, a Blantyre coma score (BCS) ≥ 2 with no improvement following correction of hypoglycemia) and an available plasma sample with sufficient volume that had not undergone a previous freeze-thaw. Exclusion criteria included evidence of meningitis or any other identifiable cause for coma [9, 23]. All participants received standard treatment, including antimalarial and/or antibacterial therapy as indicated, according to Malawian national guidelines.

Ophthalmological Examination

After admission of a child with altered consciousness, the patient's pupils were dilated by application of mydriatic eyedrops (tropicamide and phenylephrine) and the fundi were examined by direct and indirect ophthalmoscopy. The findings of an ophthalmologist or experienced clinician were recorded on standardized forms. Retinopathy was defined by the presence of any one of the following retinal findings: hemorrhages, retinal whitening, or vessel changes with or without papilloedema, as previously described [24, 25]. Clinical grading of retinal changes were recorded as follows: 0-4 for hemorrhages (0=none, 1= mild (1-5 hemorrhages), 2= moderate (>5 & 20 hemorrhages), 3= severe (>20 & 50 hemorrhages), 4= very severe (>50 hemorrhages)), and 0-3 for papilloedema, retinal whitening, orange vessels, white vessels, and white capillaries (0=none, 1=mild, 2=moderate, 3=severe) [26]. Papilloedema alone did not constitute retinopathy.

Quantification of Biomarkers

Plasma concentrations of biomarkers Ang-1, Ang-2, and sTie-2 (DuoSets, R&D Systems, Minneapolis, MN) were measured by ELISA, "blinded" to other patient data, as previously described [27].

Statistics

Comparisons of continuous variables were performed using the Mann-Whitney U test and Spearman rank correlation coefficient. Comparisons of proportions were performed using Pearson chi-square test, linear-by-linear association, or Fisher's exact test. Odds ratios (OR) were calculated using Pearson Chi-square or logistic regression models to adjust for covariates. Bonferroni adjustment was used to account for multiple comparisons. Variables were included in logistic regression models as covariates if there was a significant association between the independent and dependent variable. Variables were excluded from multivariate models if >5% of the data was missing (to limit bias and reduction of power); the exception was thrombocytopenia, as platelets are a known source of Ang-1. Classification trees (CRT) were used to identify the biomarker cut-offs. The prognostic accuracy of biomarkers and individual laboratory findings were assessed using receiver operating characteristic (ROC) curves. The area under the ROC curves (AUC) or c-index was compared using the DeLong-DeLong Clarke Pearson method [28].

Multivariable logistic regression and CRT were used to generate predictive models of mortality. All variables except age were dichotomized prior to inclusion in the logistic regression models to circumvent issues of non-linearity and multicollinearity. Linearity of age with the log odds of the dependent variable (retinopathy and mortality) was confirmed by including a Box-Tidwell transformation into the model and ensuring that this term was not significant. Models were validated by ensuring the Hosmer-Lemeshow goodness-of-fit test was not significant ($p > 0.10$).

CRT was performed including all significant univariable predictors with the following settings: 50 cases for parent nodes and 25 for child nodes; a prior probability of death of 23.8% and a misclassification cost (i.e. misclassifying deaths as survivors) of 10; and cross-

validation with 10 sample folds to generate an estimate of the misclassification rate (when available). Surrogates were used to classify cases for variables with missing values. Analysis was performed with GraphPad Prism v5.0, SPSS v16.0 and MedCalc.

RESULTS

Patient Characteristics

155 children between the ages of 8 months and 14 years were included in the study. All the children met the WHO case definition for CM. 50 subjects were retinopathy negative, 103 were retinopathy positive, and 2 died before fundoscopic exams could be performed. Fifty-nine (38.1%) of the children included in this study died. All subjects had plasma samples collected and clinical characteristics recorded at admission. The demographic and clinical characteristics and laboratory findings at admission are summarized in Table 1.

Biomarkers of endothelial activation are associated with malaria retinopathy

Based on the hypothesis that endothelial activation and dysfunction are central to the pathophysiology of cerebral malaria, markers of endothelial activation were measured in children with retinopathy compared to those without. Venous lactate, a validated biochemical marker of disease severity, was included as a comparator [29, 30]. Median Ang-1 levels were decreased ($p=0.018$), and median Ang-2 and sTie-2 levels were increased ($p<0.0001$) in subjects with retinopathy compared to those without retinopathy (Figure 1). Venous lactate was also more elevated in children with malarial retinopathy than without ($p=0.004$).

The relationship between endothelial biomarkers and retinopathy was further examined following dichotomization of biomarkers. Biomarker levels (Ang-1 $>6.76\text{ng/mL}$, Ang-2 $>3.85\text{ng/mL}$, sTie-2 $>67.8\text{ng/mL}$) were associated with an increased odds of having retinopathy (Table 2: Odds ratio (OR) (95% CI), p -value: Ang-1, 5.9 (2.7-12.8), $p<0.001$; Ang-2, 10.6 (4.6-24.6), $p<0.001$; sTie-2, 11.7 (3.9-35.0), $p<0.001$). Ang-2 and sTie-2 remained independent predictors of retinopathy after adjusting for covariates (Table 2: age, respiratory distress, severe anemia, thrombocytopaenia, history of convulsions).

Relationship between specific retinal changes and the angiotensin-Tie2 axis

The distribution of retinal changes for this population is described in Figure 2. Whitening (94.5%) was the most common retinal change followed by vessel changes (76.9%) and hemorrhages (70.3%). Less than 10% of children had a single retinal change recorded, while 51.6% of children had all three retinal changes. Papilloedema alone did not constitute retinopathy; only one child in the retinopathy-negative group had papilloedema alone.

We assessed the severity of retinal changes using a standardized clinical score (see Methods) [26, 31]. Ang-2 and sTie-2 concentrations correlated positively with the number of hemorrhages, the severity of retinal whitening, and the extent of white capillaries (Table 3: $p<0.05$ following Bonferroni adjustment for multiple comparisons). Venous lactate concentrations correlated with retinal whitening ($p<0.05$). Overall, Ang-2 and sTie-2 levels correlated with the severity of hemorrhage, retinal whitening, and white vessels (non-perfusion) but were not related to orange vessels (which probably represent parasite sequestration).

Biomarkers of endothelial activation are associated with mortality

We next investigated the hypothesis that markers of endothelial activation would be associated with a poor prognosis. Increased median levels of Ang-2 and sTie-2 at admission, but not Ang-1, were associated with a fatal outcome (Figure 3, top panel A-D: $p<0.0001$).

Venous lactate was also elevated in children who subsequently died ($p=0.002$). In the subset of children who were retinopathy positive, Ang-2 alone was significantly more elevated in fatal cases than in survivors ($p=0.027$). We generated receiver operating characteristic (ROC) curves to assess the prognostic accuracy of the biomarkers. Ang-2, sTie-2, and venous lactate had comparable areas under the ROC curve (AUC): Ang-2 (AUC (95% CI), 0.71 (0.63-0.78); sTie-2, 0.64 (0.55-0.73); and venous lactate, 0.67 (0.59-0.78) (Figure 3, E-H). Decision plots were constructed to visualize the trade-off between sensitivity and specificity of the biomarkers at the various cut-offs along the ROC curve (Figure 3, I-L). The cut-points for each marker, as determined by CRT, are represented in the decision plots (Ang-1 5.89ng/mL, Ang-2 > 3.85ng/mL, sTie-2 >67.8ng/mL).

The relationship between endothelial biomarkers and mortality were further explored using logistic regression. Biomarker levels were associated with increased odds of death (Table 2; Odds ratio (OR) (95% CI), p-value: low Ang-1, 2.4 (1.2-5.1), $p=0.017$; high Ang-2, 7.9 (2.6-23.6), $p<0.001$; high sTie-2, 3.2 (1.6-6.3), $p=0.001$). Ang-2 and Ang-1 remained independent predictors of mortality after adjusting for covariates (Table 2: age, respiratory distress, BCS, severe anemia) with adjusted odds ratios of 7.9 (2.6-23.6) and 2.5 (1.0-5.9) respectively. Venous lactate and leukocyte count were excluded due to incomplete data.

Incorporating Ang-Tie2 biomarkers into prognostic clinical models

We used logistic regression and classification and regression tree (CRT) analysis to generate prognostic models using routine clinical parameters and the plasma biomarkers. A clinically predictive model of mortality was generated using parameters that are readily available to clinicians (age, BCS, respiratory distress, severe anemia). We used the predicted probabilities from this clinical model to generate a c-index (equivalent to the AUC) of 0.73 (95% CI: 0.65-0.79). Then, using the clinical model as a foundation, we added biomarker tests to determine whether they would significantly improve predictive accuracy. The clinical model including all three biomarkers had a c-index of 0.79 (0.72-0.84), which was significantly better than the clinical model alone ($p=0.03$). We assessed whether a more parsimonious model could be achieved using a single biomarker. Inclusion of Ang-2 alone (but not Ang-1 or sTie-2 alone) led to a model that was significantly better than the clinical model alone (c-index (95% CI), p-value: 0.78 (0.68-0.82), $p=0.02$).

CRT was used as an alternative method to develop a prognostic model. This intuitive approach generates a decision tree that is easy to interpret. CRT divides data into two mutually exclusive groups ("nodes") in order to maximize the homogeneity within each node. When all variables significantly associated with death were entered into the analysis, a decision tree was generated using Ang-2 at a cut-off of 3.85ng/mL to discriminate between survivors and non-survivors. This model has 93.2% sensitivity to predict death and a misclassification rate of 23.1% (Figure 4A). Next, we explored models in which a clinical sign (BCS or respiratory distress) was entered as the first variable to integrate clinical parameters and reduce the number of biomarkers required for testing. In both clinically informed models, Ang-2 was useful for identifying non-survivors in comatose children considered lower risk (BCS 1 or 2 (as opposed to 0); Figure 4B, or children without respiratory distress; Figure 4C).

DISCUSSION

In this study we provide evidence linking the angiopoietin-Tie-2 system to the clinical and pathological findings of cerebral malaria, and further suggest that the angiopoietins may be useful prognostic biomarkers in severe malaria. We demonstrate that perturbations in the angiopoietin-Tie-2 system are associated with retinopathic findings, which are likely reflective of pathological changes in the brain [32]. Consistent with a role in malaria

pathophysiology, elevated admission levels of Ang-2 and sTie-2, and decreased Ang-1 were associated with increased odds of subsequent death. Together, these data demonstrate an association between the angiopoietins and the microvascular disturbances involved in cerebral malaria pathogenesis.

Angiopoietins engage the Tie family of receptor tyrosine kinases to regulate the vascular endothelium. Altered levels of Ang-1, Ang-2 and sTie-2 in this sample of children with severe malaria provide insights into a possible pathophysiologic role of the angiopoietin-Tie-2 system in mediating disease severity and outcome. In this study, elevated Ang-2 was associated with retinopathy (Figure 1, Table 2) and levels were positively correlated with the number of hemorrhages, the degree of retinal whitening, and the extent of white capillaries (Table 3). Ang-2 is stored in intracellular vesicles called Weibel-Palade bodies and is rapidly mobilized and released following endothelial activation [33]. Ang-2 is thought to amplify endothelial activation by opposing the effects of Ang-1 (although it can exert partial agonist activity in the absence of Ang-1 [19]). Previous studies have demonstrated that Ang-2 sensitizes the endothelium to sub-threshold levels of TNF [34], increases endothelial cell permeability [18], and promotes the upregulation of cellular adhesion molecules such as ICAM-1 [18]. We hypothesize that Ang-2 promotes a positive feedback loop of endothelial activation, inflammation, and parasite sequestration. However, Ang-2 may simply be a reflection of endothelial activation and hypoxia [35] [36] and further studies will be required to determine its role in severe malaria.

sTie-2 was also associated with retinopathy and malaria severity. sTie-2 has been identified in supernatants from cultured endothelial cells, is present in normal human serum and plasma [37], and has been identified as a biomarker in cancer [38-40], sepsis [41], and ischemia [42]. It is shed from endothelial cells as a result of ectodomain cleavage by matrix metalloproteinases [43], and this cleavage can be stimulated by a variety of physiological factors, including basic fibroblast growth factor and vascular endothelial growth factor [37, 42, 44]. To date, the function of sTie-2 is incompletely understood. sTie-2 can inhibit Ang-2 activity [45], and thus it is plausible that sTie-2 produced during malaria infection counters the potentially detrimental effects of Ang-2.

Ang-1 is released from mural cells (vascular smooth muscle cells and pericytes) and activated platelets, and helps promote endothelial cell survival and maintain the integrity of endothelial tight junctions [46, 47]. Like Ang-2 and sTie-2, Ang-1 was associated with retinopathy; however, this relationship was lost following adjustment for thrombocytopenia. Platelet counts were reduced in retinopathy positive children, consistent with other reports [25, 48]. Thus, decreased Ang-1 levels in retinopathy may reflect a decrease in platelet number and/or platelet Ang-1 content. Together, these data implicate the angiopoietin-Tie-2 system in the vascular dysfunction seen in cerebral malaria, but further studies will be required to investigate a possible causal role.

An additional aim of this study was to investigate the prognostic value of endothelial biomarkers in comatose children with CM. Admission levels of sTie-2 and Ang-2 were elevated in cases that subsequently progressed to a fatal outcome, and these biomarkers had discriminative value comparable to venous lactate, currently the best available biochemical marker for severe malaria in children (Figure 3) [29, 30]. Admission plasma Ang-2 concentration remained a significant independent predictor of mortality after controlling for covariates (age, BCS, respiratory distress, severe anemia; Table 2) and Ang-2, not lactate, was considered the best predictor of death using classification analysis. This suggests that Ang-2 may have clinical utility as a biomarker. These data could be further used to add prognostic value above and beyond clinical parameters (Figure 4). These results are in

agreement with those of Yeo et al. which show a significant association between Ang-2 levels and outcome in Asian adults [20].

Our data, together with previous reports, suggest that Ang-2 is a robust, quantitative and objective marker of malaria severity that may have utility in clinical practice to triage patients and monitor response to therapy. Ang-2 is elevated in severe compared to uncomplicated malaria and healthy controls across a range of ethnicities and ages [20, 22], and correlates with clinical progression or deterioration when measured longitudinally over the course of illness [20]. Here we show that Ang-2 predicts mortality independently of clinical signs. Given its recognized role as a mediator of endothelial activation, Ang-2 may be useful as a surrogate endpoint in clinical trials of adjunctive therapies for malaria that target the host endothelium [20], as well as affording a promising molecular target for intervention[49].

The results of this study, while promising, must be interpreted with caution. The retrospective case-control design may introduce bias. This study only includes children with well-defined cerebral malaria. While this homogeneity in study participants enables us to better investigate the potential role of the biomarkers in disease pathogenesis, it may reduce the generalizability of the data. In addition, the case-control nature of the study precludes us from investigating whether Ang-2 is independently associated with retinopathy, mortality or both. These results will need to be confirmed in larger prospective studies. Nonetheless, these data suggest that: i) the angiotensin-Tie-2 system may be involved in CM pathogenesis; and ii) Ang-2 is a promising prognostic biomarker in severe malaria. Predictive markers that could identify children with severe malaria at greatest risk of poor outcomes could enable better triage, allocation of resources, and selection of high-risk patients for evaluation of new adjunctive interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Endothelial biomarkers and lactate are associated with retinopathy in children with cerebral malaria

Bar graphs showing the median and scatter of endothelial biomarkers and venous lactate. (A) Angiopoietin (Ang)-1 (ng/mL), (B) Ang-2 (ng/mL), (C) sTie-2 (ng/mL), and (D) venous lactate (mmol/L) were measured in children with clinically defined cerebral malaria by ELISA with (n= 103) or without (n= 50) malarial retinopathy as defined by the presence of hemorrhage, retinal whitening or vessel changes. Analysis by Mann-Whitney (U statistic, p-value), †significant after Holms correction for 4 pair-wise comparisons: *p<0.05. **p<0.01, ***p<0.001. (A) Ang-1 (1969, p=0.018) †, (B) Ang-2 (1288, p<0.0001) †, (C) sTie-2 (1258, p<0.0001) †, and (D) venous lactate (1358, p=0.004) †.

Figure 2. Venn-diagram depicting the distribution of retinal changes in the CM patients with retinopathy

A Venn diagram depicting the distribution of retinal changes according to the number of children with hemorrhages (H), retinal whitening (W), or vessel abnormalities (V: orange vessels, white vessels, white capillaries). Data are presented as percentages followed by the number of children in each group. Detailed classification of retinal changes was available for 91 of the 103 retinopathy positive children. A total of 70.3% children had hemorrhages (n=64), 94.5% had retinal whitening (n=86) and 76.9% had vessel abnormalities (n=70).

Figure 3. Endothelial markers and venous lactate predict mortality

(A-D) Graphs showing the median and scatter of endothelial biomarkers and venous lactate, with the associated (E-F) receiver operating characteristic (ROC) curves and (I-L) decision plots of sensitivity and specificity generated from the ROC curves. Prognostic accuracy was assessed using receiver operating characteristic (ROC) curves and comparing the area under the ROC curve (AUC: 95% CI). Levels of Angiopoietin (Ang)-2 (B, F, J), sTie-2 (C, G, K) and venous lactate (D, H, L) were significantly elevated in children who died compared to those who survived (AUC (95% CI), p-value: Ang-2, 0.71 (0.63-0.78), $p < 0.0001$; sTie-2, 0.64 (0.55-0.73), $p = 0.003$; venous lactate, 0.67 (0.59-0.78), $p = 0.002$). There was no difference in Ang-1 levels (A, E, I) between children who survived and children who died (AUC (95% CI), p-value: 0.56 (0.47-0.65), $p = 0.20$). The AUC were compared between lactate, sTie-2, and Ang-2 and there were no significant differences (method of DeLong et al.). The vertical line represents the cut-point for dichotomizing the biomarkers by CRT or established criteria (hyperlactatemia).

Figure 4. Classification and regression tree models for the prediction of mortality in children with cerebral malaria

We used classification and regression tree analysis (CRT) to generate an intuitive decision tree based on significant predictors of mortality according to the following conditions: misclassification cost, 10; cross-validation, 10-fold (if appropriate); prior probability of death, 23.8%; minimum number per child node, 25; and impurity measure, gini criterion. (A) Model generated from all significant independent variables (Angiotensin (Ang)-2 (cut-point $>3.85\text{ng/mL}$): sensitivity- 93.2%, specificity- 36.5%, cross-validated misclassification rate (standard error)- 0.231 (0.032); Clinically informed models: (B) Blantyre Coma Score plus Ang-2 (sensitivity- 94.9%, specificity- 34.4%, misclassification rate (standard error)- 0.198 (0.038); (C) Respiratory distress plus Ang-2 (sensitivity- 93.2%, specificity- 27.1%, misclassification rate (standard error)- 0.228 (0.035).

Table 1

Demographic and clinical characteristics of population at admission

Characteristic	All (n= 155)	Retinopathy Negative (n= 50)	Retinopathy Positive (n= 103)	P value	Survivors (n=96)	Deaths (n= 59)	P value
Demographic							
Age (months), median (IQR)	34 (27-51)	43 (31- 79)	32 (25- 44)	0.003 <i>f</i>	37 (29-51)	30 (22-51)	0.032 <i>g</i>
Male sex, %	55.6%	58.0%	53.4%	0.727	55.2%	54.2%	0.996
Malnutrition, <2SD	36.2%	32.0%	39.0%	0.402	37.9%	33.3%	0.571
Clinical							
Hypotension	4.1% <i>a</i>	4.1%	4.2%	0.981	3.2%	5.7%	0.468
Respiratory Distress	45.2%	28.0%	52.4%	0.004 <i>f</i>	34.4%	62.7%	0.001 <i>g</i>
History of Convulsions	20%	10.0%	33.8%	0.032 <i>f</i>	15.6%	27.1%	0.082
Blantyre Coma Score							
0	12.9%	12.0%	12.6%		6.2%	23.7%	
1	43.2%	44.0%	42.7%		42.7%	44.1%	
2	43.9%	44.0%	44.7%	0.997	51.0%	32.2%	0.001 <i>g</i>
Laboratory							
Hyperparasitemia	12.3% <i>b</i>	4.0%	15.5%	0.115	12.5%	11.9%	0.732
Severe anemia	21.9%	4.0%	31.1%	<0.0001 <i>f</i>	16.7%	30.5%	0.043 <i>g</i>
Leukocytosis	36.8% <i>c</i>	27.7%	40.0%	0.149	28.0%	52.9%	0.003
Thrombocytopenia	77.3% <i>d</i>	52.3%	89.7%	<0.0001 <i>f</i>	75.6%	81.0%	0.491
Hypoglycemia	5.8%	2.0%	7.8%	0.155	4.2%	8.5%	0.265
Hypertactemia	62.6% <i>e</i>	49.0%	70.0%	0.017	54.7%	83.3%	0.003

a n=147,
b n=154,
c n=144 (excluded from multivariable analysis as >5% missing),
d n=132 (included in multivariable analysis despite >5% missing data because platelets are a known source of Ang-1),
e n=131 (excluded from multivariable analysis as >5% missing)

$f_{i,g}$ included in multivariable logistic regression models as covariates

Variables were dichotomized based on the following: hypotension (pediatric advanced lifesupport guidelines); malnutrition, weight for age z-scores (WHO 2007 reference values); hyperparasitemia ($> 500,000$ parasites/ μL); severe anemia ($< 15\%$ hematocrit); leukocytosis ($> 12,000$ leukocytes/ μL); thrombocytopenia ($< 150,000 \times 10^3$ platelets/ μL); hypoglycemia ($< 2.2\text{mmol/L}$); hyperlactatemia, $> 5\text{mmol/L}$; and retinopathy (any one of: hemorrhage, whitening, vessel changes).

Table 2

Odds ratios for positive biomarker tests in retinopathy and mortality

	Retinopathy			Mortality		
	OR (95% CI)	P value	OR (95% CI) ^a	P value ^a	OR (95% CI)	P value ^b
Low Ang-1	5.9 (2.7-12.8)	<0.001	2.8 (0.8-9.4)	0.099	2.4 (1.2-5.1)	0.017
High Ang-2	10.6 (4.6-24.6)	<0.001	4.3 (1.3-14.7)	0.020	7.9 (2.6-23.6)	<0.001
HighTie-2	11.7 (3.9-35.0)	<0.001	9.7 (2.1-45.9)	0.004	3.2 (1.6-6.3)	0.001

Odds ratios (OR), Endothelial biomarkers dichotomized by CRT: Ang-1 (Cut-point: ≤ 6.76 ng/mL Retinopathy, ≤ 5.89 Mortality), Ang-2 (Cut-point: >3.85 ng/mL), sTie-2 (Cut-point: >67.80 ng/mL).^a Adjusted P values: multivariable logistic regression model adjusting for covariates; retinopathy - age, respiratory distress, severe anemia, thrombocytopenia, history of convulsions;^b Adjusted P values: multivariable logistic regression model adjusting for covariates; mortality - age, respiratory distress, Blantyre coma score, severe anemia.

Table 3

Plasma concentrations of the Angiopoietin-Tie-2 system and lactate correlate with the severity scores of retinal abnormalities

Biomarker	Hemorrhage	Whitening	Orange Vessels	White vessels	White capillaries	Papilloedema
Ang-1	-0.082	-0.161	-0.188	0.015	-0.051	-0.038
Ang-2	0.328**	0.448**	0.081	0.254*	0.296**	-0.212
sTie-2	0.309**	0.431**	0.111	0.222	0.284*	-0.107
Lactate	0.135	0.270*	0.180	0.131	0.138	-0.117

Spearman's rho correlating biomarker levels with clinical grading score of retinal components. Hemorrhage (0=none, 1=mild, 2=moderate, 3=severe, 4=very severe); Whitening, vessel changes (orange vessels, white vessels, white capillaries), and papilloedema (0=none, 1=mild, 2=moderate, 3=severe).

Orange vessels represent irregular narrowing of vessels and white vessels represent no perfusion.

*
p<0.05 or

**
p<0.01 following Bonferroni adjustment for multiple comparisons (cut-off at p<0.008 for 24 comparisons for alpha=0.05)