HHS Public Access

Author manuscript

Am J Hematol. Author manuscript; available in PMC 2016 January 06.

Published in final edited form as:

Am J Hematol. 2012 August ; 87(8): 782–789. doi:10.1002/ajh.23253.

Reduced Systemic Bicyclo-Prostaglandin-E_2 and Cyclooxygenase-2 Gene Expression are Associated with Inefficient Erythropoiesis and Enhanced Uptake of Monocytic Hemozoin in Children with Severe Malarial Anemia

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Abstract

In holoendemic *Plasmodium falciparum* transmission areas, severe malaria primarily occurs in children <48 mos. and manifests as severe malarial anemia [SMA; hemoglobin (Hb)<6.0 g/dL]. Induction of high levels of prostaglandin-E_2 (PGE_2) through inducible cyclooxygenase-2 (COX-2) is an important host defense mechanism against invading pathogens. We have previously shown that COX-2-derived PGE_2 levels are reduced in children residing in hyperendemic transmission regions with cerebral malaria and in those with mixed sequelae of anemia and hyperparasitemia. Our *in vitro* studies further demonstrated that reduced PGE_2 was due to down-regulation of COX-2 gene products following phagocytosis of malarial pigment (hemozoin, *Pf*Hz). However, since COX-2-PGE_2 pathways and the impact of naturally acquired *Pf*Hz on erythropoietic responses have not been determined in children with SMA, plasma and urinary bicyclo-PGE_2/creatinine and leukocytic COX-2 transcripts were determined in parasitized children (<36 mos.)

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The study was approved by the Ethics Committees of the Kenya Medical Research Institute and University of New Mexico Institutional Review Board. Informed written consent was obtained from the parent or legal guardian of all children participating in the study.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

stratified into SMA (n=36) and non-SMA (Hb≥6.0 g/dL; n=38). Children with SMA had significantly reduced plasma (P=0.001) and urinary (P<0.001) bicyclo-PGE_2/creatinine, and COX-2 transcripts (P=0.007). There was a significant positive association between Hb and both plasma (r=0.363, P=0.002) and urinary (r=0.500, P=0.001)] bicyclo-PGE_2/creatinine. Furthermore, decreased systemic bicyclo-PGE_2/creatinine was associated with inefficient erythropoiesis (i.e., reticulocyte production index; RPI<2.0, P=0.026). Additional analyses demonstrated that plasma (P=0.031) and urinary (P=0.070) bicyclo-PGE_2/creatinine and COX-2 transcripts (P=0.026) progressively declined with increasing concentrations of naturally acquired *Pf*Hz by monocytes. Results presented here support a model in which reduced COX-2-derived PGE_2, driven in part by naturally acquired *Pf*Hz by monocytes, promotes decreased erythropoietic responses in children with SMA.

Keywords

PGE_2; COX-2; mRNA; *P. falciparum*; SMA; PCM

INTRODUCTION

Approximately 85% of the fatalities from malaria occur in children less than five years of age in sub-Saharan Africa and are due to infection with *Plasmodium falciparum* (1). Severe malaria can present as single or overlapping clinical features, including severe anemia, metabolic acidosis, respiratory distress, acute renal failure, hypoglycemia, hyperparasitemia, and cerebral malaria (CM) (2). In holoendemic *P. falciparum* transmission regions such as Siaya, western Kenya, severe malarial anemia (SMA) is the most common cause of malaria-associated morbidity and mortality and primarily occurs in children less than four years of age (3–5).

The etiology of SMA can occur through one (or a combination) of pathophysiological mechanisms, including lysis of infected and uninfected red blood cells (RBCs) (6–9), splenic sequestration of RBCs (10), dyserythropoiesis and suppression of erythropoiesis (11, 12). In addition, the pathogenesis of SMA is frequently complicated by co-infections with HIV-1, bacteremia, upper respiratory tract viral infections, and hookworm infections (13–19). In infants and young children residing in holoendemic regions, some or all of these factors, along with constant, year-round malaria transmission can culminate in chronically low Hb concentrations. Our recent study showed that insufficient erythropoiesis was important in the etiology of SMA in children in the Siaya community (20).

A central feature that mediates the pathogenesis of SMA is the release of soluble mediators of inflammation (e.g., cytokines, chemokines, and effector molecules) as part of the host-immune response (21). During a malaria infection, this process is largely driven by phagocytosis of malarial pigment (hemozoin, *Pf*Hz) by monocytes, neutrophils, and resident macrophages (21). *Pf*Hz is formed during the intraerythrocytic asexual replication cycle in which *P. falciparum* metabolizes host Hb as a source of amino acids (22, 23). The remaining iron-rich heme portion [i.e., ferriprotoporphyrin IX (FP-IX)] is then aggregated into an insoluble product, *Pf*Hz, by the action of heme polymerase (24–29). Monocytes, neutrophils,

and macrophages acquire *Pf*Hz through phagocytosis of parasitized RBCs and by taking up free *Pf*Hz released upon lysis of infected RBCs (21).

Previous *in vitro* studies from our laboratories showed that ingestion of *Pf*Hz by blood mononuclear cells caused suppression of prostaglandin- E_2 (PGE_2) through suppression of cyclooxygenase-2 (COX-2; prostaglandin endoperoxide H2 synthase-2) gene products (30). COX-2 is an inducible enzyme predominantly expressed in cells involved in inflammatory reactions, such as macrophages, endothelial cells, and fibroblasts (31–33). Increased expression of COX-2 by pro-inflammatory mediators generates high levels of PGE_2 production as part of the host-immune response to infections (34–39). Since PGE_2 and its metabolites are unstable *in vivo*, levels of PGE_2 are measured as bicyclo-PGE_2 (the stable breakdown product of PGE_2 and 13,14-dihydro-15-keto PGE_2) and can be expressed in a ratio with creatinine levels to account for differences in hydration status (40, 41).

Our previous studies in Gabonese children with *P. falciparum* malaria demonstrated an inverse relationship between circulating bicyclo-PGE_2/creatinine, peripheral blood mononuclear cell (PBMC) COX-2 mRNA and protein (42) expression, and disease severity. Consistent with these results, our follow-up study in Tanzanian children with CM demonstrated that systemic levels of bicyclo-PGE_2/creatinine decreased with increasing disease severity such that children with neurological sequelae and/or those who eventually died had the lowest bicyclo-PGE_2/creatinine levels (43). Furthermore, we have shown that high levels of naturally acquired *Pf*Hz were associated with decreased PGE_2 production in cultured intervillous blood mononuclear cells (IVBMCs) from Kenyan women with placental malaria (44). Taken together, these results demonstrate that suppression of COX-2-derived PGE_2 is associated with enhanced severity of falciparum malaria.

Although unexplored in children with SMA, the COX-2-PGE_2 pathway may be important since COX-2 plays an important role in erythroid maturation (45–49), and PGE_2 can cause reduced RBC deformability (50) and volume (51). As such, we examined plasma and urinary levels of bicyclo-PGE_2/creatinine and leukocytic COX-2 transcripts in extensively phenotyped children with *P. falciparum* infections (age<36 mos.; n=74) stratified into non-SMA (Hb≥6.0 g/dL) and SMA (Hb<6.0 g/dL). Since co-infection with HIV-1 and/or bacteremia alters the host-immune response in children with SMA (16, 18), all co-infected children were excluded from the study. The current study explores the relationship between the COX-2-PGE_2 pathway and erythropoiesis, and the impact of naturally acquired *Pf*Hz on COX-2-derived plasma and urinary levels of bicyclo-PGE_2/creatinine.

MATERIALS AND METHODS

Study site

The study was carried out at the Siaya District Hospital (SDH) in western Kenya. Falciparum malaria prevalence more than a decade ago was 83% in children between 1–4 years (52) and has remained stable with an increase in pediatric malaria admissions, particularly from mid-2006 to date (53). Consequently, SMA remains a significant contributor to hospital-associated morbidity and mortality (3). Details of the study site and malarial anemia in the pediatric population are described in our previous report (4).

Study participants

Children with malaria (n=74) of both genders (age, <36 mos.; male [n=45], female [n=29]) visiting SDH for their first hospital contact were enrolled, after obtaining written informed consent from the parent/legal guardian, to participate in the study. Hb levels (g/dL) were used to group children with falciparum malaria into two groups: (i) non-SMA (Hb≥6.0 g/dL, n=38) and (ii) SMA (Hb<6.0 g/dL, n=36) based on the description of anemia determined by greater than 14,000 longitudinal Hb measurements in age- and gender-matched children from the same geographic location (54). In addition, to place the current findings into a global context, results are also presented for children grouped according to the World Health Organization (WHO) definition of SMA: non-SMA (Hb≥5.0 g/dL, n=52) and SMA (Hb<5.0 g/dL, n=22) (55). Children were excluded from the study if they had mixed malaria species infections, HIV-1, bacteremia, prior hospitalization (for any reason), antimalarial and/or antipyretic treatment within two weeks prior to enrollment, and CM. Patients were treated and provided supportive care according to the Ministry of Health (MOH)-Kenya guidelines. The study was approved by the Ethics Committees of the Kenya Medical Research Institute and University of New Mexico Institutional Review Board.

Clinical laboratory evaluation

Venipuncture blood samples (<3.0 mL) were collected from enrolled participants before any treatment interventions. Complete blood counts were determined using the Beckman Coulter A^{c}T diff2^{TM} (Beckman-Counter Corporation, Miami, FL, USA). Asexual malaria trophozoites in thick and thin peripheral blood smears, and reticulocyte count were determined according to previous methods (16). Since there are known cofounders of anemia in this malaria endemic region, co-infections (i.e., HIV-1 and bacteremia), and sickle-cell status were determined according to our previous methods (16). Parents/guardians of the study participants received pre- and post-test HIV/AIDS counseling and provided informed consent.

Determination of bicyclo-PGE$_2$ and creatinine levels

To measure bicyclo-PGE$_2$ levels in plasma and urine samples, a commercial prostaglandin E metabolite (PGEM) kit (Cayman Chemical Company, MI, USA) was used according to the manufacturer's instructions. Since PGE$_2$ has a high turnover rate in peripheral circulation, the PGE$_2$ metabolites (13,14-dihydro-15-keto PGA$_2$ and 13,14-dihydro-15-keto PGE$_2$) were converted to single derivatives (stable end product bicyclo-PGE$_2$). Briefly, 250 μL of either plasma or urine samples were precipitated in 95% ethanol. Organic solvents were eliminated by passing samples through C-18 (containing 500 mg sorbent) solid phase extraction (SPE) cartridges (Supelco Analytical, PA, USA) coated with octadecyl silica as the packing material. Samples were eluted from the columns in 5mL ethyl acetate (Sigma-Aldrich Co., MO, USA) containing 1% methanol and evaporated to dryness under a stream of nitrogen gas. Samples were then re-suspended in 500μL of commercial 1× enzyme immunoassay (EIA) buffer (Cayman Chemicals Company, MI, USA). Resulting PGE$_2$ and the intermediary metabolites were derivatized overnight at 37°C to bicyclo-PGE$_2$ in 150 μL of 1 M carbonate buffer and thereafter 200 μL of 1M phosphate buffer and 150 μL of 1× EIA buffer added (Cayman Chemicals Company, MI, USA). Bicyclo-PGE$_2$ levels were then

determined by quantitative sandwich EIA as described by the manufacturer (Cayman Chemicals Company, MI, USA). Sensitivity of detection for bicyclo-PGE_2 levels was ≥2 pg/mL.

Plasma and urine creatinine levels were determined using the creatinine determination kit (Cayman Chemicals Company, MI, USA). Plasma and urine samples were diluted 1:20 with ultra-pure water and creatinine was quantified by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Cayman Chemicals Company, MI, USA).

Total RNA isolation and COX-2 gene expression analyses

Total RNA was isolated from cryo-preserved white blood cell pellets (preserved in commercial RNAlater RNA stabilization reagent [Qiagen, CA, USA]) by the acid guanidinium thiocyanate-phenol-chloroform extraction method (56). Resulting RNA concentrations were determined by measuring absorbance (A260nm/A280nm) and the quality assessed by checking for contaminating salts and proteins at A230nm/A320nm, using a GeneQuant pro spectrophotometer (Biochrom Ltd. Cambridge, England).

Reverse transcription of RNA to complementary DNA (cDNA) was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's protocol. Briefly, 2 μg of total RNA was reverse transcribed in a 20 μL reaction mix, containing as final concentrations, 1× reverse transcription (RT) buffer, 1mM dNTP mix, 1× RT random hexamers, 5 units MultiScribe™ reverse transcriptase and 20 units RNase inhibitor. Reverse transcription steps were performed using a GeneAmp PCR system 9700 (Applied Biosystems, CA, USA), with the thermal cycler conditions set at an initial 65°C, hold for 5 minutes, followed by a 25°C hold for 10 minutes, 48°C hold for 45 minutes, and a final enzyme denaturing step at 95°C for 5 minutes.

To quantify COX-2 mRNA expression, the resulting complementary DNA (cDNA) was amplified for 30 cycles using oligonucleotides spanning the exon-intron junction in the COX-2 gene, with the sense (5′-GAC TCC CTT GGG TGT CAA AGG TAA-3′) and antisense (5′-GTG AAG TGC TGG GCA AAG AAT G-3′) sequence used to generate a 138 bp product. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (endogenous control) was amplified to yield a 381 bp fragment in a 30 cycle reaction using the following oligo sequences; sense (5′-CTA CTG GCG CTG CCA AGG CTG T-3′) and antisense (5′-GCC ATG AGG TCC ACC ACC CTG T-3′). Resulting polymerase chain reaction (PCR) fragments were resolved on a 2% agarose gel stained with 0.5 mg/mL ethidium bromide (Sigma Chemicals Co. MO, USA) and visualized under UV light (Sprectroline® Corporation, NY, USA). Electrophoretic gel films were analyzed using the ImageJ software (57) and PCR product mean band intensities quantified. The COX-2 mRNA expression mean values (arbitrary units; AU) were normalized by expressing them relative to GAPDH mRNA mean values.

Determination of pigment containing monocytes (PCMs), reticulocyte production index (RPI) and erythrophagocytosis

Pigment containing monocyte (PCM) levels were determined in thin Giemsa-stained blood smears, with a total of 30 monocytes examined per slide, and the number of PCM expressed as a percentage of the total number of cells examined. Total PCM/μL was calculated according to previous methods (58, 59). PCM levels were grouped as follows; PCM (−) = no pigment-containing monocytes; Low = ≤10%; Moderate = >10 and <26.7%; and High = ≥26.7% (19). The RPI was similarly determined according to our previous methods (60, 61). To approximate the rates of erythrophagocytosis among the clinical groups, methanol-fixed Giemsa stained thin blood smears were used, where 100 monocytes/macrophages were counted per slide, and the number of monocytes/macrophages with phagocytosed RBCs expressed as a percentage of the total number of cells examined. In addition, erythrophagocytosis/μL was estimated using the coulter analyzer generated total monocyte counts.

Statistical Analyses

Analyses were performed with SPSS® statistical software package version 19 (IBM® IL, USA). Comparisons of demographic, clinical, and parasitological variables between the groups were computed using Pearson's Chi-square (χ^2) and Mann-Whitney U tests. Relationships between plasma or urinary bicyclo-PGE_2/creatinine levels and Hb concentrations were determined using Spearman's correlation co-efficient. The relationship between plasma and urinary bicyclo-PGE_2/creatinine levels and malaria clinical groups (and PCM levels) was examined using Mann-Whitney U tests for pair-wise comparisons and Kruskal-Wallis tests for across group comparisons, respectively. COX-2 mRNA expression data were normalized by expressing COX-2 as a ratio over GAPDH (endogenous control) with comparisons between the non-SMA and SMA groups performed using Student's *t*-test. Comparison of COX-2/GAPDH across the various PCM groups was performed by ANOVA. Statistical significance was set at $P \leq 0.050$.

RESULTS

Clinical and laboratory characteristic of study participants

Parasitemic children (n=74; <36 mos. of age) were grouped according to previously defined criterion (54) into non-SMA (Hb≥6.0 g/dL; n=38) and SMA (Hb<6.0 g/dL; n=36). The clinical, demographic, and laboratory characteristics of the participants are presented in Table 1. Although the distribution of females and males in the clinical groups was comparable (P=0.671), children with SMA were significantly younger (P=0.010). Enrollment temperature (°C) and glucose (mmol/L) levels were comparable (P=0.637 and P=0.278, respectively) between the groups. Given the *a priori* classification of the clinical phenotypes, hematological indices, including median Hb levels (P<0.001), hematocrit (P<0.001) and total red blood cells (RBCs; P<0.001) were significantly lower in children with SMA. The red cell distribution width (RDW; P=0.008) and white blood cell count (WBC; P=0.035) were higher in the SMA group. However, the mean corpuscular volume (MCV; P=0.516), mean corpuscular hemoglobin (MCH; P=0.390) and mean cell hemoglobin concentration (MCHC; P=0.660) were comparable between the groups. The

significant elevation in the RDW in the SMA group in the context of a "normal" (non-significant change) in the MCV may suggest the beginning stages of a vitamin B12 or folic acid deficiency and/or the initial stages of iron deficiency anemia. In addition, the total lymphocyte (P=0.066) and monocyte (P=0.065) were marginally increased in children with SMA. Although the total granulocyte counts were higher in children with SMA (P=0.015), platelet counts were comparable between the two groups (P=0.764). Since reticulocyte counts and hematocrit levels are required to determine the absolute reticulocyte number (ARN) and reticulocyte production index (RPI), children (n=6) with missing data on these variables were excluded from analyses. Although reticulocyte counts were significantly higher among children with SMA (P=0.006), the ARN (P=0.070) and RPI (P=0.165) were marginally lower in the SMA group. However, insufficient erythropoiesis (i.e., RPI<2) was significantly more frequent in children with SMA (P=0.030). Furthermore, erythrophagocytosis (% and /μL) was elevated in children with SMA (P=0.036 and P=0.140, respectively) relative to those with non-SMA, suggesting increased destruction of erythrocytes as a potential cause of reduced Hb concentrations in children with SMA. Peripheral parasite densities (P=0.875), geometric mean parasitemia, and high-density parasitemia (HDP, ≥10,000/μL; P=0.811) did not differ between the groups. In addition, distribution of sickle-cell trait (P=0.927) and G6PD deficiency (P=0.788) were comparable between the groups. Children with SMA had a higher prevalence of PCMs (P=0.001) and higher plasma and urinary creatinine levels compared to those with non-SMA (P=0.007 and P=0.047, respectively).

Bicyclo-PGE_2 and COX-2 transcripts are suppressed in children with SMA

To investigate the association between *in vivo* systemic PGE_2 concentrations, COX-2 gene expression, and clinical outcomes, we examined plasma (n=74) and urinary (n=44) bicyclo-PGE_2/creatinine levels (pg/mg/mL) and WBC COX-2 transcripts in the two groups. To account for potential differences in hydration status, bicyclo-PGE_2 (pg/mL) levels were expressed per unit creatinine (mg/dL). Children with SMA had significantly reduced plasma (P=0.001; Figure 1A) and urinary (P<0.001; Figure 1B) bicyclo-PGE_2/creatinine levels relative to non-SMA. Consistent with these results, WBC COX-2 transcripts were significantly lower in the SMA group (n=14) relative to those with non-SMA (n=9, P=0.007; Figure 1C).

Upon classification according to the WHO criteria (55), children with SMA (Hb<5.0 g/dL) had significantly lower bicyclo-PGE_2/creatinine levels in plasma (P=0.004) and urine (P=0.011). Similarly, COX-2 transcripts were also significantly reduced in the SMA group (n=14) compared to children with non-SMA (n=9, P=0.003). Thus, reduced leukocytic COX-2 transcripts and lower systemic bicyclo-PGE_2/creatinine levels are associated with more severe clinical manifestations of malaria.

Additional analyses were performed with the inclusion of healthy children to explore bicyclo-PGE_2/creatinine production in non-infected vs. malaria-infected individuals. There was a significant across group difference in plasma bicyclo-PGE_2/creatinine levels in healthy controls [median (IQR) 2,371 (8,576), n=10], children with non-SMA [3,667 (7235), n=38], and those with SMA [2,122 (2,552), n=36, P=0.005, Kruskal Wallis test). Additional

post hoc analyses showed no difference in bicyclo-PGE_2/creatinine levels between healthy controls and those with SMA (P=0.236). However, bicyclo-PGE_2/creatinine levels were elevated in the non-SMA group compared to healthy controls (P=0.050).

Suppression of bicyclo-PGE_2 is associated with insufficient erythropoiesis

Next, we determined the association between systemic bicyclo-PGE_2/creatinine and Hb concentrations. These analyses revealed a significant positive correlation between Hb levels and bicyclo-PGE_2/creatinine in both plasma (r=0.363, P=0.002 (Figure 2A) and urine (r=0.500, P=0.001 (Figure 2B). Since children with SMA were younger than those with non-SMA [12.5 (13.0) vs. 8.0 (7.0) mos.)], and the COX-2-PGE_2 pathway could (potentially) be affected by age, we examined the relationship between bicyclo-PGE_2/creatinine and age. There was no relationship between age and bicyclo-PGE_2/creatinine in either plasma (r=-0.079, P=0.503) or urine (r=0.168. P=0.287) in parasitemic children. Additional analyses in children stratified according to disease severity (i.e., non-SMA and SMA) also failed to show a relationship between age and bicyclo-PGE_2/creatinine in plasma and urine in the non-SMA (r=−0.075, P=0.653 and r=−0.058, P=0.798, respectively) and SMA (r=−0.002, P=0.989 and r=0.094, P=0.687, respectively) groups.

To explore the relationship between PGE_2 and erythropoiesis, bicyclo-PGE_2/creatinine levels were compared between individuals with insufficient erythropoiesis (RPI<2.0) and those with appropriate (RPI≥3.0) erythropoiesis (60, 61). As shown in Figure 2C, children with insufficient erythropoiesis (RPI<2.0) had significantly lower plasma bicyclo-PGE_2/creatinine levels compared to those with appropriate erythropoiesis (RPI≥3.0, P=0.026). No significant differences in erythropoiesis were reflected for urinary bicyclo-PGE_2/creatinine (P=0.371), likely due to the small number of samples available (n=3) in the RPI≥3.0 group (data not presented).

Suppression of bicyclo-PGE_2 and COX-2 transcripts is associated with increasing deposition of monocytic PfHz

To investigate the impact of naturally acquired *Pf*Hz on COX-2-PGE_2 pathways, children were stratified according to the level (%) of PCM. With increasing levels of PCM, bicyclo-PGE_2/creatinine concentrations progressively declined in plasma (P=0.031; Figure 3A) and urine (P=0.070; Figure 3B). Compared to the PCM (−) group, plasma bicyclo-PGE_2/creatinine levels were significantly lower in the moderate (P=0.050; Figure 3A) and high (P=0.013) PCM groups. Additional analyses of urinary bicyclo-PGE_2/creatinine levels showed a significant decrease in children with moderate PCM compared to the PCM (−) group (P=0.029; Figure 3B). Consistent with the results obtained for the systemic levels of bicyclo-PGE_2, COX-2 transcripts decreased progressively with increasing deposition of *Pf*Hz in monocytes (P=0.026; Figure 3C). Pair-wise analyses demonstrated a significant decrease in the moderate (P=0.039) and high (P=0.010) PCM groups compared to the PCM (−) group. Taken together, these results show that increasing deposition of *Pf*Hz in monocytes is associated with reduced systemic bicyclo-PGE_2 production and leukocytic COX-2 gene expression.

DISCUSSION

The primary objective of this study was to determine the relationship between the COX-2-PGE_2 pathway, erythropoiesis, and naturally acquired monocytic *Pf*Hz. As such, we examined the COX-2-PGE_2 pathway in a pediatric population living in a *Plasmodium falciparum* holoendemic region of western Kenya in which the primary manifestation of severe malaria is SMA (3, 4, 16). To eliminate the potential influence of co-infection on the host-immune response, all co-infected children were excluded from the study. Results presented here demonstrate that systemic bicyclo-PGE_2/creatinine and WBC COX-2 mRNA transcripts were significantly suppressed in children with SMA. Consistent with this finding, there was a positive correlation between Hb concentrations and both plasma and urinary bicyclo-PGE_2/creatinine levels with decreased bicyclo-PGE_2/creatinine being associated with inappropriate erythropoiesis (i.e., RPI<2.0). Furthermore, suppression of systemic bicyclo-PGE_2/creatinine and COX-2 transcripts were associated with increasing levels of monocytic *Pf*Hz acquired during the acute infection.

Our previous studies (42–44) have consistently shown that COX-2-derived PGE_2 production is suppressed during severe malaria infections. These results are consistent with a study in adults (15–70 years) with *P. vivax* malaria in the Brazilian Amazon (62), and experimental models of murine malaria in which reductions in PGE_2 are associated with more severe clinical outcomes (63–65). Results presented here extend these previous findings by showing that perturbations in the COX-2-PGE_2 pathway are also important for influencing the erythropoietic cascade in children with SMA. In the current study, we observed that children with SMA were significantly younger than the non-SMA group. However, analyses of the association between bicyclo-PGE_2 levels and age revealed no significant relationships, suggesting that decreased levels of bicyclo-PGE_2 are not simply a product of age, but rather a true pathophysiologic process.

Data presented here are consistent with the fact that PGE_2 is an important soluble factor for promoting efficient erythropoiesis (66–68). In addition to erythropoietin (EPO) (69), PGE_2 plays a critical role in human erythroid development by augmenting both cellular maturation and Hb formation (70–74). Previous studies also showed that PGE_2 is the predominant prostanoid released by human erythroblasts (75), and that RBCs both release and respond to physiological concentrations of PGE_2 (48, 50, 51, 76). Thus, the association between suppression of the COX-2-PGE_2 pathway, more severe anemia, and reduced erythropoietic responses reported here in children with malaria parallels the known actions of PGE_2 on erythroid maturation. Although previous studies show that EPO is elevated in children with malarial anemia, and not a likely the cause of ineffective erythropoiesis (77–80) in these children, this cannot be definitively ruled out since EPO was not determined in the current study.

The etiology of SMA is complex and multifactorial (21, 67, 81, 82) and is characterized by increased lysis of infected and uninfected erythrocytes (6, 7, 9), inefficient erythropoiesis or dyserythropoiesis (10), and erythrocyte sequestration in the spleen (11, 12). A recent case-control study investigating hemolysis in Gabonese children with malaria found that both extravascular and intravascular hemolysis are important causal factors for reduced Hb

concentrations in children with SMA (83). In addition, consistent with our previous publication in Kenyan children (19), they also found that SMA was characterized by a low RPI (83). In the current study, we found that a low RPI (<2) was associated with reduced systemic PGE_2 levels. Based on the lack biological materials that could be obtained from these young, anemic children with serious health complications, we were unable to perform comprehensive investigations on the link between the COX-2-PGE_2 pathway and intravascular and extravascular hemolysis. However, indirect markers of extravascular hemolysis such as significantly elevated spleen size (20) and monocytic pigment deposition in our cohort of children with SMA suggest that extravascular hemolysis is an important etiology of SMA in this holoendemic transmission region. Furthermore, the rates of erythrophagocytosis were enhanced among children with SMA in this study, consistent with a previous report (83). We are currently performing studies that include measures of erythrocyte turnover (e.g., LDH and neopterin) and erythrophagocytosis (measurement of CD35, 55, 59, C3c, Annexin V) to expand the knowledge about how the COX-2-PGE_2 pathway mediates these important etiological causes of SMA.

A primary challenge in the current study was the lack of bone-marrow biopsies from the children, based on practical and ethical considerations. As such, we cannot definitively determine if the low RPI scores in the context of decreased PGE_2 levels is truly indicative of a suppressed erythropoietic response. Although we will likely not be able to obtain bone-marrow biopsies in the future, we are currently investigating the direct effects of PGE_2 on erythroid maturation in a novel *in vitro* model of erythropoiesis we have developed using $CD34^+$ stem cells (84). These studies should provide important insight about the direct effects of PGE_2 on erythroid development.

Results from our laboratory (61, 84) and others (85–87) have shown that *Pf*Hz and *Pf*Hz-derived inflammatory mediators suppress erythropoiesis. In addition, *in vitro* studies from our laboratories demonstrated that phagocytosis of *Pf*Hz suppresses COX-2 gene products and PGE_2 in a time- and dose-dependent manner (88). The current study extends these findings by showing a progressive decrease in COX-2 transcripts and systemic bicyclo-PGE_2/creatinine levels with increasing deposition of naturally acquired monocytic *Pf*Hz, suggesting that accumulation of *Pf*Hz in monocytic cells may be an important mechanism through which COX-2 and PGE_2 levels are suppressed during a malaria infection. Although reduced COX-2 expression in tissue macrophages could contribute to the lower levels of systemic bicyclo-PGE_2/creatinine observed here, it was impossible to determine the impact of these cellular populations on PGE_2 levels since tissue biopsies were unavailable. In addition, based on the fact that there was exceedingly limited biological sample available from these anemic infants, we opted not to measure COX-1 transcriptional since this isoform is a constitutively expressed and produces PGEs in the context of physiological homeostasis (89). In addition, our previous results indicate that COX-1 transcriptional expression is not altered following the phagocytosis of *Pf*Hz by mononuclear cells (30). Although it is unlikely that COX-1 contributed to the differences observed in systemic bicyclo-PGE_2/creatinine levels in the clinical groups, this possibility cannot be definitively ruled out.

Since salicylates and acetaminophen (paracetamol) can suppress urinary production of PGE_2 (90, 91), the current study did not include children with reported antipyretic use two weeks prior to enrollment. Although it is possible that some of the children were given antipyretics prior to seeking treatment at the hospital, and this was not accurately reported by their caregivers, it is important to note that salicylates and acetaminophen primarily reduce PGE_2 production through steric hindrance of the COX enzymatic site and have minimal effects on *de novo* COX-2 gene expression (92, 93). Thus, data presented here showing the reduction of COX-2 transcripts in children with SMA, and the dose-dependent reduction in COX-2 message with increasing levels of monocytic *Pf*Hz accumulation would not be affected by antipyretic use. Additional studies aimed at measuring the exact concentrations of antipyretic metabolites and their association with PGE_2 production in children with malaria, however, is warranted and may offer further insight into potential mechanisms that could affect the COX-2-PGE_2 pathway.

In conclusion, based on the results presented here in Kenyan children from a holoendemic *P. falciparum* transmission region, along with our previous studies conducted in other geographic regions with differing malaria endemicities, and in individuals with distinct genetic backgrounds (30, 42–44, 88), we propose that suppression of systemic PGE_2 is a universal mediator of malaria pathogenesis. We further propose that reduced levels of systemic PGE_2 during a malaria infection are mediated, at least in part, by phagocytosis of *Pf*Hz by leukocytes. Recent *in vitro* and *in vivo* results from our laboratory showed that suppression of COX-2-mediated PGE_2 production is associated with over-production of TNF-α in children with malaria (88). Interestingly, measurement of 25 cytokines and chemokines in the plasma of the children investigated here, failed to show any significant associations between bicyclo-PGE_2/creatinine levels and inflammatory mediators. Thus, the exact means by which reduced production of PGE_2 alters the erythropoietic cascade in malaria remains to be determined.

Acknowledgments

We offer our sincere gratitude and appreciation to all parents, guardians, and children from the Siaya District community for their participation in this study. We also thank the staff at University of New Mexico/KEMRI laboratories and the Siaya District Hospital management for their support during the study. These data presented are published with the permission and approval of the Director, Kenya Medical Research Institute. The study was funded from a National Institutes of Health (NIH) Grant 1 R01A151305 (DJP) and a Fogarty International Center (FIC) Training Grant 1 D43TW05884 (DJP).

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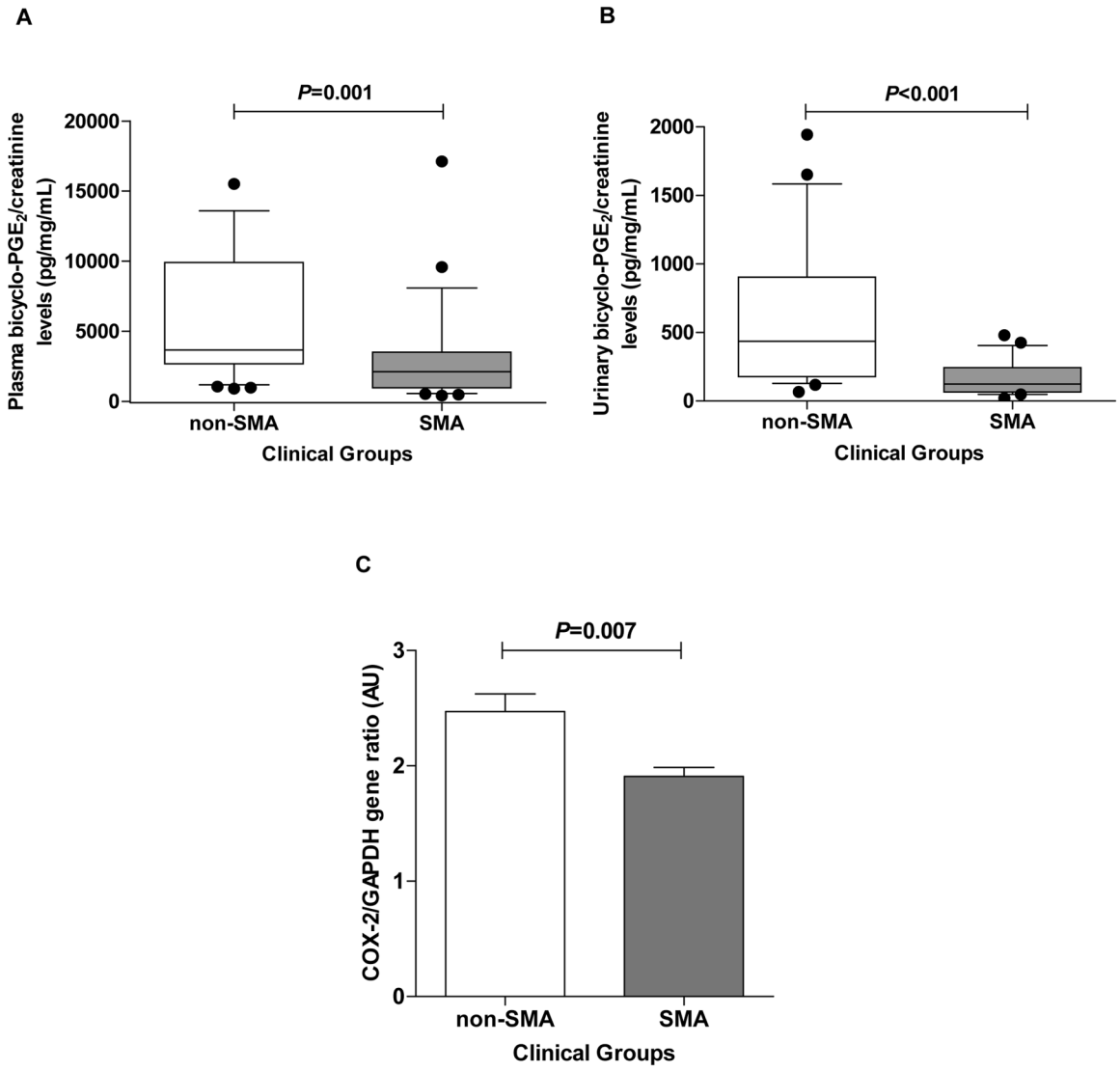

Figure 1. Bicyclo-PGE$_2$/creatinine concentrations and COX-2 transcripts in children with and without SMA

Concentrations of bicyclo-PGE$_2$/creatinine and COX-2 transcripts in children with non-SMA (Hb≥6.0 g/dL, with any density parasitemia) and SMA (Hb<6.0 g/dL, with any density parasitemia). (**A**) Plasma bicyclo-PGE$_2$/creatinine levels (pg/mg/mL) in the non-SMA (n=38) and SMA (n=36) groups. Differences between the groups were determined by Mann Whitney U test. (**B**) Urinary bicyclo-PGE$_2$/creatinine levels (pg/mg/mL) in the non-SMA (n=22) and SMA (n=22) groups. Differences between the groups were determined by Mann Whitney U test. (**C**) Semi-quantitative COX-2 transcript expression in children presenting with either non-SMA (n=9) or SMA (n=14). COX-2 mRNA expression mean values (arbitrary units, AU) were normalized by expression over GAPDH mRNA mean values (endogenous control). Differences between the groups were determined by Student's *t*-test.

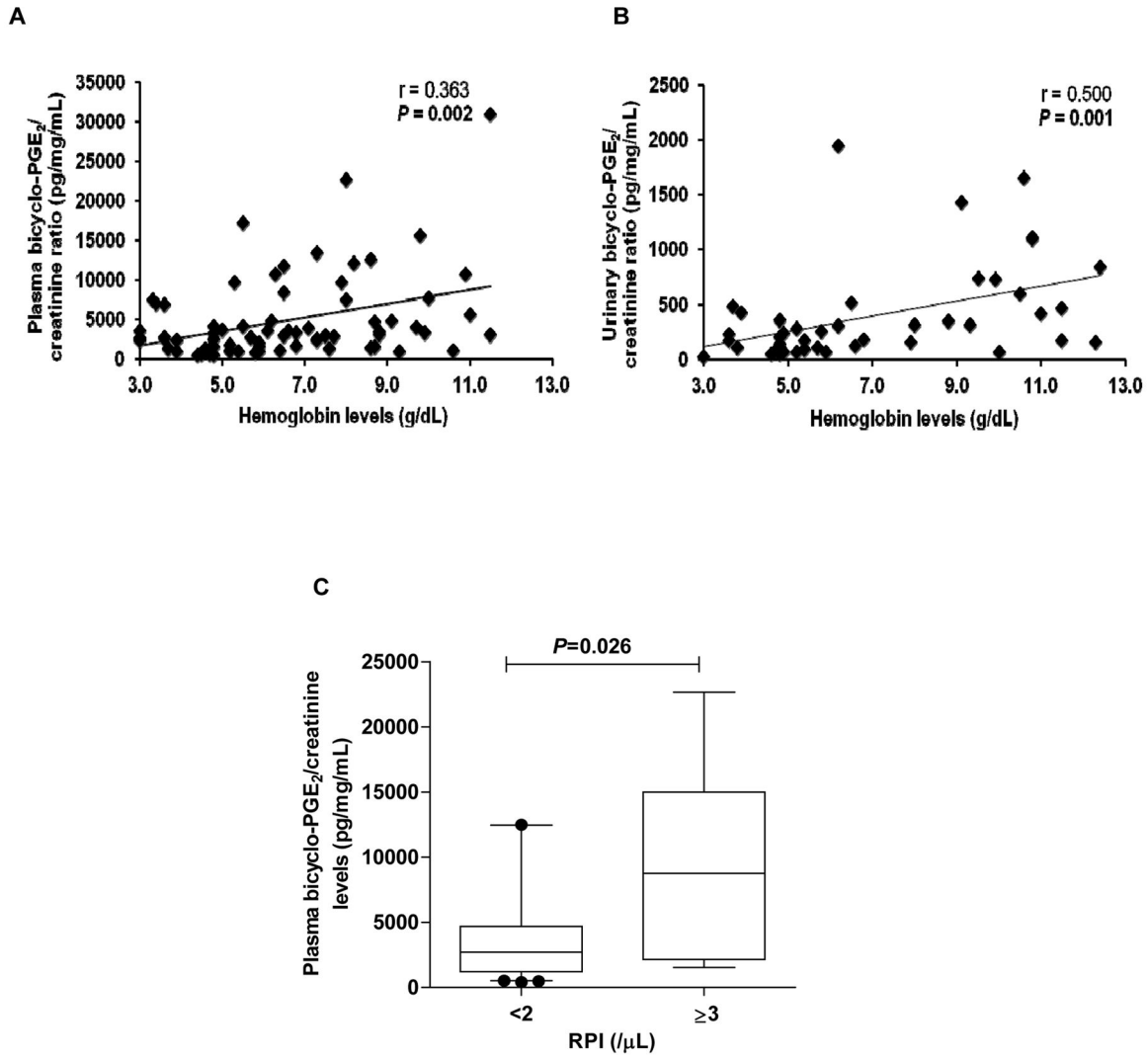

Figure 2. Relationship between bicyclo-PGE$_2$/creatinine levels, hemoglobin concentrations, and reticulocyte production index

(**A**) Relationship between plasma bicyclo-PGE$_2$/creatinine (pg/mg/mL) and Hb concentrations (g/dL) in children with malaria (n=74). Correlation coefficient (r) and statistical significance determined by Spearman's rank correlation test. (**B**) Relationship between urinary bicyclo-PGE$_2$/creatinine (pg/mg/mL) and Hb levels (g/dL) in children with malaria (n=44). Correlation coefficient (r) and statistical significance determined by Spearman's rank correlation test. (**C**) Plasma bicyclo-PGE$_2$/creatinine (pg/mg/mL) in children with insufficient (RPI<2.0) and appropriate (RPI≥3.0) erythropoiesis. Differences between the groups were determined by Mann Whitney U test.

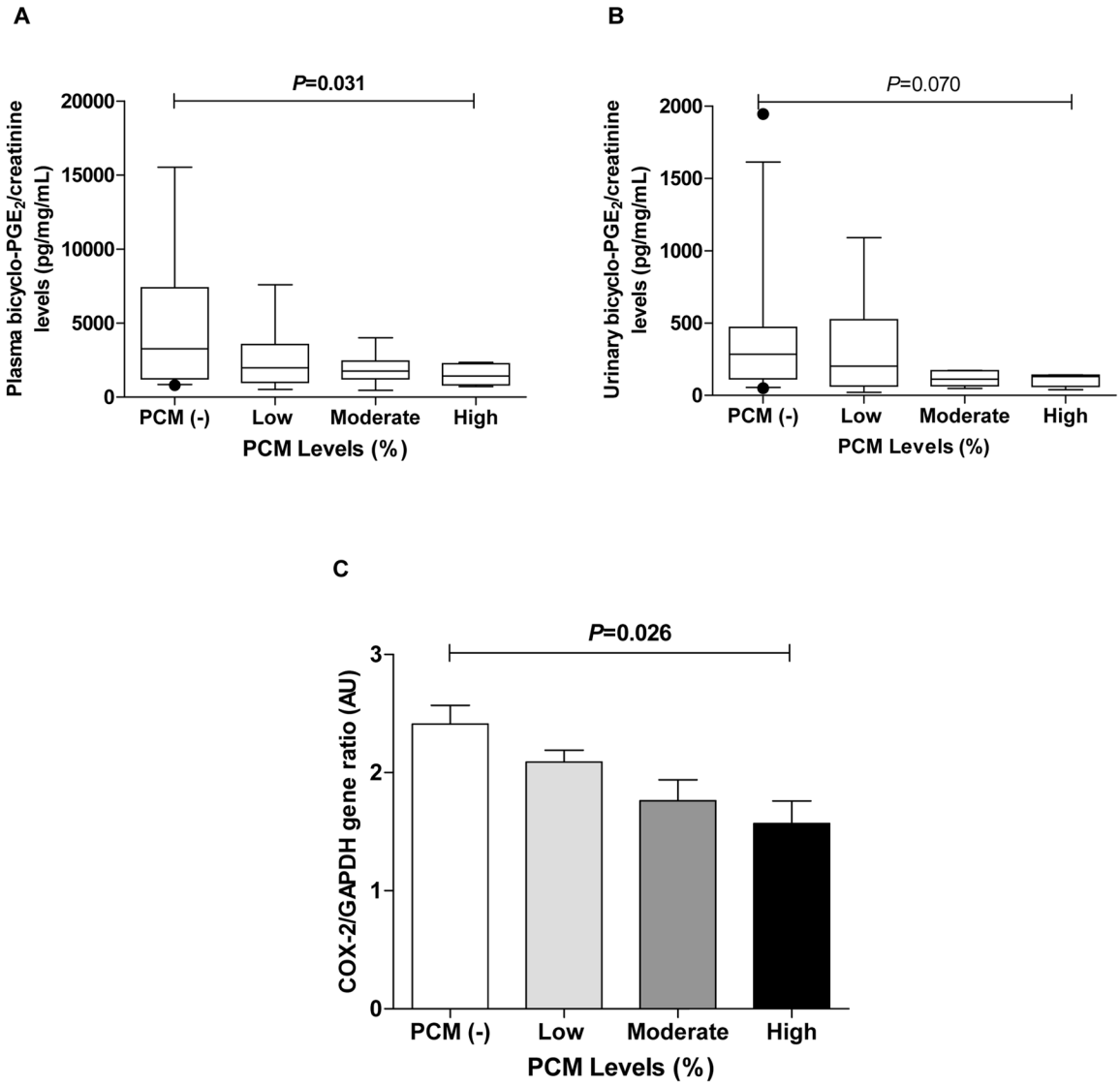

Figure 3. Bicyclo-PGE$_2$/creatinine concentrations and COX-2 transcripts stratified according pigment-containing monocytes

(**A**) Plasma bicyclo-PGE$_2$/creatinine (pg/mg/mL) in children with malaria grouped according to PCM levels. PCM (−) = no pigment-containing monocytes; Low = ≤10%; Moderate = >10<26.7%; and High = ≥26.7%. Differences across the groups were determined by Kruskal-Wallis test with post-hoc comparisons performed by Mann-Whitney U-test. (**B**) Urinary bicyclo-PGE$_2$/creatinine (pg/mg/mL) in children with malaria grouped according to PCM levels. PCM (-) = no pigment-containing monocytes; Low = ≤10%; Moderate = >10<26.7%; and High = ≥26.7%. Differences across the groups were determined by Kruskal-Wallis test with post-hoc comparisons performed by Mann-Whitney U-test. (**C**) Semi-quantitative COX-2 transcript expression in children with malaria grouped according to PCM levels (n=23). COX-2 mRNA expression mean values (arbitrary units, AU) were normalized by expression over GAPDH mRNA mean values (endogenous

control). Multivariate analyses performed by ANOVA with post hoc bivariate comparisons performed using Student's *t*-test.

Table 1

Clinical, demographic and laboratory characteristics of the study participants.

Characteristics	non-SMA (Hb ≥6.0g/dL)	SMA (Hb<6.0g/dL)	*P* value
No. of participants	**38**	**36**	
Gender, n (%)			
Male	24 (63.2)	21 (58.3)	0.671[a]
Female	14 (36.8)	15 (41.7)	
Age, months	12.5 (13.0)	8.0 (7.0)	**0.010**[b]
Admission temperature, °C	37.6 (2.0)	37.5 (2.0)	0.637[b]
Glucose, mmol/L	5.6 (2.0)	5.6 (1.0)	0.278[b]
Hematological Indices			
Hemoglobin, g/dL	8.0 (2.6)	4.8 (1.5)	**<0.001**[b]
Hematocrit, %	24.2 (7.1)	14.7 (4.4)	**<0.001**[b]
RBCs, $\times 10^6/\mu L$	3.5 (1.4)	2.1 (0.9)	**<0.001**[b]
RDW, %	20.6 (2.8)	23.4 (5.8)	**0.008**[b]
MCV, fL	70.8 (14.0)	70.4 (11.0)	0.516[b]
MCH, fL/cell	23.0 (5.1)	22.6 (3.8)	0.390[b]
MCHC, g/dL	32.6 (2.1)	32.2 (2.0)	0.660[b]
WBCs, $\times 10^9/L$	9.7 (6.4)	13.9 (11.6)	**0.035**[b]
Lymphocytes, $\times 10^3/\mu L$	5.3 (2.6)	6.9 (5.3)	0.066[b]
Monocytes, $\times 10^3/\mu L$	1.0 (0.8)	1.4 (1.1)	0.065[b]
Granulocytes, $\times 10^3/\mu L$	3.6 (3.0)	5.8 (6.0)	**0.015**[b]
Platelets, $\times 10^3/\mu L$	159.0 (132.0)	145.5 (76.0)	0.764[b]
Erythropoietic Indices			
Reticulocyte count, %	1.9 (3.1)	4.4 (5.6)	**0.006**[a]
Absolute reticulocyte number (ARN), $\times 10^9/L$	43.8 (78.2)	36.7 (54.2)	0.070[b]
Reticulocyte production index (RPI), /μL	1.0 (2.0)	0.9 (2.0)	0.165[b]
RPI<2, n (%)	31 (47.0)	35 (53.0)	**0.030**[a]
Erythrophagocytosis, %	2.5 (6.0)	4.0 (12.0)	**0.036**[b]
Erythrophagocytosis, $\times 10^3/\mu L$	0.02 (0.07)	0.05 (0.13)	0.140[b]
Parasitological Indices			
Parasite density, MPS/μL	8,717.6 (34,823.4)	11,320.8 (31,339.1)	0.875[b]
Geomean parasitemia, /μL	9,358. 7	9,787.5	-
HDP (≥ 10,000 Parasites/μL), n (%)	19 (50.0)	19 (53.0)	0.811[a]

Characteristics	non-SMA (Hb ≥6.0g/dL)	SMA (Hb<6.0g/dL)	*P* value
Genetic Variants			
Sickle cell trait, n (%)	5 (13.2)	5 (13.9)	0.927[a]
G6PD deficiency, n (%)	5 (13.2)	4 (11.1)	0.788[a]
Additional Laboratory Measures			
Pigment containing monocytes (PCM), n (%)	11 (31.4)	24 (68.6)	**0.001**[a]
Plasma creatinine, mg/dL	0.3 (0.4)	0.6 (0.5)	**0.007**[b]
Urinary creatinine, mg/dL	32.1 (32.9)	42.8 (67.0)	**0.047**[b]

Data are presented as the median (interquartile range; IQR) unless otherwise noted. Parasitemic children (n=74) were categorized according to a modified definition of SMA based on age and geographically-matched hemoglobin concentrations [i.e., Hb<6.0 g/dL, with any density parasitemia (54)], into non-SMA (n=38) and SMA (n=36). However, for the determination of the absolute reticulocyte number (ARN) and the reticulocyte production index (RPI) in the clinical groups, six samples with missing data on reticulocyte count and/or hematocrit levels were excluded from analyses. As such, comparison of the erythropoietic indices between non-SMA (n=37) and SMA (n=31) was carried out on 68 children. ARN and RPI were calculated, based on previous procedures (19, 60), as follows: reticulocyte index (RI) = (reticulocyte count × hematoctrit) ÷ 30.7 (average hematocrit of children < 5 years of age in Siaya district); maturation factor (MF) = 1 + 0.05 (30.7 − hematocrit); RPI = RI/MF; ARN = (RI × RBC count /L) ÷ 100. Erythrophagocytosis was determined in thin smear Giemsa-stained blood slides, with 100 monocytes counted per slide, and the number of monocytes/macrophages with phagocytosed RBCs expressed as a percentage of the total number of cells examined. In addition, erythrophagocytosis/μL was estimated using the coulter analyzer generated total monocyte counts. All subjects positive for HIV-1 or bacterial infections were excluded from the analyses.

[a] Statistical significance was determined by Pearson's Chi-square test.

[b] Statistical significance was determined by Mann-Whitney U test.

Abbreviations: Hb - Hemoglobin; non-SMA - non-severe malarial anemia (Hb≥6.0g/dL, with any density parasitemia); SMA - severe malarial anemia (Hb<6.0g/dL, with any density parasitemia) (54); RDW - Red cell distribution width; MCV - Mean corpuscular volume; MCH - Mean corpuscular hemoglobin; MCHC - Mean corpuscular hemoglobin concentration; MPS - malaria parasites; HDP - high density parasitemia (MPS≥10,000/μL).