Regular Article

RED CELLS, IRON, AND ERYTHROPOIESIS

Circulatory hepcidin is associated with the anti-inflammatory response but not with iron or anemic status in childhood malaria

Florence Burté,[1] Biobele J. Brown,[2,3] Adebola E. Orimadegun,[2] Wasiu A. Ajetunmobi,[2] Nathaniel K. Afolabi,[2] Francis Akinkunmi,[2] Olayinka Kowobari,[2] Samuel Omokhodion,[2,3] Kikelomo Osinusi,[2,3] Felix O. Akinbami,[2,3] Wuraola A. Shokunbi,[3,4] Olugbemiro Sodeinde,[1-3] and Delmiro Fernandez-Reyes[1-3]

[1]Division of Parasitology, Medical Research Council National Institute for Medical Research, London, UK; [2]Department of Paediatrics, College of Medicine, University of Ibadan, University College Hospital, Ibadan, Nigeria; [3]Childhood Malaria Research Group, University College Hospital, Ibadan, Nigeria; and [4]Department of Haematology, College of Medicine, University of Ibadan, University College Hospital, Ibadan, Nigeria

Key Points

- Hepcidin rises more dramatically in mild malaria than in severe malaria.
- Hepcidin levels are linked to inflammation, not anemia, in severe malarial anemia and cerebral malaria.

Cerebral malaria (CM) and severe malarial anemia (SMA) are the most serious life-threatening clinical syndromes of *Plasmodium falciparum* infection in childhood. Therefore, it is important to understand the pathology underlying the development of CM and SMA as opposed to uncomplicated malaria (UM). Increased levels of hepcidin have been associated with UM, but its level and role in severe malarial disease remains to be investigated. Plasma and clinical data were obtained as part of a prospective case-control study of severe childhood malaria at the main tertiary hospital of the city of Ibadan, Nigeria. Here, we report that hepcidin levels are lower in children with SMA or CM than in those with milder outcome (UM). While different profiles of pro- and anti-inflammatory cytokines were observed between the malaria syndromes, circulatory hepcidin levels remained associated with the levels of its regulatory cytokine interleukin-6 and of the anti-inflammatory cytokine inerleukin-10, irrespective of iron status, anemic status, and general acute-phase response. We propose a role for hepcidin in anti-inflammatory processes in childhood malaria. (*Blood*. 2013;121(15):3016-3022)

Introduction

Human malaria, caused by the parasite *Plasmodium spp.*, has an estimated annual global disease burden of 216 million clinical episodes, leading to 655 000 deaths.[1] Nigeria, a site where the principal causative agent of malaria is the most deadly species *Plasmodium falciparum,* has a quarter of the global cases and a third of the malaria-attributable childhood deaths.[2,3] Several complications in malaria-infected children can arise, with cerebral malaria (CM) and severe malarial anemia (SMA) being the leading causes of morbidity and mortality.[4]

During the period between 2000 and 2005, SMA, the most common severe malarial disease, accounted for 9% of all admissions at the University College Hospital (UCH) of Ibadan and 75% of severe malaria cases. It affected mostly malaria-infected younger children (95% of SMA occurs in children younger than 5 years).[3] It is a systemic syndrome characterized by peripheral parasitemia and severe anemia (hemoglobin levels <5 g/dL) believed to be due to the destruction of infected and uninfected erythrocytes, splenic sequestration of erythrocytes, and dyserythropoiesis.[5] CM is the most fatal syndrome, mainly characterized by coma.[6] Cytoadherence of infected erythrocytes to endothelial cells in the microvasculature is thought to play a role in CM. Sequestration of infected erythrocytes can lead to clogging in these sites, a phenomenon particularly observed in the brain.[6] However, this process does not fully explain the syndrome and other pathological events are

thought to be involved.[6] Although the physiopathologies of both severe syndromes have been widely investigated, the exact molecular and cellular events leading to severe childhood malaria cases have not been completely explained.

Human host response studies have suggested that an imbalance between pro- and anti-inflammatory cytokines could play a role in the establishment of severe malaria syndromes.[5,6] In particular, a prolonged proinflammatory response and inadequate anti-inflammatory response might contribute to persistent anemia.[7] However, circulatory inflammatory events in severe malaria remain elusive, particularly in CM, due to contradictory observations in different cohort studies.[8-12]

Hepcidin is a 25-amino-acid cysteine-rich peptide derived from an 84-amino-acid preprohormone produced in hepatocytes. It was first discovered as an antimicrobial agent in urine[13] but was soon observed to have a major role in iron homeostasis.[14,15] In a non-inflammatory environment, hepcidin leads to the sequestration of iron in enterocytes and macrophages by binding to the iron exporter membrane protein ferroportin.[16] Hepcidin is also considered as an acute-phase protein induced by interleukin-6 (IL-6) during infection and inflammation processes that decreases circulating iron and iron supplies to cells.[17] It can also be downregulated by hypoxia and high levels of erythropoietin.[18] Because of hepcidin's role in iron homeostasis and inflammatory

---

processes, there is considerable interest in its involvement in the pathogenesis of malaria. A recent study in a mouse malaria model suggested that hepcidin mediates host regulation of malaria superinfections.[19] In humans, several studies have reported an increase in urine or serum hepcidin during uncomplicated and asymptomatic childhood malaria.[20-22] Hepcidin has also been proposed to play a role in the pathogenesis of anemia and in the establishment of anemia in patients with SMA.[7] However, the hepcidin levels in other manifestations of severe malaria have not been measured and mechanisms of hepcidin regulation in malaria infection need further investigation.

Because hepcidin has been linked to inflammation, iron-regulatory processes, and anemia,[16] we hypothesized that one of these mechanisms could explain differences between severe and mild cases. We conducted a case-control study on the role of hepcidin in the major syndromes of childhood malaria. Hepcidin levels in CM pediatric patients were investigated for the first time. Our study suggests that hepcidin has a particular role in the host response to malaria infection alongside IL-6 and interleukin-10 (IL-10) anti-inflammatory cytokines.

Methods

Ethics statement

Parents or guardians of study participants gave informed written consent in accordance with the Declaration of Helsinki. This research was approved by the joint ethics committee of the College of Medicine of the University of Ibadan and the UCH Ibadan.

Study site

All study participants were recruited under the auspices of the Childhood Malaria Research Group (CMRG) at the 600-bed tertiary hospital UCH in the city of Ibadan, Nigeria, in west sub-Saharan Africa. Ibadan is a densely populated urban setting with a population of 2.5 million inhabitants. Ibadan has a lengthy 8-month rainy season from March to October, with malaria transmission and severe disease present year round.

Study design and case definitions

The UCH Ibadan Department of Pediatrics screens about 12 000 children attending the hospital for malaria parasites per year and diagnoses 11.3% SMA and 19.7% CM in the parasitized children younger than 5 years of age.[3]

The participants in this study were recruited from 2008 to 2010 as part of a larger prospective case-control study of severe childhood malaria currently ongoing under the auspices of the CMRG.[23]

Malaria parasites were detected and counted by microscopy following Giemsa staining of thick and thin blood films.[24] Children with severe malaria were recruited on admission from the Otunba Tunwase Children's Emergency Ward. Children with uncomplicated malaria were recruited as part of a daily routine malaria parasite screening at the Children's Outpatient Clinics. Malaria-negative ill children were recruited either at admission from Otunba Tunwase Children's Emergency Ward or from the Department of Pediatrics inpatient wards. Malaria-negative healthy community control children were recruited from local vaccination clinics as well as during school visits across several Ibadan districts.

We recruited children aged 6 months to 12 years using 5 participant definitions. The malaria-positive children (the cases) included uncomplicated malaria (UM), SMA, and CM. The malaria-negative children (the controls) were disease controls (DC) and community controls (CC). We followed the World Health Organization criteria for severe *P. falciparum* malaria.[25] CM cases were defined as children in unarousable coma for at

least 1 hour in the presence of asexual *P. falciparum* parasitemia with normal cerebrospinal fluid. A Blantyre coma score <2 was used to assess coma status. Children with hypoglycemia were excluded from the study. Added to the strict clinical and laboratory definitions of CM, our study patients recovered consciousness after effective antimalarial therapy. We excluded from this study those CM patients who died. Our overall mortality rate for CM was of the order of 10%. SMA cases were defined as conscious children with packed cell volume (PCV) <16% in the presence of *P. falciparum* parasitemia. We excluded from this study those SMA patients who died. Our overall mortality rate for SMA was <1%. UM cases were defined as febrile children with *P. falciparum* parasitemia who did not require hospital admission. Our study only included those children with CM and UM with PCV >20%. We excluded from the study blood-culture–positive cases. Although we did not carry out blood cultures in all severe malaria patients, the cases recruited into this study are those in whom septicemia was not suspected and who were successfully treated with antimalarial therapy alone.

The DC group consisted of malaria-negative children with infectious diseases such as meningitis, otitis media, diarrhea, and upper respiratory tract infections. It also included mild to moderately anemic children and children admitted for surgery.

Clinical data and sample collection

Participants' clinical data were collected using a malaria-tailored questionnaire designed by the CMRG. Two blood samples were obtained from each participant: 1 in an EDTA blood collection tube for subsequent plasma separation and 1 in a serum separator tube for serum collection. Blood samples were kept on ice and transferred to the central malaria laboratory. Plasma for this study was harvested by centrifugation (1000g, 10 minutes). Both plasma and serum samples were aliquoted and frozen at −80°C no later than 4 hours following collection.

Clinical laboratory analysis

PCV was measured using the microhematocrit method.[24] Briefly, blood was obtained in capillary tubes. Tubes were centrifuged at 12 000g for 5 minutes. The percentage cell volume compared with the whole tube volume was calculated (ie, PCV). Median (first, third interquartile) PCVs for each clinical group were compared using a Mann-Whitney U test ($P < .05$).

Malaria parasites (MPs) were detected and counted by microscopy following Giemsa staining of thick and thin blood films.[24] MP densities were calculated as follows: MP/μL = [(number MPs/number white blood cells) × 8000] for each malaria-positive clinical group (Table 1). The microscopic criterion for declaring a participant to be free of malaria was the absence of parasites in 100 high-power (×1000) fields. Then 1 in 10 thick blood films were randomly selected and independently reviewed by local experienced microscopists not part of the research team.

Identification of hepcidin in SELDI-TOF mass spectra

Immunoprecipitations were carried out for the identification of hepcidin-25 (using rabbit polyclonal anti–hepcidin-25; Abcam UK). Antibodies and plasma samples were mixed overnight at 4°C. As controls, plasma replicates were incubated with a nonspecific antibody (anti-serum amyloid A; Abcam UK). A 50% protein G-Sepharose slurry was added and incubated at room temperature for 2 hours before being pelleted. The supernatants were collected and pellets were washed 3 times in phosphate-buffered saline before being resuspended in 50% ACN/0.5% TFA (v/v). After pelleting, supernatants and eluates were subjected to surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry analysis using a copper-charged immobilized metal affinity solid-phase fractionation (IMAC30 ProteinChip arrays; Bio-Rad Laboratories) surface as detailed in the following paragraph. The 2790-Da peak cluster was positively identified as hepcidin (supplemental Figure 1). The 2790-Da peak was depleted in the plasma supernatant (supplemental Figure 1B) compared with plasma sample prior immunoprecipitation (supplemental Figure 1A) and was present in the immunoprecipitate pellet (supplemental Figure 1C).

Table 1. Clinical characteristics of children in the different clinical groups

Clinical group	CC	DC	UM	CM	SMA
N	68	21	49	19	22
Age (mo), median (IQR)	96 (48-108)	60 (36-108)	48 (26-86)	56 (34-69)	58 (17-136)
Sex, ratio female:male	34:34	12:9	19:30	7:12	9:13
PCV (%), median (IQR)	35 (33-37)	34 (29-36)	32 (30-34)*†	30 (26-33)*‡	11 (10-13)*†‡§
Parasite density (MP/μL), median (IQR)	N/A	N/A	38 214 (2693-63 212)	13 806 (1700-56 606)	5330 (462-76 154)‡

IQR, interquartile range; N, number of samples in each group; N/A, not applicable.

*Statistically different compared with CC ($P < .0001$, Mann-Whitney U test).

†Statistically different compared with DC ($P < .0001$, Mann-Whitney U test).

‡Statistically different compared with UM ($P < .01$, Mann-Whitney U test).

§Statistically different compared with CM ($P < .0001$, Mann-Whitney U test).

Differential expression of plasma and serum hepcidin

Crude plasma and serum samples were coded, blinded, randomized, and diluted 10-fold in denaturation buffer (50 mM Tris-HCl [pH 9.0], 9M urea, 2% CHAPS) for 30 minutes. Denatured samples were applied onto an immobilized copper affinity solid-phase fractionation surface (IMAC30 ProteinChip array; Bio-Rad Laboratories). Mass spectra were generated on a System 4000 Bio-Rad ProteinChip mass spectrometer using laser energies 2000, 2400, and 3000 nJ with the focus mass setting set to 2790 Da to improve resolution around the protein of interest (hepcidin-25: 2789 Da).

Data analysis for hepcidin differential expression was first carried out using ProteinChip Datamanager Client 4.1 software. Spectra were normalized by total ion current starting with a minimum m/z of 1500 Da. Outliers, spectra with a normalization factor outside of mean ± 2 SD, were discarded. The 2789 Da (±1 Da) peaks corresponding to hepcidin-25 were labeled. To compare directly to the IL-6 enzyme-linked immunosorbent assay (ELISA) data, for the plasma samples, the median of peak cluster intensities (m/z) for each study group were directly plotted on a boxplot with 5 to 95 percentile whiskers and compared between groups using Mann-Whitney U test. In the case of serum hepcidin-25, peak intensity values were standardized to $\mu = 0$ and $\sigma^2 = 1$ and medians (first, third interquartiles) were tabulated and compared between groups using the Mann-Whitney U test ($P < .05$).

Circulatory levels of IL-6, CRP, ferritin, transferrin, and sTfR

The levels of human plasma IL-6, plasma C-reactive protein (CRP), plasma and serum ferritin, plasma transferrin and plasma soluble transferrin receptor (sTfR) were assessed using Quantitative sandwich enzyme immunoassays (ELISA). The ELISA assays were performed following the manufacturers' instructions (human IL-6 and sTfR, R&D Systems Quantikine assays; CRP, ferritin, and transferrin, α Diagnostic International). The data were represented as median with 5th to 95th percentile whiskers and compared between disease groups using the Mann-Whitney U test ($P < .05$).

Bead immunoassay of serum samples

Serum samples from the same patients were used to measure inflammatory status. The levels of 10 different circulating inflammatory cytokines (granulocyte macrophage–CSF [GM-CSF], interleukin-1β [IL-1β], interleukin-2, interleukin-4 [IL-4], interleukin-5, IL-6, interleukin-8 [IL-8], IL-10, interferon-γ, and tumor necrosis factor [TNF-α]) were simultaneously measured. The 179 serum samples were subjected to the human cytokine 10-plex multiplex beads immunoassay kit according to the manufacturer's instructions (Invitrogen, Life Technologies). Plates were read using the Bio-Plex 200 Systems (Bio-Rad Laboratories; powered by xMAP Technology) and analyzed using the Bio-Plex Manager 6.0 software (Bio-Rad Laboratories). Data for interleukin-2, interleukin-5, and interferon-γ were discarded because more than 25% of the results were under the linear range of the assay. For each analyte (IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, and GM-CSF), medians (first, third interquartiles) were calculated for each study group, tabulated, and compared between groups using the Mann-Whitney U test ($P < .05$).

Regression and correlation analyses

To ensure uniformity of the results from plasma and serum samples, ferritin levels were repeated in serum samples using the same assay (ELISA) and serum IL-6 levels were measured using the multiplex bead immunoassay and compared with plasma IL-6 levels measured by ELISA. Both assays showed very good linear regressions with slopes and r^2 closed to 1 ensuring harmony between plasma and serum analyte levels and between the two different types of immunoassays (supplemental Figure 2).

To correlate hepcidin, serum cytokines, and clinical data, each data set for each serum analyte (inflammatory cytokines, ferritin, and hepcidin) was compared with using the nonparametric Spearman r test ($P < .01$).

Results

Study participants

A total of 179 children from Ibadan, Nigeria, participated in this study, comprising 90 malaria-positive children with CM, SMA, or UM and 89 malaria-negative children who were either DC or CC. Clinical data acquired for these patients are shown in Table 1.

Circulatory hepcidin was lower in children with severe malaria irrespective of anemic status

Hepcidin levels were measured in plasma samples and compared between study groups. Plasma hepcidin-25 levels were low in the malaria-negative groups but increased significantly in the malaria-positive groups (Figure 1A). We found no significant correlation between plasma hepcidin levels and parasitemia in any of the malaria-positive groups. The levels of plasma hepcidin were dramatically lower in both the CM and SMA severe malaria groups when compared with the UM group (Figure 1A). Interestingly, the CM group, comprising very mildly anemic patients, had levels of hepcidin similar to the SMA group, comprising severe anemic patients (Table 1; supplemental Figure 3).

Circulatory hepcidin was not associated with iron status or the major acute-phase reaction

We first assessed the acute-phase reaction status by measuring IL-6, inducer of hepcidin hormone, and CRP, one of the major acute-phase proteins. The plasma levels of IL-6 showed very similar trends to plasma hepcidin (Figure 1B). CRP levels were significantly higher in all ill-children (malaria-positive and malaria-negative) groups when compared with CC with no major differences in levels whether children had malaria or another illness (Figure 1C).

We also assessed the iron circulatory status by measuring plasma transferrin, sTfR (a surrogate of cellular iron intake[9]), and ferritin (a marker of iron stores, also increased in inflammatory
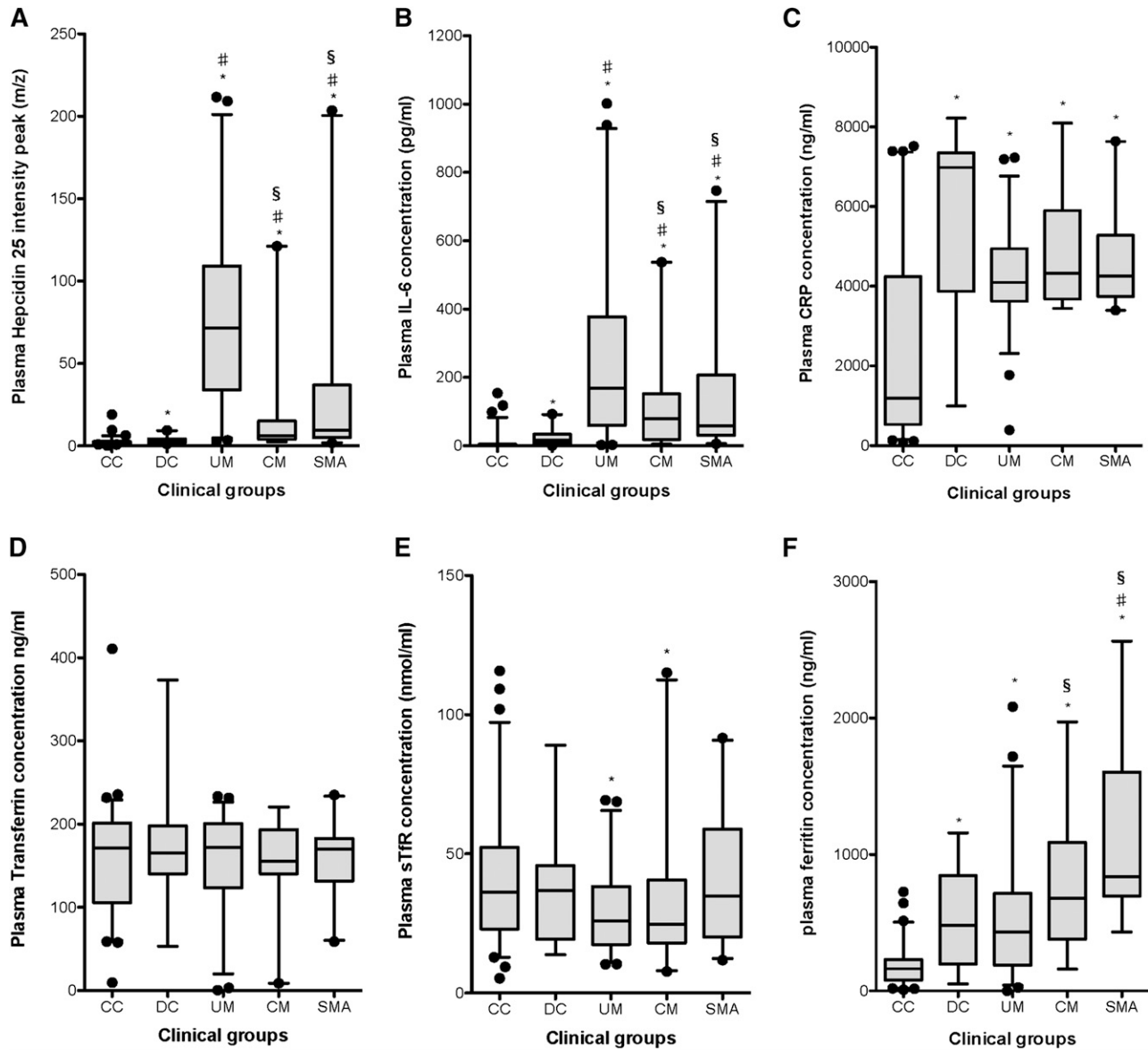
Figure 1. Plasma levels of proinflammatory and iron-regulatory proteins. (A) Plasma hepcidin levels. Hepcidin-25 (m/z 2789) peak intensities were measured by SELDI-TOF and compared between groups. *Statistically different compared with CC ($P < .0001$); #statistically different compared with DC ($P < .001$); §statistically different compared with UM ($P < .001$). (B) Plasma IL-6 levels. *Statistically different compared with CC ($P < .001$); #statistically different compared with DC ($P < .01$); §statistically different compared with UM ($P < .05$). (C) Plasma CRP levels. *Statistically different compared with CC ($P < .001$). (D) Plasma transferrin levels. (E) Plasma sTfR levels. (F) Plasma ferritin levels. *Statistically different compared with CC ($P < .001$); #statistically different compared with DC ($P < .05$); § statistically different compared with UM ($P < .01$). Boxplot with median and 5th to 95th percentile whiskers.

or/and oxidative conditions[18]). All clinical groups (malaria-positive, disease, and CC children) showed comparable transferrin and sTfR levels (Figure 1D-E), showing different trends to hepcidin. Malaria-positive children had higher levels of ferritin than the malaria-negative CC group (Figure 1F). Children with severe malaria (CM, SMA) showed statistically significant higher levels of ferritin among the malaria-positive groups (Figure 1F).

Circulatory hepcidin was associated with the inflammatory cytokines IL-6 and IL-10 in all children

Levels of inflammatory cytokines (IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, and GM-CSF) were further investigated. Medians and interquartile ranges of a variety of inflammatory cytokines and the acute-phase proteins ferritin and hepcidin were measured in serum

(Table 2). In general, all measured cytokines had higher levels in UM compared with other malaria-positive study groups. On the contrary, levels of measured cytokines were the lowest in the CM group compared with other malaria-positive study groups. However, compared with other illnesses (DC group), the CM group showed similar levels of inflammatory cytokines, except for IL-6 and IL-10, which showed higher medians. As a general observation, in the SMA study group, the levels of proinflammatory cytokines (IL-1β and TNF-α) were similar to those in the UM group, whereas the levels of IL-6 and IL-10 cytokines were low, similar to CM cases. The exception was for GM-CSF levels, which were in majority between the levels and not significantly different from UM and CM study groups, indicating a probable dual role for GM-CSF in SMA. Except for IL-4 and IL-8, all other cytokines had higher levels in all malaria-positive groups compared with CC.

Table 2. Inflammatory status of children in the different clinical groups

	CC	DC	UM	CM	SMA
IL-1β, pg/mL	0.89 (0.22-3.81)	2.01 (0.48-2.90)	3.79* (1.45-5.81)	1.12† (0.36-2.44)	2.88*‡§ (1.94-5.97)
IL-4, pg/mL	5.64 (1.61-25.04)	3.22* (1.61-3.76)	3.76* (1.88-5.64)	1.88*† (1.61-1.88)	3.22§ (1.61-3.76)
IL-6, pg/mL	6.80 (2.99-19.82)	21.35* (9.82-73.67)	195.57*‡ (77.46-433.10)	75.30*† (21.53-140.27)	55.39*‡ (35.39-163.73)
IL-8, pg/mL	392.59 (103.44-1127.22)	51.29* (21.50-418.11)	69.95* (30.79-141.90)	39.04* (13.47-153.80)	45.68* (27.78-227.82)
IL-10, pg/mL	7.44 (3.70-13.45)	15.94* (5.31-37.19)	730.18*‡ (240.77-1900.77)	76.55*†‡ (45.80-489.80)	199.69*†‡ (112.37-397.60)
TNF-α, pg/mL	1.80 (0.90-6.12)	1.80 (0.66-2.70)	4.60*‡ (2.70-11.68)	1.80† (0.66-3.29)	3.44‡ (0.98-6.57)
GM-CSF, pg/mL	1.04 (0.93-2.87)	0.52* (0-0.93)	2.78*‡ (0.93-4.63)	0.93† (0.51-1.70)	1.24‡ (0.93-2.73)
Ferritin, ng/mL	99.35 (55.07-154.10)	536.50* (144.10-755.30)	313.40* (176.70-509.80)	539.70*‡ (363.40-985.90)	583.20*† (472.10-985.90)
Serum hepcidin, standardized peak intensity (m/z)	−0.43 (−0.46 to −0.37)	−0.44 (−0.48 to −0.36)	0.11*‡ (−0.38 to 1.037)	−0.22*† (−0.43 to 0.62)	−0.38† (−0.48 to 0.25)

Level of serum inflammatory molecules shown as median (interquartile range).
*Statistically different (P < .05) compared with CC.
†Statistically different (P < .05) compared with UM.
‡Statistically different (P < .05) compared with DC.
§Statistically different (P < .05) compared with CM.

Standardized serum hepcidin levels showed very similar trends to IL-6 and IL-10 levels for all groups (including DC). Accordingly, serum hepcidin levels correlated only with IL-6 and IL-10 and correlated best with the latter (supplemental Table 1).

In the UM group, all the pro- and anti-inflammatory cytokines (IL-1β, TNF-α, GM-CSF, IL-6, and IL-10) correlated between themselves (Table 3). Interestingly, the anti-inflammatory cytokine IL-10 correlated with IL-6 in both the UM and SMA study groups (Table 3) but not in the CM group. GM-CSF correlated with all other cytokines in the UM and SMA groups but only correlated with IL-6 in the CM group.

Discussion

Since the first observation that the iron-regulatory peptide hepcidin was linked to malaria,[22] a growing interest for the role of hepcidin in malaria pathogenesis has developed. However, the exact mechanisms and role of host regulation of hepcidin in the pathogenesis of malaria and more particularly in the establishment of the different phenotypes of the disease remain obscure.

Despite the fact that hepcidin levels were on average higher in children affected by various diseases compared with CC, the levels were strikingly higher in malaria-affected children. This suggested a more pronounced host hepcidin response in malaria compared with other illnesses. Contrary to previous reports,[22] we found no significant correlation between plasma hepcidin levels and parasitemia in any of the malaria-positive groups. Most of the previous studies have concentrated on the levels of hepcidin in mild and asymptomatic malaria cases.[20-22,26] These studies proposed that high hepcidin levels could lead to limited iron availability for erythropoiesis and eventually anemia. This observation also raises the question of the necessity of iron supplementation in malaria cases because elevated levels of hepcidin can lead to the sequestration of iron in enterocytes, preventing the release of newly absorbed dietary iron.[16,27]

In the present study, we also showed that hepcidin levels were lower in SMA than in milder UM. We also observed that hepcidin levels were not associated with circulatory iron status fluctuations. These results were consistent with a recent study[27] showing that levels of hepcidin did not correlate with circulatory iron status and that lower levels of hepcidin were found in severely anemic patients compared with mild or nonanemic patients.[27] Nevertheless, in our study, we also observed that hepcidin levels were similarly low in CM patients, characterized by mild or no anemia, compared with SMA. This finding therefore undermines the link between hepcidin and anemia in severe malarial disease.

The acute-phase reaction was high in all children with illness, as supported by high levels of CRP and ferritin. Despite the general acute-phase response in all malaria syndromes, the inflammatory status of malaria-infected patients showed different trends in the various study groups. In children with mild disease, the levels of all studied inflammatory cytokines were elevated compared with children with other illnesses or severe malaria syndromes, indicating a strong inflammatory response in these patients.

The balance between pro- and anti-inflammatory cytokines in mild disease seemed to be disturbed in each of the two severe study groups. In SMA, the cytokine IL-6 and the anti-inflammatory cytokine IL-10 showed lower levels compared with mild disease,

Table 3. Correlation between inflammatory cytokines in the different malaria clinical groups

	IL-10			IL-6			GM-CSF			TNF-α		
	UM	CM	SMA	UM	CM	SMA	UM	CM	SMA	UM	CM	SMA
IL-1β	r = 0.359	r = 0.333	r = 0.842	r = 0.532	r = 0.614	r = 0.482	r = 0.483	r = 0.345	r = 0.6276	r = 0.524	r = 0.384	r = 0.535
	P = .011	P = .150	P = .015	P < .0001	P = .004	P = .015	P = .004	P = .137	P = .0008	P = .001	P = .011	P = .006
TNF-α	r = 0.423	r = 0.585	r = 0.577	r = 0.633	r = 0.583	r = 0.7347	r = 0.638	r = 0.5365	r = 0.571			
	P = .003	P = .007	P = .001	P < .0001	P = .004	P < .0001	P < .0001	P = .015	P = .002			
GM-CSF	r = 0.808	r = 0.453	r = 0.4857	r = 0.844	r = 0.762	r = 0.5667						
	P < .0001	P = .045	P = .009	P < .0001	P < .0001	P = .002						
IL-6	r = 0.801	r = 0.297	r = 0.801									
	P < .0001	P = .179	P < .0001									

r, nonparametric Spearman correlation coefficient (statistically significant if P < .01 according to the nonparametric Spearman r test).

while proinflammatory cytokines IL-1β and TNF-α had similar levels in both study groups. The strong correlation between IL-6 and IL-10 suggested an anti-inflammatory role for the dual IL-6 cytokine. This observation is in accordance with the consensus that an imbalance between pro- and anti-inflammatory cytokines is linked with the establishment of severe disease in malaria,[28] with IL-6 acting alongside IL-10 as an anti-inflammatory cytokine.[11]

The inflammatory status of CM patients resembled more the one of patients with other illnesses (DC). Compared with UM, all cytokine levels were consistently lower in the CM group but no clear-cut imbalance between pro- and anti-inflammatory cytokines could be observed. In our study, CM TNF-α levels were elevated when compared with CC but lower than in the UM group. Our findings conflict with those of studies from Malawi[29] and Gambia[30] that showed that TNF-α was higher in CM than in SMA or UM. However, a more recent study[12] showed no difference in TNF-α levels between CM and non-CM. While there are conflicting data regarding TNF-α levels in severe malaria and syndrome outcome, several studies conducted in children infected with P. *falciparum* are in agreement regarding the association of proinflammatory cytokines with other clinical variables.[28-31]

Here, we report for the first time that the association between circulatory hepcidin levels and the anti-inflammatory cytokine IL-10, alongside the acute-phase–regulatory cytokine IL-6, occurs irrespective of the level of anemia and inflammatory status of the host. It could be suggested that hepcidin behaves as an atypical acute-phase protein in an anti-inflammatory dependent way, irrespective of the malaria syndrome. The anemic status of patients with SMA did not seem to be solely due to hepcidin or IL-10 effects as previously reported.[27] The main difference observed in the present study between the two severe syndromes was the higher proinflammatory response compared with anti-inflammatory response. TNF-α has long been linked to anemia.[31,32] Proinflammatory cytokines and resulting nitric oxide signaling might downregulate erythropoiesis.[33] Moreover, TNF-α itself has apoptotic effects on erythrocyte precursors.[33] The observation that ferritin, transcriptionally regulated by oxidative stress,[34] was significantly higher in the SMA group also agrees with the hypothesis that oxidative signaling, probably induced by the prominent proinflammatory response, is involved in the establishment of the syndrome. It can be hypothesized that the imbalance observed between pro- and anti-inflammatory response in SMA patients might be at the basis of anemia rather than iron-related events controlled by hepcidin. This imbalance does not occur in CM patients, who are not severely anemic, and is not directly linked to the anti-inflammatory response and hepcidin.

The present study not only emphasizes the role of iron-regulatory and pro- and anti-inflammatory mechanisms in the establishment of the different severe syndromes, but also supports the hypothesis that iron supplementation might not be required in the management of severe malaria. Also, hepcidin, although not a component of anemic status in the present study, could potentially be used to predict disease severity, which urges the need for well-characterized reference ranges and harmonized methodologies[35-37] to measure plasma hepcidin.

Acknowledgments

Authorship

Contribution: D.F.-R. and O.S. conceived, designed, deployed, and directed the case-controlled study at the Childhood Malaria Research Group, Ibadan, Nigeria. B.J.B., A.E.O., W.A.A., N.K.A., F.A., O.K., S.O., K.O., F.O.A., W.A.S., O.S., and D.F.-R. carried out patient recruitment, sample collection, storage, and transport. F.B. and D.F.-R. carried out hepcidin experiments. F.B. and D.F.-R. analyzed mass spectra. F.B. and D.F.-R. performed statistical analysis. D.F.-R., F.B., O.S., B.J.B., A.E.O., W.A.A., and O.K. processed, reviewed, and analyzed demographical and clinical data. F.B. carried out iron status, CRP, and inflammatory status determination. F.B., O.S., and D.F.-R. produced figures and tables and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Delmiro Fernandez-Reyes, Division of Parasitology, MRC National Institute for Medical Research, The Ridgeway, London NW7 1AA, UK; e-mail: dfernan@nimr.mrc.ac.uk.

References

1. WHO. World Malaria Report: 2010; 2010.

2. Amzat J. Assessing the progress of malaria control in Nigeria. *World Health Popul*. 2011; 12(3):42-51.

3. Orimadegun AE, Fawole O, Okereke JO, et al. Increasing burden of childhood severe malaria in a Nigerian tertiary hospital: implication for control. *J Trop Pediatr*. 2007;53(3):185-189.

4. Chang KH, Tam M, Stevenson MM. Inappropriately low reticulocytosis in severe malarial anemia correlates with suppression in the development of late erythroid precursors. *Blood*. 2004;103(10):3727-3735.

5. Perkins DJ, Were T, Davenport GC, et al. Severe malarial anemia: innate immunity and pathogenesis. *Int J Biol Sci*. 2011;7(9):1427-1442.

6. Pamplona A, Hanscheid T, Epiphanio S, et al. Cerebral malaria and the hemolysis/methemoglobin/heme hypothesis: shedding new light on an old disease. *Int J Biochem Cell Biol*. 2009;41(4):711-716.

7. Nweneka CV, Doherty CP, Cox S, et al. Iron delocalisation in the pathogenesis of malarial anaemia. *Trans R Soc Trop Med Hyg*. 2010; 104(3):175-184.

8. Grau GE, Mackenzie CD, Carr RA, et al. Platelet accumulation in brain microvessels in fatal pediatric cerebral malaria. *J Infect Dis*. 2003; 187(3):461-466.

9. Muñoz M, García-Erce JA, Remacha AF. Disorders of iron metabolism. Part II: iron deficiency and iron overload. *J Clin Pathol*. 2011;64(4):287-296.

10. Akanmori BD, Kurtzhals JA, Goka BQ, et al. Distinct patterns of cytokine regulation in discrete clinical forms of Plasmodium falciparum malaria. *Eur Cytokine Netw*. 2000;11(1): 113-118.

11. Day NP, Hien TT, Schollaardt T, et al. The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. *J Infect Dis*. 1999;180(4):1288-1297.

12. Lyke KE, Burges R, Cissoko Y, et al. Serum levels of the proinflammatory cytokines interleukin-1 beta (IL-1beta), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and IL-12(p70) in Malian children with severe Plasmodium falciparum malaria and matched uncomplicated malaria or healthy controls. *Infect Immun*. 2004; 72(10):5630-5637.

13. Park CH, Valore EV, Waring AJ, et al. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem.* 2001;276(11):7806-7810.

14. Nicolas G, Bennoun M, Devaux I, et al. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proc Natl Acad Sci USA.* 2001; 98(15):8780-8785.

15. Pigeon C, Ilyin G, Courselaud B, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem.* 2001;276(11):7811-7819.

16. Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood.* 2003;102(3):783-788.

17. Ganz T. The role of hepcidin in iron sequestration during infections and in the pathogenesis of anemia of chronic disease. *Isr Med Assoc J.* 2002;4(11):1043-1045.

18. Lasocki S, Longrois D, Montravers P, et al. Hepcidin and anemia of the critically ill patient: bench to bedside. *Anesthesiology.* 2011;114(3): 688-694.

19. Portugal S, Carret C, Recker M, et al. Host-mediated regulation of superinfection in malaria. *Nat Med.* 2011;17(6):732-737.

20. de Mast Q, Nadjm B, Reyburn H, et al. Assessment of urinary concentrations of hepcidin provides novel insight into disturbances in iron homeostasis during malarial infection. *J Infect Dis.* 2009;199(2):253-262.

21. de Mast Q, Syafruddin D, Keijmel S, et al. Increased serum hepcidin and alterations in blood iron parameters associated with asymptomatic P. falciparum and P. vivax malaria. *Haematologica.* 2010;95(7):1068-1074.

22. Howard CT, McKakpo US, Quakyi IA, et al. Relationship of hepcidin with parasitemia and anemia among patients with uncomplicated Plasmodium falciparum malaria in Ghana. *Am J Trop Med Hyg.* 2007;77(4):623-626.

23. Burté F, Brown BJ, Orimadegun AE, et al. Severe childhood malaria syndromes defined by plasma proteome profiles. *PLoS ONE.* 2012;7(12): e49778.

24. Bain B, Bates I, Laffan M, et al. *Dacie and Lewis Practical Haematology.* 11th ed. London: Churchill Livingstone ELSEVIER; 2012

25. Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster. *Trans R Soc Trop Med Hyg.* 2000;94(Suppl 1): S1-S90.

26. de Mast Q, van Dongen-Lases EC, Swinkels DW, et al. Mild increases in serum hepcidin and interleukin-6 concentrations impair iron incorporation in haemoglobin during an experimental human malaria infection. *Br J Haematol.* 2009;145(5):657-664.

27. Casals-Pascual C, Huang H, Lakhal-Littleton S, et al. Hepcidin demonstrates a biphasic association with anemia in acute Plasmodium falciparum malaria. *Haematologica.* 2012;97(11): 1695-1698.

28. Kurtzhals JA, Adabayeri V, Goka BQ, et al. Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. *Lancet.* 1998;351(9118): 1768-1772.

29. Grau GE, Taylor TE, Molyneux ME, et al. Tumor necrosis factor and disease severity in children with falciparum malaria. *N Engl J Med.* 1989; 320(24):1586-1591.

30. Kwiatkowski D, Hill AV, Sambou I, et al. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated Plasmodium falciparum malaria. *Lancet.* 1990;336(8725):1201-1204.

31. Nyakundi JN, Warn P, Newton C, et al. Serum tumour necrosis factor in children suffering from Plasmodium falciparum infection in Kilifi District, Kenya. *Trans R Soc Trop Med Hyg.* 1994;88(6): 667-670.

32. Shaffer N, Grau GE, Hedberg K, et al. Tumor necrosis factor and severe malaria. *J Infect Dis.* 1991;163(1):96-101.

33. Nairz M, Sonnweber T, Schroll A, et al. The pleiotropic effects of erythropoietin in infection and inflammation. *Microbes Infect.* 2012;14(3): 238-246.

34. Kakuta K, Orino K, Yamamoto S, Watanabe K. High levels of ferritin and its iron in fetal bovine serum. *Comp Biochem Physiol A Physiol.* 1997; 118(1):165-169.

35. Kroot JJ, van Herwaarden AE, Tjalsma H, et al. Second round robin for plasma hepcidin methods: first steps toward harmonization. *Am J Hematol.* 2012;87(10):977-983.

36. Delaby C, Beaumont C. [Serum hepcidin assay in 2011: where do we stand?] *Ann Biol Clin (Paris).* 2012;70(4):377-386.

37. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, et al. Serum hepcidin: reference ranges and biochemical correlates in the general population. *Blood.* 2011;117(25):e218-e225.