Assessment of Urinary Concentrations of Hepcidin Provides Novel Insight into Disturbances in Iron Homeostasis during Malarial Infection

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Disturbances in iron homeostasis are frequently observed in individuals with malaria. To study the effect of malaria and its treatment on iron homeostasis and to provide a mechanistic explanation for observed alterations in iron distribution, we studied the course of the iron regulatory hormone hepcidin in anemic Tanzanian children with febrile Plasmodium falciparum malaria. Before initiation of antimalarial treatment, urinary concentrations of hepcidin were strongly elevated and were associated with iron maldistribution, as was suggested by the presence of hypoferremia and high serum concentrations of ferritin. Antimalarial treatment resulted in a rapid decrease in urinary concentrations of hepcidin and reversal of the hypoferremia. Exploration of regulatory pathways of hepcidin production by analysis of iron, erythropoietic, and inflammatory indices suggested that reduced erythropoietic activity and inflammation stimulated hepcidin production. We conclude that high concentrations of hepcidin explain the observed disturbances in host iron homeostasis associated with malaria and may contribute to malarial anemia and an impaired erythropoietic response to iron supplementation.

Iron deficiency and malaria are among the most common causes of anemia in African children, and the 2 conditions frequently coexist and interact in a way that is currently poorly understood [1, 2]. Recently, the importance of unraveling the complex interaction between malaria and iron metabolism was highlighted by an iron supplementation trial performed in Pemba Island, Tanzania; in this trial, routine iron and folic acid supplementation resulted in an increased incidence of severe adverse events among iron-replete children in an area where malaria was hyperendemic [3]. To date, studies of iron and malaria have been hampered by insufficient insight into the mechanism underlying disturbances in iron homeostasis induced by Plasmodium falciparum malaria [4]. The recently discovered peptide hormone hepcidin is, however, a new tool that may contribute to better understanding of the changes in host iron homeostasis in malarial infection.

Hepcidin controls extracellular iron concentration and distribution of iron in tissue by binding and subsequent degradation of the sole transmembrane iron exporter ferroportin [5, 6]. High concentrations of hepcidin restrict intestinal absorption of iron and the release of iron from macrophages and hepatocytes, resulting in low plasma concentrations of iron and limited availability of iron for erythropoiesis.
In febrile *P. falciparum* malaria, hepcidin production may be up-regulated by inflammatory cytokines [7–9] and bone marrow suppression [10, 11], whereas hypoxia and anemia may down-regulate production [12]. Increased hepcidin concentrations could induce or worsen malaria-associated anemia, and, by causing iron-restricted erythropoiesis, they might impair the recovery of hemoglobin (Hb) levels after antimalarial treatment. In addition, the efficacy of iron supplementation therapy may be impaired by high concentrations of hepcidin. Finally, hepcidin concentrations may influence susceptibility to malaria and other infections by regulating the supply of iron to microorganisms [13].

Recently, our group of investigators developed urine and serum assays for the measurement of hepcidin concentrations by use of mass spectrometry [14–16], and subsequent studies showed a strong correlation between the hepcidin concentrations measured by both assays [15]. With the use of a recent quantitative update of the urinary hepcidin assay, the aim of our present study was to investigate the effect of febrile *P. falciparum* malaria and its treatment on iron homeostasis in anemic Tanzanian children. In addition, determination of the indicators of iron status, inflammation, and erythropoietic activity enabled us to explore the putative regulatory pathways of hepcidin expression in malaria. The urine assay was chosen (1) to minimize the volume of blood collected from young children and (2) because urinary levels of hepcidin are less affected by a diurnal variation [15]. Anemic children were included in the study because disturbances in iron homeostasis might have especially severe consequences in this group.

**PATIENTS AND METHODS**

**Patients and study design.** The patients recruited for the present study were a subgroup of children enrolled in a 1-year study investigating the causes of acute illness in children (age, 2 months to 13 years) who had a current or recent history of fever and were admitted to a district hospital in an area of intense *P. falciparum* transmission in Northeast Tanzania (the SFI [Severe Febrile Illness] Study). Children enrolled in the study underwent a standard examination; they then had blood samples drawn for culture, HIV testing, and slide examination for malaria and were provided routine clinical care according to hospital and national guidelines. Children were eligible to participate in the hepcidin study if they were febrile at admission (axillary temperature, >37.5°C), had a positive result of blood slide examination for *P. falciparum* (i.e., with detection of at least 125 parasites/200 white blood cells [WBCs]), a negative HIV test result, a negative blood culture result, anemia (Hb level, <10 g/dL), and no history of iron therapy in the preceding 3 weeks. Serum and urine samples obtained from these children at admission were stored at −80°C, pending laboratory analysis with additional serum and urine samples obtained at 48 h and at 4 weeks after admission; the samples obtained at week 4 were available for only a minority of children, because of logistical constraints. At the time of discharge from the hospital, all children were prescribed a 2-week course of iron supplementation (with the dose determined according to body weight, as per Tanzanian guidelines).

At the follow-up visit at week 4, a short questionnaire documented the intake of iron supplementation after discharge and any current illness. The child’s temperature and Hb level were determined, and a thick blood smear was obtained to check for *Plasmodium* parasitemia. The urinary concentration of hepcidin in the sample obtained at week 4 was determined for those children without current or recent (within <48 h) fever and with a negative result of blood smear examination for the presence of asexual *P. falciparum* parasites. Urine samples were collected from 11 A.M. to 6 P.M., to minimize the effect of circadian variation in hepcidin secretion [15]. For children from whom no sample could be obtained before 6 P.M., urine was collected the following morning.

**Laboratory analyses.** Thick and thin blood smears were stained with Giemsa and were examined independently by 2 experienced microscopists. A third microscopist reviewed the slides in case of discordant results. The number of parasites was quantified against 200 WBCs, and parasite density was calculated under the assumption that the WBC count was 8000 WBCs/μL. Blood cultures were performed using BacT/Alert PF bottles and the BacT/Alert 3D automated blood culture system (bioMérieux). The Capillus rapid assay test (Trinity Biotech) was used for detection of HIV-1/HIV-2 antibodies. A full blood count was determined using a hematologic analyzer (Beckman Coulter) at admission; the Hb level at week 4 was determined using Hemocue. Lactate levels in capillary blood were determined using a Lactate Pro analyzer (Arkay).

The concentrations of serum iron and total iron-binding capacity (TIBC) were determined using a colorimetric method; the serum C-reactive protein (CRP) level was determined using immunologic agglutination detection, and the urinary concentration of creatinine was determined using enzymatic colorimetric detection, with the use of an Abbott Aeroset analyzer (Abbott Laboratories). The serum transferrin saturation was calculated as a percentage of the total iron concentration in serum divided by the TIBC. The serum concentration of ferritin was measured using an immunometric assay with the use of an Immulite 2500 (Diagnostic Products). The serum concentration of soluble transferrin receptor (sTfR) was measured immunonephelometrically on a BN II System (Dade Behring). Serum concentrations of interleukin (IL)–1 receptor antagonist (IL-1Ra), IL-6, IL-10, and tumor necrosis factor (TNF)–α were analyzed in one batch with the use of a multiplex assay (Luminex). The lower limits of detection for CRP, IL-1Ra, IL-6, IL-10, and TNF–α were 5 mg/L, 40 pg/mL, 7 pg/mL, 7 pg/mL, and 18 pg/mL, respectively. For all analyses, the value of the lower limit of detection was used in samples with values below the limit of detection.
Urinary concentrations of hepcidin were measured using surface-enhanced laser desorption/ionization–time-of-flight mass spectrometry, as reported elsewhere [15, 16]. A custom-made synthetic hepcidin-24 peptide (Peptide International) was used as an internal reference standard. The urine concentrations of hepcidin were normalized to urine creatinine values and were expressed as the number of nanomoles per millimoles of creatinine.

**Statistical analyses.** Data are the median value (interquartile range), unless otherwise stated. Changes in serial laboratory parameter concentrations noted at the 3 time points when each patient was evaluated were examined with linear mixed models. The association between urinary concentrations of hepcidin and concentrations of other laboratory parameters was assessed using Spearman’s correlation coefficient. Predictors of urinary concentrations of hepcidin were assessed in a multivariate linear regression model with log-transformed data, because the distributions of these variables were skewed. Biologically plausible variables reported to affect hepcidin concentrations were entered in a multivariate linear regression model with backward stepwise elimination of nonsignificant ($P < .1$) factors, regardless of their univariate status. These variables were the Hb level, parasite density, the serum concentration of ferritin (as a measurement of iron sequestration in macrophages, inflammation, and iron stores in the body), $s$TfR (as a measurement of erythropoietic activity and iron demand in the erythron [17]), and IL-6 (as a marker of inflammation). IL-6 was specifically chosen because this proinflammatory cytokine has predominantly been implicated in stimulation of hepcidin production [7, 9]. All analyses were performed using SPSS software for Windows (version 15.0; SPSS).

**Ethics.** Written, informed consent was obtained for all children. The ethics committees of the National Institute for Medical Research, Tanzania, and the London School of Hygiene and Tropical Medicine approved the study.

**RESULTS**

**Characteristics at baseline.** From September 2006 through January 2007, a total of 104 children were enrolled in the study. Patient characteristics and laboratory parameters at baseline, with reference values as reported by the assay’s manufacturers or as reported in the literature, are shown in table 1. Urinary concentrations of hepcidin were very high and associated with the presence of hypoferremia, as was indicated by low serum iron concentrations and low transferrin saturations. In contrast, serum concentrations of the iron storage protein ferritin and the acute-phase protein CRP were high. The median $s$TfR concentration was slightly above the upper reference limit for adults.

**Temporal changes in urinary concentrations of hepcidin and in related laboratory parameters after antimalarial treatment.** Urine and blood samples obtained on day 2 after treatment were collected from 53 of 104 patients; 46 of these 53 patients returned for the follow-up visit at week 4. Ten of these 46 patients were excluded because they had a positive result of blood slide examination for malaria or had signs of a systemic infection at week 4. For the remaining 36 patients, the course of urinary concentrations of hepcidin after the start of antimalarial treatment and the temporal associations with concentrations of iron status indicators, $s$TfR, and inflammatory markers were determined. There were no significant differences in characteristics at baseline between these 36 children and the children for whom only a sample obtained at baseline was available (data not shown). A blood transfusion had been given to 6 (16.7%) of 36 children during hospitalization, and all children were reported by mothers or guardians to have used iron supplements after discharge. The median Hb level increased from 7.5 g/dL (range, 6.1–8.7 g/dL) at admission to 10.2 g/dL (range, 8.9–11.5 g/dL) at week 4, with 25 (69.4%) of 36 children remaining anemic (Hb level, <11.0 g/dL). Figure 1 shows the course of urinary concentrations of hepcidin, iron status indicators, and $s$TfR. Urinary concentrations of hepcidin decreased rapidly after the start of antimalarial treatment, from a median level of 229.0 nmol/mmol creatinine (range, 125.8–330.2 nmol/mmol creatinine) at baseline to 34.7 nmol/mmol creatinine (range, 10.2–93.9 nmol/mmol creatinine) and 9.8 nmol/mmol creatinine (range 2.5–19.3 nmol/mmol creatinine) at day 2 and week 4, respectively. This rapid decrease in urinary concentrations of hepcidin was associated with a concurrent increase in the serum concentration of iron and transferrin saturation, whereas marked changes in concentrations of serum ferritin, TIBC, and $s$TfR were apparent only at week 4. As shown in figure 2, changes in the concentrations of CRP, the proinflammatory cytokines IL-1Ra, IL-6, and TNF-$\alpha$, and the anti-inflammatory cytokine IL-10 paralleled changes in urinary concentrations of hepcidin. Concentrations of all inflammatory markers were high at baseline, but they decreased rapidly after initiation of antimalarial treatment. TNF- $\alpha$ was only detectable in serum at baseline. Concentrations of IL-1Ra, IL-6, and IL-10 at week 4 were very low and were below the detection limit of the assay in 6%, 26%, and 38% of samples, respectively.

**Association between urinary concentrations of hepcidin and selected laboratory parameters.** Spearman’s correlations between selected laboratory parameters and urinary concentrations of hepcidin at baseline and at week 4 are shown in table 2. There was a positive correlation of urinary concentrations of hepcidin with Hb concentrations, as well as with concentrations of the cytokines IL-6, IL-10, and TNF-$\alpha$. Hepcidin concentrations were inversely associated with extracellular iron concentrations, as was reflected by the strong negative Spearman’s correlations with serum iron concentration, transferrin saturation, and TIBC. There also was a negative correlation between urinary concentrations of hepcidin...
and sTfR concentrations. Spearman’s correlations determined at week 4 had changed considerably, compared with correlations noted at baseline. The strength of the correlation between the urinary concentration of hepcidin and the serum concentration of ferritin was notably stronger, whereas the strong inverse correlation between serum iron, transferrin saturation, and sTfR noted at baseline had weakened.

Predictors of urinary concentrations of hepcidin. Predictors of urinary concentrations of hepcidin at baseline and week 4 were investigated in a multivariate linear regression model with backward stepwise elimination of nonsignificant factors (table 3). Parasite density and concentrations of Hb, serum ferritin, sTfR, and IL-6 were included in the initial model as putative indicators or mediators of the various regulatory pathways of hepcidin production. At baseline, Hb concentrations and parasite density were positively associated with urinary concentrations of hepcidin, whereas sTfR concentrations were negatively associated. In accordance with Spearman’s correlation analysis, serum concentrations of ferritin were not significantly associated with urinary concentrations of hepcidin in the multivariate model. In contrast to Spearman’s correlation analysis, IL-6 was eliminated from the model, and inclusion of one of the other inflammatory cytokines (IL-1Ra, IL-10, or TNF-α) or CRP in the initial model (instead of IL-6) also resulted in their elimination (data not shown). Pairwise comparison revealed significant Spearman’s correlations between IL-6 concentrations and other variables in the model: parasite density (r = 0.398; P < .001), ferritin concentration (r = 0.333; P < .001), and sTfR concentration (r = −0.386; P < .001). At week 4 after treatment initiation, the serum concentration of ferritin was the sole independent variable associated with the urinary concentration of hepcidin.

DISCUSSION
In the present study, we showed that urine concentrations of the iron-regulatory hormone hepcidin were strongly elevated in
anemic children with febrile *P. falciparum* infection, and they were associated with iron maldistribution, as was indicated by observations of hypoferremia and high serum concentrations of ferritin. Antimalarial treatment resulted in a rapid decrease in urinary concentrations of hepcidin and reversal of the hypoferremia. The latter outcome probably resulted from a restored functional capacity of the iron transporter ferroportin to export sequestered iron from macrophages, enterocytes, and hepatocytes.

These findings provide a mechanistic explanation for the observed disturbances in iron homeostasis in patients with malaria, and hepcidin may prove to be a new tool with which to quantify and determine the duration of these disturbances. In addition, as discussed in the following paragraphs, induction of hepcidin expression during malaria may (1) prove to be a new and important pathogenic factor in malarial anemia and in the impaired recovery of Hb levels after antimalarial treatment, (2) reduce the erythropoietic response to iron supplementation, and (3) pro-
tect against proliferation of malaria parasites and explain the
high incidence of infections with intracellular bacteria in pa-
tients with malaria.

Increased hepcidin production may contribute to the
pathogenesis of malarial anemia by limiting the availability of
iron for erythropoiesis and, possibly, also by directly inhibit-
ing erythroid progenitor proliferation and survival [20, 21].
Proinflammatory cytokines also exert a direct [21, 22] and
indirect suppressive effect on the bone marrow through a
decrease in erythropoietin production or erythroblast recep-
tivity to erythropoietin [23, 24]. Because Hb levels invariably
decrease during episodes of malaria, it is crucial that, as soon
as the infection is under control, concentrations of both hep-
cidin and proinflammatory cytokines decrease rapidly so the
bone marrow can regain its activity. Failure to do so may
result in severe anemia as a result of ongoing bone marrow
suppression. In our patients, urinary hepcidin and proin-
flammatory cytokine concentrations decreased rapidly after
initiation of antimalarial treatment, suggesting that bone
marrow suppression in patients with febrile malaria is rapidly
reversible after initiation of efficacious antimalarial therapy.
Indeed, previous studies noted a reticulocyte response within
3–5 days after the start of antimalarial treatment [25, 26]. In
the present study, Hb concentrations increased markedly by
week 4 after treatment initiation, although an Hb level >11
g/dL was noted in only a minority (30.6%) of children.

Our current findings may also contribute to a more ratio-
nal use of iron supplementation for children with malaria
anemia. High hepcidin concentrations will compromise in-
testinal iron absorption, and the nonabsorbed iron might
cause gastrointestinal complaints. The initial iron supply for
erthropoiesis after antimalarial treatment might also pre-
dominantly be derived from the release of sequestered iron.
Our current findings suggest that, in patients with febrile ma-
laria, iron therapy might only be effective until after the com-
pletion of antimalarial treatment, when hepcidin concentra-
tions have decreased. Recently, using stable isotope iron
incorporation studies, Doherty et al. [27] observed reduced
iron incorporation in children with postmalaria anemia, and
they also suggested that the early need for erythropoietic iron
is met by iron recycling rather than by iron absorption from
the intestine.

Like all living organisms, microorganisms (including P. fal-
ciparum) require iron for vital cell functions [13, 28]. The im-

Figure 2. Box-whisker plots of temporal changes in concentrations of C-reactive protein and inflammatory cytokines noted in patients with febrile Plasmodium falciparum malaria at baseline, as well as on day 2 and at 4 weeks after initiation of antimalarial treatment. Boxes span the interquartile range, and lines denote the location of the first quartile, the median, and the third quartile. Whiskers extend to the last data point within 1.5 times the interquartile range in either direction. Outliers are indicated by open circles and denote patients for whom values were between 1.5 and 3 box-lengths from the first and third quartiles. Extremes, denoting patients for whom values were >3 box-lengths from first and third quartiles, are not shown in the figure. P values were determined using linear mixed models. CRP, C-reactive protein; IL, interleukin; IL-1Ra, IL-1 receptor antagonist.
Importance of iron in malaria is supported by the antimalarial effect of iron chelators [29] and by a recent trial showing an increased risk of death and of hospital admission among iron-replete children receiving iron and folic acid supplementation in an area of endemicity for malaria [3]. Malaria parasites may use several sources of iron, including a labile intraerythrocytic iron pool, heme iron from Hb breakdown [30], and extracellular iron [31, 32]. We speculate that high hepcidin concentrations might

Table 2. Spearman’s rank correlation coefficient ($\rho$) of urinary concentrations of hepcidin with hematologic, iron status, erythropoietic activity, and inflammatory indicators at baseline and at week 4.

<table>
<thead>
<tr>
<th>Factor</th>
<th>At baseline</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>At week 4</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Patients, no.</td>
<td>$\rho$</td>
<td>$P$</td>
<td>Patients, no.</td>
<td>$\rho$</td>
<td>$P$</td>
<td>Patients, no.</td>
<td>$\rho$</td>
<td>$P$</td>
</tr>
<tr>
<td>Parasite density</td>
<td>104</td>
<td>0.183</td>
<td>.63</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Hb level</td>
<td>104</td>
<td>0.202</td>
<td>.039</td>
<td>34</td>
<td>$-0.203$</td>
<td>.242</td>
<td>33</td>
<td>$-0.100$</td>
<td>.547</td>
</tr>
<tr>
<td>Serum transferrin saturation</td>
<td>96</td>
<td>$-0.227$</td>
<td>.026</td>
<td>33</td>
<td>$-0.100$</td>
<td>.547</td>
<td>33</td>
<td>$-0.183$</td>
<td>.301</td>
</tr>
<tr>
<td>Serum level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Of iron</td>
<td>96</td>
<td>$-0.326$</td>
<td>.001</td>
<td>33</td>
<td>$-0.183$</td>
<td>.301</td>
<td>33</td>
<td>$-0.183$</td>
<td>.301</td>
</tr>
<tr>
<td>Of ferritin</td>
<td>91</td>
<td>0.067</td>
<td>.527</td>
<td>34</td>
<td>0.372</td>
<td>.028</td>
<td>34</td>
<td>0.372</td>
<td>.028</td>
</tr>
<tr>
<td>Of sTfR</td>
<td>88</td>
<td>$-0.266$</td>
<td>.007</td>
<td>33</td>
<td>$-0.132$</td>
<td>.456</td>
<td>33</td>
<td>$-0.132$</td>
<td>.456</td>
</tr>
<tr>
<td>Of CRP</td>
<td>97</td>
<td>0.119</td>
<td>.245</td>
<td>33</td>
<td>$0.252$</td>
<td>.145</td>
<td>33</td>
<td>$0.252$</td>
<td>.145</td>
</tr>
<tr>
<td>Of IL-6</td>
<td>83</td>
<td>0.286</td>
<td>.008</td>
<td>33</td>
<td>$0.209$</td>
<td>.118</td>
<td>33</td>
<td>$0.209$</td>
<td>.118</td>
</tr>
<tr>
<td>Of IL-1Ra</td>
<td>82</td>
<td>0.193</td>
<td>.080</td>
<td>33</td>
<td>$0.275$</td>
<td>.058</td>
<td>33</td>
<td>$0.275$</td>
<td>.058</td>
</tr>
<tr>
<td>Of IL-10</td>
<td>83</td>
<td>0.256</td>
<td>.019</td>
<td>33</td>
<td>$-0.060$</td>
<td>.372</td>
<td>33</td>
<td>$-0.060$</td>
<td>.372</td>
</tr>
<tr>
<td>Of TNF-α $^b$</td>
<td>83</td>
<td>0.255</td>
<td>.019</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
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<td>...</td>
</tr>
<tr>
<td>Of TIBC</td>
<td>97</td>
<td>$-0.251$</td>
<td>.013</td>
<td>33</td>
<td>$-0.218$</td>
<td>.215</td>
<td>33</td>
<td>$-0.218$</td>
<td>.215</td>
</tr>
</tbody>
</table>

**NOTE.** CRP, C-reactive protein; Hb, hemoglobin; IL, interleukin; IL-1Ra, IL-1 receptor agonist; NA, not applicable; sTfR, soluble transferrin receptor; TIBC, total iron-binding capacity; TNF, tumor necrosis factor.

$^a$ Concentrations of CRP, IL-6, IL-1Ra, and IL-10 at week 4 were below the lower limit of detection of the assay in 59%, 26%, 6%, and 38% of patients, respectively. The value of the lower limit of detection was used in analyses of these patients.

$^b$ No $\rho$ was calculated at week 4, because the TNF-α concentration was above the lower limit of detection of the assay in only one sample.

Table 3. Backward stepwise multivariate linear regression analysis of putative predictors (log transformed) of urinary hepcidin levels (log transformed) at baseline and at week 4 after admission.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Full model</th>
<th></th>
<th></th>
<th>Final model</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$B$ (95% CI)</td>
<td>$\beta$</td>
<td></td>
<td>$B$ (95% CI)</td>
<td>$\beta$</td>
<td></td>
</tr>
<tr>
<td>Hb level, g/dL</td>
<td>1.078 (0.120 to 2.332)</td>
<td>0.215</td>
<td></td>
<td>1.169 (0.111 to 2.227)</td>
<td>0.234</td>
<td></td>
</tr>
<tr>
<td>Parasite density, parasites/μL</td>
<td>0.331 (0.120 to 0.783)</td>
<td>0.168</td>
<td></td>
<td>0.392 (0.020 to 0.805)</td>
<td>0.198</td>
<td></td>
</tr>
<tr>
<td>sTfR level, mg/L</td>
<td>$-0.761$ (0.064)</td>
<td>$-0.227$</td>
<td></td>
<td>$-0.862$ (0.153)</td>
<td>$-0.257$</td>
<td></td>
</tr>
<tr>
<td>Serum ferritin level, μg/L</td>
<td>0.014 (0.353 to 0.382)</td>
<td>0.010</td>
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<tr>
<td>IL-6, μg/L</td>
<td>0.068 (0.140 to 0.277)</td>
<td>0.085</td>
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<tr>
<td>At week 4</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Serum ferritin level, μg/L</td>
<td>0.438 (0.125 to 1.002)</td>
<td>0.296</td>
<td></td>
<td>0.511 (0.017 to 1.039)</td>
<td>0.345</td>
<td></td>
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<tr>
<td>Hb level, g/dL</td>
<td>$-0.925$ (0.220)</td>
<td>$-0.118$</td>
<td></td>
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<td>...</td>
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</tr>
<tr>
<td>sTfR level, mg/L</td>
<td>$-1.021$ (1.141)</td>
<td>0.185</td>
<td></td>
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<td>...</td>
<td></td>
</tr>
<tr>
<td>IL-6, μg/L</td>
<td>0.741 (0.362 to 1.844)</td>
<td>0.263</td>
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</table>

**NOTE.** The full model with all entered variables and the final model after elimination of nonsignificant ($P < .1$) variables are shown. Data are expressed as unstandardized coefficients ($B$) with 95% confidence intervals (95% CIs) and as standardized coefficients ($\beta$). In this model, a log$_{10}$ increase in the hemoglobin (Hb) concentration was associated with an estimated increase of 1.078 in the log$_{10}$ value for the urinary concentration of hepcidin. Tolerance values (1/variance inflation factor) were between 0.959 and 0.983 in the final model at baseline, indicating that multicollinearity did not have a major influence on the final model. CRP, C-reactive protein; Hb, hemoglobin; sTfR, soluble transferrin receptor.
inhibit multiplication of malaria parasites by depriving the parasites from the iron supply of the host. However, in contrast to a potential protective effect against malaria, sequestration of iron in macrophages may prove to be a risk factor for intracellular bacterial infections. Hepcidin-induced down-regulation of ferroportin expression was recently reported to enhance the growth of *Salmonella enterica* [33], as well as that of other intracellular bacteria [34], in macrophages in vitro, and this finding may explain the observed association between malaria and non-serotype Typhi *Salmonella* infections [35–37].

Regulatory pathways of hepcidin production in febrile malaria were explored by Spearman’s correlation analysis and multivariate linear regression analysis. Several putative upstream and interlinked pathways have been identified to date [38], including regulation by erythropoietic activity or anemia/hypoxia [10–12, 39], iron store–related [7, 40] and holotransferrin [41] regulation, and inflammation–related regulation [7, 8].

Suppressed erythropoietic activity (as reflected by low sTfR concentrations) was associated with higher hepcidin concentrations at baseline. Use of sTfR for the evaluation of erythropoietic activity has been reviewed elsewhere [17]. At baseline, sTfR concentrations were in the upper limit of the range considered to be normal for adults, but these concentrations might have been inappropriately low, considering the degree of anemia and the effect of age, whereby the highest sTfR concentrations are found in infants [18, 19]. Suppressed erythropoiesis with reticulocytopenia is a well-known characteristic of febrile malaria [42], and the negative association between concentrations of hepcidin and sTfR in the present study is in line with the findings of recent studies in mice, which have shown increased hepcidin production after bone marrow suppression by irradiation or cytotoxic chemotherapy [10, 11].

IL-6 has previously been demonstrated to stimulate expression of hepcidin [7–9]. IL-6 concentrations correlated signifi-
canty with hepcidin concentrations in the present study, and the kinetics of hepcidin and inflammatory cytokine concentrations showed a strong resemblance. However, IL-6 was eliminated as an independent predictor in the multivariate linear regression model. The fact that inflammation was also represented by other variables in the multivariate model—as reflected by significant Spearman’s correlations between IL-6 and these other variables—may explain the elimination of IL-6.

There was also a trend for a positive association between parasite densities and hepcidin concentrations. Higher parasite densities were associated with higher IL-6 concentrations, although a direct effect on hepatic and/or macrophage hepcidin production by malaria parasites or their products (e.g., hemozoin or glycosylphosphatidylinositol) cannot be excluded.

Finally, our findings of a positive correlation between Hb and hepcidin on one hand and a negative correlation between the erythropoietic marker sTfR and hepcidin on the other hand may seem contradictory, because bone marrow activity might be expected to determine Hb levels. However, in patients with acute malaria, Hb levels may be more influenced by the extent of hemolysis and the duration of malaria than by the degree of bone marrow suppression, especially given the long half-life of erythrocytes. Instead, the positive association between Hb and hepcidin levels in our patients with anemia probably resulted from the suppressive effects of anemia and secondary hypoxia on hepcidin expression, as a result of the cellular excretion of soluble hemoujuvelin that acts as a negative regulator of hepcidin [12, 43]. Figure 3 summarizes the putative regulatory pathways of hepcidin production in children with febrile P. falciparum malaria at the time of admission to the hospital. At week 4, after receipt of successful antimalarial treatment, hepcidin concentrations appeared to be most strongly associated with iron status (as reflected by serum concentrations of ferritin).

To our knowledge, only one other study has measured hepcidin concentrations in patients with malaria. Howard et al. [45] reported semiquantitative urinary concentrations of hepcidin noted in a cross-sectional study of patients presenting for evaluation of possible P. falciparum malaria in Ghana. They found that patients with malaria had higher hepcidin concentrations than did healthy volunteers in other studies. However, neither iron status indicators, inflammatory markers, nor longitudinal data were reported in the study from Ghana. In addition, reports of hepcidin concentrations in individuals with other infectious diseases are sparse. We previously reported urinary concentrations of hepcidin in healthy volunteers injected with lipopolysaccharide [9], and the urinary concentrations of hepcidin found in the current study were several fold higher.

The present study was performed in a lowland region of Tanzania characterized by intense P. falciparum transmission and a high prevalence of asymptomatic parasite carriage. To minimize the chance of enrolling children with causes of fever other than P. falciparum malaria, we used stringent case definitions of febrile malaria with a high cutoff for parasite density that has been shown to be appropriate for this region [46]; we also performed blood culture and HIV testing for all children. The influence of diurnal variation in hepcidin concentrations on our results was probably limited, because reported diurnal fluctuations in urinary concentrations of hepcidin [15] were negligible, compared with the very high urinary concentrations of hepcidin found in our patients.

In summary, we showed that hepcidin concentrations were strongly elevated in febrile P. falciparum malaria, probably as a result of suppressed erythropoietic activity, inflammation, and, perhaps, also by a direct stimulatory effect of malaria parasites and their products. Malaria-induced hepcidin expression provides a mechanistic explanation for the observed maldistribution of iron in patients with malaria, and we hypothesize that it may contribute to the high incidence of infections with intracellular bacteria in regions where malaria is endemic. Our results might provide leads for the introduction of novel diagnostic biomarkers of iron homeostasis and the design of novel therapeutic interventions.

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References

11. Vokurka M, Krijt J, Sulc K, Necas E. Hepcidin mRNA levels in mouse Umbilical Hepcidin Levels in Febrile Malaria • JID 2009;199 (15 January) • 261


