Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children

Short title: Hepcidin regulation of oral iron utilization

Andrew M Prentice1, Conor P Doherty2, Steven A Abrams3, Sharon E Cox1, Sarah H Atkinson4, Hans Verhoef1,5, Andrew E Armitage*6 and Hal Drakesmith*6

1  MRC International Nutrition Group, London School of Hygiene & Tropical Medicine, London, UK and MRC Keneba, The Gambia
2  Royal Hospital for Sick Children, Glasgow, UK
3  USDA/ARS Children’s Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, USA
4  Dept of Paediatrics, University of Oxford, UK
5  Cell Biology and Immunology Group, Wageningen University, Netherlands
6  Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford, UK

Corresponding author: Andrew M Prentice, PhD, MRC International Nutrition Group, London School of Hygiene & Tropical Medicine, Keppel St, London, WC1E 7HT, UK. Tel:+44-20-7958-8140. Fax:+44-20-7958-8111.
E-mail: andrew.prentice@lshtm.ac.uk

* - AEA and HD contributed equally to this manuscript
Abstract
Iron supplementation strategies in the developing world remain controversial owing to fears of exacerbating prevalent infectious diseases. Understanding the conditions in which iron will be absorbed and incorporated into erythrocytes is therefore important. We studied Gambian children with either post-malarial or non-malarial anemia, who were given oral iron supplements daily for 30 days. Supplements administered on days 1 and 15 contained the stable iron isotopes $^{57}$Fe and $^{58}$Fe respectively, and erythrocyte incorporation was measured in blood samples drawn 14 days later. We investigated how the iron regulatory hormone hepcidin and other inflammatory/iron-related indices, all measured on the day of isotope administration, correlated with erythrocyte iron incorporation. In univariate analyses, hepcidin, ferritin, C-reactive protein (CRP) and soluble transferrin receptor (sTfR) strongly predicted incorporation of $^{57}$Fe given on day 1, while hepcidin, ferritin and sTfR / log Ferritin correlated with $^{58}$Fe incorporation. In a final multivariate model, the most consistent predictor of erythrocyte isotope incorporation was hepcidin. We conclude that under conditions of competing signals (anemia, iron deficiency and infection) hepcidin powerfully controls utilisation of dietary iron. We suggest that low-cost point-of-care hepcidin assays would aid iron supplementation programs in the developing world.
Introduction
Children living in poor areas with high infection rates in the developing world face conflicting challenges with respect to iron status. Iron deficiency and iron deficiency anemia are widespread\(^1\), so that raising iron status through iron supplementation would be highly desirable, facilitating optimal cognitive and physiological development, and alleviating the risks associated with iron deficiency anemia\(^2\). However, iron is also a critical mediator of host-pathogen interactions, and supplementation may have serious adverse consequences in areas of high infectious burden. Elevated iron status may increase vulnerability to bacterial\(^3,4\), protozoal\(^5\), and viral\(^6\) infections. Currently available data do not support definitive guidelines as to when it is safe and efficacious to administer iron in such settings, particularly in the context of malaria\(^7,8\).

The regulatory systems controlling iron absorption and localisation reflect this conflict of priorities. Erythroid drive, iron deficiency, and hypoxia are all associated with increased uptake of dietary iron, while certain infections and inflammation serve to abrogate iron absorption. Extensive evidence suggests that a key molecular contributor to these effects is the liver-derived circulating peptide hepcidin, itself regulated by each of these opposing signals: it is suppressed during iron deficiency, anemia and hypoxia, but stimulated by serum and hepatic iron, and during infection/inflammation\(^9-12\). Hepcidin inhibits the function of ferroportin\(^13\) the sole known mammalian iron export protein\(^14\), expressed highly on duodenal enterocytes and iron-recycling macrophages\(^15-17\). Therefore, when hepcidin levels are high, enterocyte absorption of dietary iron and release of macrophage iron to serum are blocked, resulting in hypoferremia that is thought to be anti-infective, but which also limits iron supply to the erythron and other tissues. It has recently been shown that the hepcidin-iron axis is a key component of innate immune defense against malarial super-infection in murine models\(^18\), providing proof-of-principle for a likely wider role of hepcidin in protection against potentially lethal infections.

In this study, we compared the associations between erythrocyte incorporation of orally-administered stable iron isotopes, hepcidin and other indices, using samples from a previously reported study\(^19\) of iron supplementation and utilization in rural Gambian children with either post-malarial or non-malarial anemia. Studies in a population such as this should be informative, since several of the major stimulatory and suppressive factors directing hepcidin expression are likely to be simultaneously active. We found that hepcidin was the most consistent predictor of erythrocyte stable iron isotope incorporation in this population.
Methods
Study subjects and iron supplementation schedule.
A study was previously carried out in anemic children (hemoglobin <110 g/l) aged 18-36 months recruited from the MRC Keneba clinic in the West Kiang region of The Gambia during the malaria season of 2003. Children were considered as having post-malarial anemia if they presented with fever and with peripheral parasitemia (para00). Incorporation of stable iron isotopes into erythrocytes was compared between iron supplemented post-malarial anemic children (n=37) following treatment of *Plasmodium falciparum* malaria (3 days of chloroquine/Fansidar, after which iron supplementation was initiated, on the day defined as ‘Day 1’, the fourth day after presentation with malaria) or matched anemic but non-malarial children (n=36), as previously described\(^\text{19}\). Children were given a 30-day course of iron supplementation. Stable tracer isotopes consisting of non-heme $^{57}$Fe (ferrous sulfate, 3.9 mg) at Day 1 and $^{58}$Fe (ferrous sulfate, 1.3 mg) at Day 15 of the supplementation schedule were used, with all children receiving 2 mg/kg/d iron as liquid iron glycine sulfate on all other days of the supplementation course from Day 2, as described previously\(^\text{19}\). This schedule is depicted in **Figure 1**. Children were fasted for at least 2 hours prior to tracer dosing, which was followed by administration of 50mg vitamin C. These precautions minimize the influence of local inhibitory factors such as dietary phytates and facilitate iron absorption. Here, in subjects for whom sufficient residual plasma was available, we compared the relationships between hepcidin levels, other indices and erythrocyte iron incorporation.

Ethics and consent
The study was approved by the Gambian Government/MRC Ethics Committee and parents gave written informed consent in accordance with the Declaration of Helsinki. The parents/guardians of children were approached after attendance at the MRC Keneba clinic for either routine monitoring or after emergency treatment for malaria.

Quantification of hepcidin, erythrocyte iron incorporation and other indices
Hepcidin concentrations in human plasma samples were quantified by competition ELISA using the hepcidin-25 (human) enzyme immunoassay kit (Bachem) according to the manufacturer’s protocol. Eleven post-malarial subjects (mean age 28.1 months, SD 6.2) and sixteen non-malarial anemia subjects (mean age 28.7 months, SD 6.7) had sufficient residual plasma to assay hepcidin on Days 1 and/or 15 (n=48). Plasma samples were diluted 1 in 6 in supplied standard diluent (peptide-cleared human serum) and analysed.
using a 9 or 10-point 2-fold serial dilution (maximum concentration, 25 ng/ml) standard curve. Stocks of standards, biotinylated-hepcidin tracer and anti-hepcidin primary antibody were prepared, aliquoted and frozen in sufficient quantities to cover the complete dataset; aliquots were thawed within an hour of running each plate. Samples and standards were run in triplicate; freeze-thaw cycles were kept to a minimum. Hepcidin concentrations were interpolated from standard curves generated by logistic 4-parameter non-linear curve fitting using Prism version 5.0d (GraphPad Software, Inc). Samples giving readings outside the linear region of the curve were re-run at appropriate dilutions; in cases where there was a high standard deviation between triplicate OD450 readings, samples were re-run, and the mean of all values was used in further analyses.

Hemoglobin (Hb) (Medonic CA 530 hemoglobinometer) and zinc protoporphyrin (ZnPP) (Aviv Biomedical hematofluorometer) were measured in The Gambia within 8 hours of sampling and each sample was assessed for the presence of malaria parasites. Frozen plasma was transported to the UK for the analysis of ferritin (Imx ferritin assay based on Microparticle Enzyme Immunoassay (MEIA) technology, Abbot Laboratories), soluble transferrin receptors (sTfR) (R&D systems ELISA), erythropoetin (EPO) (R&D systems Quantikine IVD ELISA) and C reactive protein (CRP) (Dade Dimension particle enhanced turbidimetric immunoassay). The sTfR-F index was defined as sTfR concentration divided by log serum ferritin (sTfR/log ferritin)$^{20}$.

For stable isotope administration, $^{57}$Fe and $^{58}$Fe were made as an aqueous solution of iron sulfate as previously described$^{21}$. Isotope ratios were measured by thermal ionization magnetic sector mass spectrometry$^{21,22}$, expressed relative to the non-administered isotope $^{56}$Fe and corrected for temperature specific differences in fractionation using the ratio of $^{54}$Fe to $^{56}$Fe. Iron isotope ratios were converted to tracer:tracee ratios as described$^{23}$ and red blood cell incorporation was expressed as the percentage of dose administered.

**Statistics**

The distributions of variables were checked for normality and log-transformed where appropriate. Geometric means and 95% confidence intervals are presented for these variables. Multi-variate regression analysis included all variables significant at $p<0.05$ in the univariate models. Regressions were calculated separately for the Day 1, Day 15 and combined data. All analysis was conducted using Datadesk 6.2.1 (Data Description Inc, Ithaca).
Results

Iron status and utilization in post-malarial and non-malarial anemic children during a course of iron supplementation

The aim of this study was to compare the predictive value of hepcidin with those of an array of indices of iron status and inflammation (CRP, ferritin, sTfR, sTfR-F index, ZnPP, serum iron, erythropoietin, and hemoglobin levels) for effective oral iron utilization, indicated by erythrocyte iron incorporation, in iron-supplemented Gambian children diagnosed with either post-malarial or non-malarial anemia. The children with post-malarial anemia presented with fever and *Plasmodium falciparum* parasitemias greater than 500 parasites per milliliter following Field’s staining of a thick blood film. They consequently completed a three-day course of anti-malarial treatment, which in every case cleared the parasitemia. Non-malarial anemic children were identified from immunization, growth monitoring or general clinics and otherwise appeared well; they had no documented fever in the previous seven days, nor a record of clinical malaria episodes or anti-malarial treatment during the ongoing malaria season. For all children, a 30-day course of oral iron supplementation was initiated, with post-malarial anemia cases commencing supplementation the day after completion of anti-malarial treatment: this day is referred to as Day 1. To investigate how effectively iron was utilized in each of these groups, the stable iron isotopes $^{57}$Fe and $^{58}$Fe were administered orally on Days 1 and 15 respectively and incorporation of each stable iron isotope into erythrocytes was quantified 14 days after its administration (*Figure 1*).

Iron indices and inflammatory markers were also measured in blood taken on the days of stable iron isotope administration. First, we compare iron status and inflammation in children from post-malarial and non-malarial anemia groups at both Day 1 and Day 15 (column 2 versus column 3, and column 4 versus column 5 in *Table 1*), and then describe changes from Day 1 to Day 15 within both these groups (column 2 versus column 4, column 3 versus column 5).

(a) Comparison of iron status and inflammation between post-malarial and non-malarial anemic children at the initiation of iron supplementation (Day 1).

On Day 1, children with post-malarial anemia had significantly lower hemoglobin levels than those in the non-malarial anemia group (*Table 1*). Despite the anti-malarial treatment clearing parasitemias in all post-malarial anemia cases, CRP and ferritin were significantly higher in these children than in non-malarial anemic cases (*Table 1*). Likewise, hepcidin, previously shown to be induced by malaria$^{24-27}$, was raised in these children compared to
non-malarial anemic children, although not statistically significantly so (Table 1). EPO levels were significantly higher in post-malarial cases than non-malarial anemic cases (Table 1). Serum iron levels were higher in post-malarial anemic children. The non-malarial anemic children were more iron deficient than the post-malarial anemic children, as indicated by sTfR levels and sTfR-F index (Table 1). Erythrocyte incorporation of $^{57}$Fe, measured in blood taken 14 days after isotope administration, was significantly suppressed in post-malarial anemic children compared to the non-malarial anemic cases, as previously described.\(^\text{19}\)

(b) Comparison of markers of iron status and inflammation between post-malarial and non-malarial anemic children following 14 days of iron supplementation (Day 15).
There were no statistically significant differences between the two groups of children on Day 15 prior to administration of $^{58}$Fe. Post-malarial children had higher hemoglobin and lower levels of EPO and ZnPP, together indicating a greater level of oxygen carriage and reduced hypoxia compared to children that were admitted to the study with non-malarial anemia. Erythrocyte incorporation of $^{58}$Fe, measured 14 days after its administration on Day 15, was less in post-malarial anemic children compared to the non-malarial anemic cases.

(c) Changes in iron status following 14 days of iron supplementation in post-malarial anemic children.
Despite the relative suppression of erythrocyte iron ($^{57}$Fe) incorporation in post-malarial anemic children, after the first 14 days of iron supplementation hemoglobin levels had risen notably (Table 1). Inflammation (CRP) in most post-malarial children had resolved and other hematological indices were normalizing by this time (Table 1). Similarly, hepcidin levels had also fallen and EPO levels had dropped markedly, indicating a correction of hypoxia probably consequent on anti-malarial treatment. Correspondingly, erythrocyte incorporation of $^{58}$Fe (administered on Day 15) was higher than $^{57}$Fe incorporation (Table 1), suggesting the relative block to oral iron utilization was being relieved.

(d) Changes in iron status following 14 days of iron supplementation in non-malarial anemic children.
In contrast to the post-malarial anemia cases, there was no significant increase in hemoglobin levels after the first 14 days of iron supplementation, and CRP levels remained low. However, serum iron and ferritin levels had risen by Day 15, and hepcidin had fallen in concert with reduced sTfR-F index (both non-significantly), indicative of a response to the
course of iron supplementation (Table 1). The efficiency of $^{58}$Fe erythrocyte incorporation administered on Day 15 was similar in these children to that of $^{57}$Fe incorporation administered on Day 1, suggesting continued efficacy of oral iron supplementation throughout the course.

In summary, these data suggest that many of the conflicting signals that affect iron handling and utilization are represented in these groups: inflammation and recent infection, iron deficiency, anemia and erythropoietic drive. There were differences in the changes over time of iron status and inflammation in children with post-malarial anemia compared with children with non-malarial anemia. Also, over the course of the study oral iron availability was maintained at a high level, so that the temporal balance of the above factors was not constant. Against this background, we wished to test how hepcidin compared with other serum indices as a predictor of iron utilization as measured by erythrocyte iron incorporation.

**Univariate associations between erythrocyte iron incorporation, hepcidin and other indices of iron status and inflammation.**

We first evaluated univariate associations between erythrocyte stable iron isotope incorporation and indices of iron status and inflammation (Table 2). Specifically, we considered associations between (a) plasma iron/inflammatory indices on the day of $^{57}$Fe administration (Day 1) and subsequent $^{57}$Fe erythrocyte incorporation; (b) plasma iron/inflammatory indices on the day of $^{58}$Fe administration (Day 15) and subsequent $^{58}$Fe erythrocyte incorporation, and (c) combined data for incorporation of both stable isotopes with associated plasma indices on the day of isotope administration. As expected there was significant covariation between hepcidin and ferritin ($R^2=23\%$, $n=48$), hepcidin and CRP ($R^2=10\%$), and ferritin and CRP ($R^2=39\%$).

(a) Predictors of $^{57}$Fe erythrocyte incorporation: assessing utilization of oral iron at the start of the course of iron supplementation (Table 2, left hand columns).

The strongest predictor of $^{57}$Fe erythrocyte incorporation was hepcidin, which was significantly negatively associated (adjusted $R^2=67\%$, $P<0.0001$; Figure 2a); CRP and ferritin were also highly significant negative predictors of $^{57}$Fe incorporation. A strong positive association of $^{57}$Fe erythrocyte incorporation with sTfR, and a weaker, but significant positive correlation with sTfR-F index were also found. These data suggest that at the start of the course of iron supplementation, there was an influence of both inflammatory stimuli and iron status on oral iron utilization.
(b) Predictors of $^{58}$Fe erythrocyte incorporation: assessing utilization of oral iron administered 2 weeks into a 30-day course of iron supplementation (Table 2, middle columns).

Despite the strong negative correlation of CRP with $^{57}$Fe erythrocyte incorporation above, CRP did not significantly predict $^{58}$Fe erythrocyte incorporation, probably because by Day 15 inflammation had waned (see Table 1). However, hepcidin remained a significant negative predictor of $^{58}$Fe incorporation (Figure 2b), suggesting it may represent a more consistent indicator of iron utilisation. Hemoglobin, ZnPP, sTfR-F index and ferritin were strongest predictors of $^{58}$Fe incorporation, consistent with iron status governing iron incorporation in the absence of inflammation.

(c) Combined data (Table 2, right hand columns).

Taking into account erythrocyte incorporation of both $^{57}$Fe and $^{58}$Fe, the strongest predictors were CRP and hepcidin. Less significant associations were found with ferritin, sTfR and ZnPP, but not with the sTfR-F index or hemoglobin. There was no significant association with EPO in any of the analyses.

**Multivariate analysis of factors predicting erythrocyte iron incorporation**

We constructed final multivariate models including all variables displaying univariate associations with erythrocyte incorporation of either $^{57}$Fe, $^{58}$Fe or the combined data (shown in Table 3). While ferritin dropped out of this analysis, hepcidin emerged as the most consistent predictor of the efficiency of incorporation of orally-administered iron into erythrocytes: hepcidin predicted incorporation of both $^{57}$Fe administered at the start of the course of iron supplementation and $^{58}$Fe given two weeks into the course; it remained a significant predictor when all data were included in the final model (Table 3). CRP was also a strong predictor when all data were considered together, but despite strongly predicting $^{57}$Fe incorporation, it did not predict $^{58}$Fe incorporation. Remarkably hepcidin, CRP and pre-treatment parasitemia (para00) explained 85% of the variance in incorporation of $^{57}$Fe, administered on day 1.
Discussion
The need to understand the conditions in which iron can be safely provided to children living in conditions of high infectious risk has become of prime public health significance following the serious adverse effects of supplementation observed in a large trial on Pemba Island.

In this study, we used incorporation of orally administered stable iron isotopes into erythrocytes as a measure of effective dietary iron utilization in Gambian children with post-malarial or non-malarial anemia. Other measures of iron status such as serum iron or sTfR-F index may be altered following iron supplementation, but do not necessarily equate to increased iron utilization for erythropoiesis. In our study population, several conflicting signals known to influence iron uptake, handling and utilization are represented: inflammation, indicated by high CRP and ferritin; iron deficiency and low iron stores, (low ferritin, high sTfR-F index and high ZnPP); strong erythropoietic drive (high EPO); and increasing iron supply (due to the prescribed 30-day course of iron supplementation).

Moreover, this population showed broad distributions of most of the indices measured, favoring the detection of true biological correlates (Hb 68-133g/l; hepcidin 0.2-94ng/ml; CRP 0.6-121mg/l; ferritin 1-211ng/ml; serum iron 6-27umol/l; sTfR 3-34mg/l; sTfR-F index 1-59; erythropoietin 4-196 mIU/ml; ZnPP 51-347mmol/mol Hb; iron incorporation, 1%-68%).

Children with post-malarial anemia demonstrated blunted erythrocyte $^{57}$Fe (Day 1) incorporation relative to non-malarial anemic children as previously described. Despite having cleared parasitemias after three days of anti-malarial treatment, they still had raised inflammatory markers (CRP and ferritin), presumably reflecting residual effects of malarial inflammation. Hepcidin likewise was raised in these individuals, but not as markedly as CRP or ferritin; this might either reflect differing kinetics of clearance, and/or that other hepcidin-suppresive factors were also in operation (e.g. erythropoietic drive, hypoxia). EPO levels were significantly higher in the post-malarial cases, potentially reflecting a compensatory response to malaria-mediated suppression of EPO responsiveness as previously described; the lower hemoglobin levels in this group could also contribute to higher EPO. Serum iron levels were raised in these children, perhaps due to residual effects of malarial hemolysis. The low levels of $^{57}$Fe incorporation despite high serum iron, and the lower levels of sTfR that were also apparent despite high EPO, together suggest that poor $^{57}$Fe incorporation in these children may at least in part be due to dyserthropyosis as well as due to the effect of high hepcidin restricting iron availability. In malaria, inflammatory cytokines and effects of the hemozoin pigment can cause bone marrow dysfunction. Despite
this relative block to incorporation of $^{57}\text{Fe}$ given on day 1, children with post-malarial anemia showed a better hemoglobin recovery after two weeks of iron supplementation than non-malarial anemic children. The rise in hemoglobin may have been fueled by the release, following resolution of inflammation, of (non $^{57}\text{Fe}$) iron sequestered into reticuloendothelial macrophages during the acute phase response to infection. The improved utilization of $^{58}\text{Fe}$, administered two weeks after $^{57}\text{Fe}$, is consistent with this idea. Together these factors may explain the faster hemoglobin recovery in the face of poorer incorporation of $^{57}\text{Fe}$ in this group.

Iron deficiency was present in children with non-malarial anemia, as indicated by low serum iron, low ferritin, relatively low hepcidin, and high sTfR-F index. These children were not inflamed and utilized dietary iron (Day 1 $^{57}\text{Fe}$) better than post-malarial anemic children. Furthermore, a response to two weeks’ iron supplementation was indicated by increased serum iron, ferritin and (non-significantly) hepcidin, and a non-significantly reduced sTfR-F index. However the unexpected lack of change to hemoglobin and ZnPP suggests a possible contribution of bone marrow dysfunction, or other factors, to anemia in this group.

We next compared how hepcidin and other plasma indices measured on the day of stable iron isotope administration predicted iron incorporation into erythrocytes. Several processes influence erythrocyte incorporation of orally administered iron. Intestinal iron uptake and iron recycling are two key factors, and are both regulated by hepcidin-dependent and independent mechanisms. Double-isotope studies (oral and intravenous) in non-pregnant Beninese women, with very similar absorption percentages to our study$^{33}$ showed that 88% of absorbed iron was incorporated into erythrocytes$^{34}$, indicating the contribution of intestinal absorption to eventual red blood cell iron. However, iron utilization may also vary post-absorption – for example iron may become trapped in macrophages, or iron may not be incorporated efficiently even if available, should erythropoiesis be inhibited (as may be the case for $^{57}\text{Fe}$ incorporation in post-malarial anemia, discussed above).

Despite all the possible causes of variable erythrocyte iron incorporation, and the fact that these processes are not regulated by hepcidin alone, in our studies hepcidin was the most consistent predictor of subsequent erythrocyte iron incorporation, significantly correlating with incorporation of both $^{57}\text{Fe}$ (administered on Day 1) and $^{58}\text{Fe}$ (administered on Day 15), and when all isotope incorporation data were considered together. Other parameters also correlated significantly with erythrocyte iron incorporation, although not in all analyses. CRP,
present at high levels immediately following the 3-day course of anti-malarial treatment prior to commencement of iron supplementation, strongly predicted incorporation of Day 1 administered $^{57}$Fe. Indeed, hepcidin, CRP and pre-treatment parasitemia explained 85% of the variance in erythrocyte incorporation of $^{57}$Fe. However, by day 15 when inflammation had subsided, CRP no longer predicted $^{58}$Fe incorporation, unlike hepcidin. This is consistent with other factors besides inflammation influencing erythrocyte iron incorporation, such that the predictive value of CRP remains limited. Ferritin (elevated by inflammation and low during iron deficiency when inflammation is absent) covaried with both hepcidin, as previously described$^{35}$, and CRP. Ferritin correlated with erythrocyte iron incorporation in univariate analyses, but was not significant in multivariate models.

Significant, but weaker, associations between hepcidin and iron absorption/utilization have also been reported in iron-replete European men ($R^2=30\%, n=33$)$^{36}$, in iron-replete non-pregnant American women ($P=0.03, n=18$)$^{37}$, and in European women with iron status ranging from replete to moderately deficient ($R^2=31\%, n=98$ from ferrous sulfate; $R^2=20\%, n=48$ from ferrous fumarate; and $R^2=10\%$ (NS) from ferric pyrophosphate)$^{38}$. A study in 23 non-pregnant Beninese women with asymptomatic *Plasmodium falciparum* parasitemia reported no association between hepcidin and iron absorption at baseline$^{34}$, but a significant association ($R^2=46\%, P<0.01$) 25 days after treatment. The strength of the association between hepcidin and erythrocyte iron incorporation in our study may be due to a greater variance of hepcidin and hepcidin-influencing factors across the study group, and/or because we assayed erythrocyte iron rather than total body iron absorption or serum iron.

That hepcidin outperforms other signals/markers of iron and infection status (Hb, ferritin, sTfR, the sTfR-F index of body iron stores, EPO and CRP) as a predictor of iron incorporation in this population has two important implications. First, it strongly suggests that hepcidin has evolved as the master regulator of host iron absorption and utilization in the face of infections, as previously inferred$^{12,39}$. In this respect, the fact that hepcidin causes rapid hypoferremia in response to infection$^{39-41}$ and that it declines rapidly after an infection or inflammatory signal has been cleared$^{26,27}$ (thus allowing rapid absorption of dietary iron and remobilization of sequestered iron) would favor safe iron acquisition by anemic children during the limited windows between repeated infections. Second, because hepcidin appears to integrate the opposing regulatory signals derived from iron deficiency and from threat of infection, it might be exploitable as an effective point-of-care index indicating ‘ready-and-safe to receive iron’ that could guide therapeutic intervention.
Acknowledgements
We gratefully acknowledge the mothers and children who participated in the study. We thank the Wellcome Trust and the MRC UK for funding. During this work H.D was funded by a Beit Memorial Fellowship for Medical Research and an MRC new investigator award.

Author Contributions
AP and CD conceived the study. CD, SC and SA conducted the study and associated analyses. AA and HD performed the hepcidin analyses and contributed to data interpretation. SA and HV contributed to data interpretation. AP, AA and HD wrote the paper with input from all authors.

Conflicts of interest
None.
References


Table 1. Iron and inflammatory markers, and erythrocyte stable isotope iron incorporation in Gambian children presenting with either post-malarial or non-malarial anemia, and supplemented with iron for 30 days.

<table>
<thead>
<tr>
<th>Status on Day 1: day of $^{57}$Fe oral administration</th>
<th>Status on Day 15: day of $^{58}$Fe oral administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-malarial Anemia</td>
</tr>
<tr>
<td>N</td>
<td>11</td>
</tr>
<tr>
<td>Hemoglobin (g/l)</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>Hepcidin (ng/ml)</td>
<td>25.5 ± 7.4</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>54 (30, 84)</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>82 (48, 164)</td>
</tr>
<tr>
<td>Serum iron (umol/l)</td>
<td>16.0 ± 1.9</td>
</tr>
<tr>
<td>sTfR (mg/l)</td>
<td>6.6 ± 0.7</td>
</tr>
<tr>
<td>sTfR-F index</td>
<td>4.8 ± 1.8</td>
</tr>
<tr>
<td>Erythropoietin (mIU/ml)</td>
<td>70 (34, 120)</td>
</tr>
<tr>
<td>ZnPP (mmol/mol Hb)</td>
<td>131 (103, 159)</td>
</tr>
<tr>
<td>Stable iron isotope incorporation 14 days post oral administration (%)</td>
<td>10.6 ± 3.5</td>
</tr>
</tbody>
</table>

Arithmetic means ± SE or geometric means (95% CI). *P<0.05, **P<0.01, ***P<0.001 by unpaired T-test comparing post-malarial to non-malarial anemia groups. †P<0.05, ††P<0.01, †††P<0.001 by Scheffé’s post-hoc test Day 15 vs Day 1.
Table 2. Univariate associations of erythrocyte incorporation of $^{57}$Fe, $^{58}$Fe or both isotopes 14 days after oral administration with serum iron and inflammatory indices on the day of isotope administration.

<table>
<thead>
<tr>
<th>Univariate predictors of iron incorporation</th>
<th>Status on Day 1: day of $^{57}$Fe oral administration</th>
<th>Status on Day 15: day of $^{58}$Fe oral administration</th>
<th>Combined data (considering both $^{57}$Fe and $^{58}$Fe erythrocyte incorporation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adjusted $R^2$ (%)</td>
<td>$P$</td>
<td>Adjusted $R^2$ (%)</td>
</tr>
<tr>
<td>Log Hepcidin</td>
<td>67</td>
<td>&lt;0.0001</td>
<td>19</td>
</tr>
<tr>
<td>Log CRP</td>
<td>55</td>
<td>&lt;0.0001</td>
<td>-</td>
</tr>
<tr>
<td>Log Ferritin</td>
<td>39</td>
<td>&lt;0.0009</td>
<td>28</td>
</tr>
<tr>
<td>sTfR</td>
<td>40</td>
<td>&lt;0.0004</td>
<td>-</td>
</tr>
<tr>
<td>sTfR-F index</td>
<td>22</td>
<td>0.014</td>
<td>37</td>
</tr>
<tr>
<td>Log ZnPP</td>
<td>-</td>
<td>NS</td>
<td>28</td>
</tr>
<tr>
<td>Hb</td>
<td>-</td>
<td>NS</td>
<td>44</td>
</tr>
</tbody>
</table>

All the listed associations had negative coefficients except for sTfR and the sTfR/logFerritin index. NS = not significant.
Table 3: Multivariate models evaluating predictors of erythrocyte incorporation of $^{57}$Fe, $^{58}$Fe or both isotopes 14 days after oral administration.

<table>
<thead>
<tr>
<th>Final multivariate models</th>
<th>Status on Day 1: day of $^{57}$Fe oral administration</th>
<th>Status on Day 15: day of $^{58}$Fe oral administration</th>
<th>Combined data (considering both $^{57}$Fe and $^{58}$Fe erythrocyte incorporation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adjusted R$^2$ (%)</td>
<td>P</td>
<td>Adjusted R$^2$ (%)</td>
</tr>
<tr>
<td>Log Hepcidin</td>
<td>&lt;0.0009</td>
<td></td>
<td>0.0064</td>
</tr>
<tr>
<td>Log CRP</td>
<td>&lt;0.0002</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Log Ferritin</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>sTfR</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Log ZnPP</td>
<td>NS</td>
<td></td>
<td>&lt;0.0004</td>
</tr>
<tr>
<td>Parasitemia</td>
<td>0.0024</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Schedule of malarial treatment and iron supplementation, including administration of stable iron isotopes.
A course of iron supplementation was initiated in anemic Gambian children. Supplementation was initiated on Day 1. Children who had presented with *Plasmodium falciparum* parasitemia 3 days previously had their parasitemias cleared by a 3 day course of chloroquine/fansidar. The stable iron isotopes $^{57}$Fe and $^{58}$Fe were administered as sulfate on Days 1 and 15 respectively. 2 mg/kg/day iron was given as liquid iron glycine sulphate on all other days of the supplementation course starting from Day 2.

Figure 2. Scatterplots of oral iron incorporation versus log$_{10}$ hepcidin.
A) Serum hepcidin measured on Day 1 is plotted against incorporation of $^{57}$Fe (which was administered on Day 1).
B) Serum hepcidin measured on Day 15 is plotted against incorporation of $^{58}$Fe (which was administered on Day 15).
Closed circles = children admitted into the study with post-malarial anemia; open circles = children admitted into the study with non-malarial anemia. Line depicts best-fit regression.
Figure 1

- **57Fe administration (3.9 mg)**
  - Anti-malarial treatment (malarial cases)
  - (standard iron supplementation 2 mg/kg/day)

- **58Fe administration (1.3 mg)**
  - (standard iron supplementation 2 mg/kg/day)

**Measurements made**
- **Day -3 1 15 30**
  - • *P. falciparum* parasitemia (para00)
  - • Iron / Inflammation indices (inc. hepcidin)
  - • Iron / Inflammation indices (inc. hepcidin)
  - • *57Fe* incorporation into erythrocytes
  - • *58Fe* incorporation into erythrocytes

**Treatments administered**
- Anti-malarial treatment (malarial cases)
- (standard iron supplementation 2 mg/kg/day)
Figure 2

A

$^{57}$Fe incorporation (%) vs. Hepcidin (ng/mL)

B

$^{58}$Fe incorporation (%) vs. Hepcidin (ng/mL)
Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children

Andrew M Prentice, Conor P Doherty, Steven A Abrams, Sharon E Cox, Sarah H Atkinson, Hans Verhoef, Andrew E Armitage and Hal Drakesmith