Anaemia, iron deficiency and infections: new perceptions of the interaction between hepcidin, iron biomarkers, anaemia and inflammation in Malawian children
Jonker, F.A.M.

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Chapter six

Unexpected low hepcidin levels in severely anaemic Malawian children with high incidence of infectious diseases and bone marrow iron deficiency


Submitted for publication
Abstract

Iron supplementation targeting only iron deficient children is preferred in infection endemic areas as supplementing iron replete children may increase their infection risk. A reliable iron biomarker for individual assessment is lacking in these populations. The iron-regulator, hepcidin, controlled by host iron status, anaemia and inflammation, is a potential candidate marker for identifying iron deficiency and guiding supplementation programs. However, circulating hepcidin levels have never been evaluated against bone marrow iron status in African children.

In this cross-sectional study, bone marrow iron status was evaluated in 237 severely anaemic (haemoglobin <5.0 g/dL) Malawian children (6-60 months) in which hepcidin levels were assessed. It was found that hepcidin levels were unexpectedly low, and compared to other peripheral iron markers, poorly predicted bone marrow iron status. Structural-equation-modelling indicated a strong down-regulating stimulus of erythropoietin, which counterbalanced up-regulation by CRP and IL-6. Furthermore CRP, not hepcidin, was negatively associated with insufficient erythroblast iron incorporation.

In these severely anaemic children hepcidin was a poor marker for bone marrow iron status. Low serum hepcidin would be an unreliable indicator for iron supplementation in these children, and may expose children to an increased infection risk.
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Introduction

Iron deficiency is a nutritional disorder which is common world-wide. Pre-school children in developing countries are one of the most important risk groups, as iron deficiency not only causes anaemia but also developmental dysfunction and is a major contributor to child morbidity and mortality in these settings. Iron supplementation is considered a cost-effective strategy to prevent and treat anaemia, although there is concern that it may increase risk of infection in iron replete children living in malaria endemic areas. The World Health Organization therefore recommends restricting iron supplementation to children with proven iron deficiency or signs of severe anaemia. In these circumstances a reliable iron biomarker is required in order to identify those iron deficient children who may benefit from iron supplementation without experiencing an increased infection risk. Such a biomarker is currently lacking.

The recently discovered key iron regulator ‘hepcidin’ plays a central role in the interaction between iron deficiency, anaemia and inflammation. In iron loaded conditions hepcidin levels increase during inflammation/infection through degradation of the cellular iron exporter ferroportin. This reduces intestinal absorption of iron and down regulates release of iron from iron-stores. Conversely, iron deficiency, anaemia/hypoxia and enhanced erythropoiesis, decrease hepcidin levels and will subsequently increase iron levels. In view of this central role, it has been suggested that serum hepcidin could be an ideal marker to guide iron supplementation requirements. Nevertheless in subjects living in areas of Africa with a high infection pressure, hepcidin has never been validated against bone marrow iron, the “gold standard” of iron status. We therefore evaluated serum hepcidin as a predictor of deficient bone marrow iron stores, as well as erythroblast iron incorporation in severely anaemic Malawian children. We further evaluated the impact of the different stimuli, iron levels, inflammation, hypoxia and erythropoiesis, on serum hepcidin levels in this population.

Methods

Methods | Study design and population

This study formed part of a large research programme investigating the etiology, pathophysiology and outcome of severe anaemia in Malawian children. A detailed description of the study methodology has been given elsewhere. In brief, 381 children (6–60 months old) presenting with severe anaemia (haemoglobin <5.0 g per decilitre) were enrolled as ‘cases’ if they had not been transfused in the previous 4 weeks. For each case, two controls were enrolled, a community-control living within 100 to 1000 meters of the case, and a hospital-control, presenting at the same hospital or outpatient facility as the case. Controls were eligible for recruitment if they were aged between 6-60 months and if their haemoglobin level was 5.0 gram per decilitre or more. At recruitment a venous blood sample was obtained and, (in cases) if the clinical condition allowed, a bone marrow aspiration was performed.

For the hepcidin study, all cases with available bone marrow aspirates were included. In addition, in order to compare hepcidin levels between severely anaemic and non-severely anaemic children, hepcidin was also assessed in a small sub-sample of controls (without available bone marrow aspirates). Written informed consent was obtained from a parent or guardian of each child. The study was approved by the ethics committees of the College of Medicine, Malawi, and the Liverpool School of Tropical Medicine, United Kingdom.
Methods | **Laboratory investigations**

Haemoglobin, mean cellular volume (MCV) and mean corpuscular haemoglobin concentration hematocrit (MCHC) were measured by a Coulter counter analyzer (Beckman Coulter, Durban, South Africa). Bone marrow slides were stained with Hematognost Fe (Merck, Darmstadt, Germany) and graded for intracellular iron content using a histological grading method which classifies iron status into six grades (0-6). In addition, all marrow smears were also assessed using an alternative grading method where utilizable iron was specifically assessed through determination of iron in the erythroblasts. At high power magnification (*×1000*) 20 fields surrounding the marrow fragments were examined whereby hundred erythroblasts were examined to enumerate the percentage containing iron granules in their cytoplasm.

Within the first hour after collection 200 μl plasma and serum aliquots were stored at -80°C. As outcome of assessments of plasma and serum hepcidin is comparable we will refer as *serum* hepcidin. These samples were used for later assessment of hepcidin-25 (the mature, active form of the peptide) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF-MS). An internal standard (synthetic hepcidin-24; Peptide International Inc., Louisville, KY) was used for quantification. Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization (MALDI) TOF-MS platform (Bruker Daltonics, Bremen, Germany). Serum hepcidin-25 concentrations are expressed as nanomoles per liter (nmol/L). The lower limit of detection of this method was 0.5 nmol/L. Samples found to have a hepcidin concentration < 0.5 nmol/L were imputed with a random value out of a uniform distribution with a minimum of 0.01 nmol/L and a maximum of 0.5 nmol/L. Ferritin was determined using electro-chemiluminescence immunoassay (Modular Analytics E170, Roche Diagnostics, Switzerland). Erythropoietin was determined using Immulite 2000 (maximum detection limit <200 and after dilution >1000 IU/L) (Siemens DPC, new Jersey, USA). Immunoturbidimetric assay (Modular P800, Roche Diagnostics, Switzerland) was used to determine serum transferrin and C-reactive protein (CRP). Soluble transferrin receptor (sTfR) was measured using an enzyme immunoassay (Ramco Laboratories, TX, USA, detection limit 1.0 μg/l). Interleukin-6 (IL-6) was measured by Cytometric Bead Array on a FACS-Calibur flow-cytometer (Becton-Dickson, South Africa). Clinical malaria was defined as a positive blood slide with concurrent fever (axillary temp >37.5°C), or history of fever within the previous 48 hours.

Methods | **Definition and cut-off values**

Deficiency of bone marrow iron stores was defined as grade 0 or 1. Erythroblast iron incorporation was used as a proxy for utilisable iron in the bone marrow; insufficient erythroblast iron incorporation (functional bone marrow iron deficiency) was defined as less than 30% of the erythroblasts having visible iron granules while having replete iron stores. Cut-off values for the peripheral iron markers were as follows: hepcidin <0.5 nmol/L; ferritin <30 μg/L; MCHC <32 g/L; MCV <67 fl (<2 years old) and <73 fl (<5 years old). Soluble transferrin log-ferritin index (sTfR-F index) was defined as [sTfR + log ferritin] in which log refers to ‘base-10 log’. Using the cut-offs sTfR >8.3 μg/L and ferritin <30 μg/L, a cut-off for sTfR-F index was calculated (>5.6).

Methods | **Statistical Methods**

Data were analyzed with use of SPSS 20.0 and AMOS 20.0 statistical computer packages (SPSS, Chicago, Illinois). Correlations with bone marrow iron grading were assessed with the Kendall rank correlation test for ordinal data. Median hepcidin levels between the different sub groups were compared using the Mann-Whitney U test. Using the dichotomous outcome of bone marrow iron status and erythroblast iron incorporation, receiver operating characteristics (ROC) curves and corresponding areas under the curve (AUCROC) were created for hepcidin and conventional iron markers. The ROC curves were used to assess
a cut-off for hepcidin providing optimal sensitivity and specificity in predicting bone marrow iron deficiency, and for all iron makers in predicting insufficient erythroblast iron incorporation. To evaluate different signalling pathways of hepcidin, all relevant available covariates relating to hepcidin were analyzed using linear regression models with bone marrow iron deficiency as outcome. These predictors of hepcidin included erythropoietin, reticulocytosis, haemoglobin, bone marrow iron grading, CRP, IL-6, malaria and bacteraemia. To evaluate the association between hepcidin and insufficient erythroblast iron incorporation, all relevant available covariates relating to erythroblast iron incorporation were analyzed using logistic regression models, these predictors of erythroblast iron incorporation included hepcidin, use of haematinics in the previous 4 weeks and CRP. Missing observations were included in analyses by creating missing-value categories. Reported p-values are two-sided; statistical significance was set at the conventional 5% level.

Structural equation model

More complex multivariate analyses allowing interaction of covariates were performed using structural equation modelling. This model was created containing all possible associations between all relevant available variables relating to hepcidin, after which all non-significant arrows or variables (p ≥0.05) were removed, unless pathophysiologically deemed relevant (dashed arrows). Skewed variables were log-transformed. Missing observations were considered as missing at random and were imputed by single imputation.

Results

From the 381 severely anaemic children, in children 237 a bone marrow aspirate was performed. In 139 of these children a serum sample with sufficient volume for hepcidin assessment was available. Haematological status of children with or without hepcidin and/or bone marrow assessment was comparable (data not shown). From the 757 controls, 43 children were randomly selected for hepcidin assessment. Haematological status of this sub-group was comparable to all controls (data not shown). In Table 1 baseline characteristics of the severely anaemic study group and the non-severely anaemic control group are presented. Bone marrow iron deficiency (30.2%), inflammation (81.2% CRP >40.0 mg/L) and high levels of erythropoietin (92.9% epo >1000 u/L), were common in the severely anaemic children.

Results | Hepcidin values in severely and non-severely anaemic children
The median value of hepcidin in the severely anaemic children was 0.40 nmol/L (IQR 0.19-2.10 nmol/L) and 1.40 nmol/L (0.31-5.60 nmol/L) in the non-severely anaemic control group (p =0.04).

Results | Hepcidin and conventional iron markers predicting bone marrow iron stores
Hepcidin levels poorly correlated with bone marrow iron grading (Kendall rank correlation coefficient 0.05, p=0.5). The ROC-curve for hepcidin identifying bone marrow iron deficiency showed an area under curve (AUCROC) of 0.598, not quite significantly higher than 0.500 (p=0.07), the cut-off to indicate an effective test. The AUCROC of ferritin, sTfR, sTfR-F index and MCHC were all greater than 0.500 (p<0.05) (Table 2). The optimal cut-off for hepcidin to predict bone marrow iron deficiency, derived from the ROC-curve, was 0.5 nmol/L. Using this cut-off for hepcidin and standard cut-offs for the conventional iron markers, sensitivity and specificity to detect bone marrow iron deficiency were calculated (Table 2). Sensitivity and specificity of hepcidin to predict bone marrow iron deficiency were 66.7 % and 49.5%, respectively.
Table 1. Baseline characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>severely anaemic cases</th>
<th>non-severely anaemic controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>207</td>
<td>43</td>
</tr>
<tr>
<td>Male</td>
<td>48.8% (101/207)</td>
<td>69.8% (30/43)</td>
</tr>
<tr>
<td>Age (months)</td>
<td>14.0 (9.7-26.3)</td>
<td>20.1 (10.6-28.3)</td>
</tr>
<tr>
<td><strong>Haematological status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>3.6 (0.8)</td>
<td>9.7 (1.9)</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>32.60 (6.8)</td>
<td>33.2 (5.4)</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>82.7 (15.4)</td>
<td>71.2 (8.0)</td>
</tr>
<tr>
<td>Reticulocytes %</td>
<td>3.6 (2.1-6.7)</td>
<td>2.3 (1.7-4.3)</td>
</tr>
<tr>
<td>Erythropoietin &gt;1000 U/L</td>
<td>95.6% (108/113)</td>
<td>0% (0/39)</td>
</tr>
<tr>
<td><strong>Iron status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deficiency of bone marrow iron stores</td>
<td>30.2% (42/139)</td>
<td>n/a</td>
</tr>
<tr>
<td>Insufficient erythroblast iron incorporation</td>
<td>41.4% (48/116)</td>
<td>n/a</td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td>0.40 (0.19-2.10)</td>
<td>1.40 (0.31-5.60)</td>
</tr>
<tr>
<td>Ferritin (µg/l)</td>
<td>404 (157-905)</td>
<td>50 (12-171)</td>
</tr>
<tr>
<td>sTR (µg/ml)</td>
<td>13.4 (8.1-22.4)</td>
<td>12.5 (7.2-17.5)</td>
</tr>
<tr>
<td>sTR-F index</td>
<td>5.1 (3.1-9.6)</td>
<td>5.9 (3.1-12.0)</td>
</tr>
<tr>
<td><strong>Inflammatory status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>12.6% (25/198)</td>
<td>0% (0/20)</td>
</tr>
<tr>
<td>Malaria parasitaemia</td>
<td>60.9% (126/207)</td>
<td>37.2% (16/43)</td>
</tr>
<tr>
<td>HIV infected</td>
<td>7.7% (15/195)</td>
<td>3.7% (2/43)</td>
</tr>
<tr>
<td>CRP &gt; 40 mg/L</td>
<td>81.2% (159/196)</td>
<td>37.7% (12/31)</td>
</tr>
<tr>
<td>Interleukin-6 pg/ml</td>
<td>41.6 (20.9-142.3)</td>
<td>25.6 (10.0-67.3)</td>
</tr>
</tbody>
</table>

Deficiency of bone marrow iron stores was defined as a bone marrow iron score of 0 or 1; Deficiency of functional bone marrow iron was defined as < 30% iron deficient erythroblasts while having replete iron stores 25,26; CRP: C-Reactive Protein; HIV: Human Immunodeficiency Virus; MCHC: mean cell haemoglobin concentration; MCV mean corpus volume; sTR: soluble transferrin receptor; sTR-F index: sTR-log ferritin index 54. Normally distributed variables are presented with their mean value (s.d.); skewed variable are presented with their median value (inter quartile range).

Table 2. Performance of biochemical iron markers to identify children with deficiency of bone marrow iron stores or insufficient erythroblast iron incorporation

<table>
<thead>
<tr>
<th>Iron marker</th>
<th>bone marrow iron deficiency</th>
<th>insufficient erythroblast iron incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUCROC</td>
<td>p value</td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td>0.598</td>
<td>0.07</td>
</tr>
<tr>
<td>Ferritin (µg/l)</td>
<td>0.855</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>sTR (µg/ml)</td>
<td>0.823</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>sTR-F index</td>
<td>0.815</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>0.727</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>0.489</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Deficiency of bone marrow iron stores was defined as a bone marrow iron score of 0 or 1; CRP: C-Reactive Protein; HIV: Human Immunodeficiency Virus; MCHC: mean cell haemoglobin concentration; MCV mean corpus volume; sTR: soluble transferrin receptor; sTR-F index: sTR-log ferritin index 54. Insufficient erythroblast iron incorporation: defined as < 30% iron deficient erythroblasts. AUC area under the curve; ROC: receiver operating characteristics. sens: sensitivity; spec: specificity.
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Results | **Hepcidin, iron status, inflammation, hypoxia and erythropoiesis**
To further explore the hepcidin levels in our population we evaluated potential predictors of hepcidin. The signalling pathways for hepcidin are theoretically classified in four main categories: iron status, inflammation, hypoxia and erythropoiesis.\(^{11}\) Associations with available variables belonging to these pathways were assessed using univariate linear regression analyses (Figure 1, colour section). The multivariate linear regression model predicting log-hepcidin included a proxy variable \( x_6 \) for each pathway (bone marrow iron status, CRP, haemoglobin and erythropoietin). To assess more complex interactions between hepcidin and the hepcidin pathways, a structural equation modelling was created (Figure 3, colour section). The iron signalling pathway, represented by bone marrow iron stores had a weak association with hepcidin (regression coefficient 0.03, \( p=0.6 \)). The inflammation signalling pathway, represented by CRP and IL-6, was strongly positively associated with hepcidin (regression coefficients of 0.23, \( p<0.0001 \) and 0.33, \( p<0.0001 \) respectively). CRP was adjusted for malaria and bacteraemia; IL-6 was adjusted for bacteraemia. The hypoxia signalling pathway reflected by haemoglobin concentration did not significantly associate with hepcidin (regression coefficient 0.02, \( p=0.7 \)). Regarding the erythropoietic signalling pathway, erythropoietin had a strong negative association with hepcidin (regression coefficient -0.56, \( p<0.0001 \)). The overall root mean square area of approximation, an indicator for model fit, was 0.288 (95% CI 0.272-0.304).

Results | **Hepcidin and conventional iron markers predicting insufficient erythroblast iron incorporation**
From the 139 available bone marrow smears, 23 were too poorly stained for erythroblast reading and were excluded from analyses concerning erythroblast iron incorporation. From the remaining samples, 49 (41.4\%) showed insufficient erythroblast iron incorporation. The value of hepcidin and the conventional iron markers to predict erythroblast iron incorporation were evaluated (Table 2). The AUC\(^{ROC} \) for hepcidin was 0.591 (\( p=0.1 \)). Using the optimal cut-off of 0.5 nmol/L, sensitivity and specificity of hepcidin to predict insufficient erythroblast iron incorporation were 45.2\% and 61.8\%, respectively (Table 2).

Results | **Hepcidin, inflammation and erythroblast iron incorporation**
To explore the association between hepcidin levels and erythroblast iron incorporation we evaluated potential predictors of erythroblast iron incorporation, which were assessed using univariate and multivariate logistic regression analyses (Figure 2, colour section). Applying these variables into the structural equation model (Figure 3, colour section), hepcidin levels were weakly associated with insufficient erythroblast iron incorporation (regression coefficient -0.04, \( p=0.6 \)). CRP was associated with insufficient erythroblast iron incorporation (regression coefficient 0.15, \( p=0.02 \)).

Discussion
This is the first study evaluating the possible role of serum hepcidin in identifying iron deficiency in children living in areas with a high infection pressure, and comparing hepcidin with bone marrow iron and other hepcidin signalling pathways. Hepcidin levels in these anaemic children were unexpectedly low in view of their high infection burden. This may result from a dominance of the erythropoietin signal for hepcidin in these severely anaemic children. As a consequence, hepcidin was found to be a poor marker for bone marrow iron stores. Furthermore hepcidin was not associated with insufficient erythroblast iron incorporation.
Bone marrow iron stores and hepcidin

Previous studies showed a positive association between hepcidin and serum and liver iron levels. With the high prevalence of bone marrow iron deficiency in our population we would have expected low hepcidin values. However, although the hepcidin values were low in this study, the structural equation model showed they were only weakly associated with bone marrow iron stores. This can be a result of the stronger effect on hepcidin production of other signalling pathways, erythropoietic drive and inflammation (Figure 3, colour section), both of which were strongly positively associated with hepcidin. As a consequence hepcidin was not a better iron marker than the previous evaluated conventional iron markers in this population from which sTfR-F index best performed. 23

Inflammation and hepcidin

In view of the strong association of hepcidin with IL-6 and CRP in our population, one would expect elevated hepcidin levels in such a population with high levels of IL-6 and CRP. Yet the hepcidin levels were low. As described below, this is explained by the strong down regulating factor of erythropoietin.

Erythropoiesis and hepcidin

Levels of erythropoietin were very high in this severely anaemic population, and strongly associated with low serum hepcidin. The regression coefficients determined in the structural equation model, and the occurrence of low hepcidin levels despite a high incidence of infection, suggested that the down-regulating stimulus due to erythropoietin exceeded that of the up-regulation due to inflammation. This could be confirmed by the higher hepcidin values in our control group with lower erythropoietin and higher CRP and IL-6 levels. Erythropoietin dominating inflammation has been reported in an infectious murine model in which low hepcidin values were observed following administration of erythropoietin. Similar findings have been reported in humans although these subjects had less inflammation compared to children in the present study. 41,42 It is unclear whether erythropoietin is directly down regulating hepatic hepcidin synthesis, or indirectly through induced production of bone marrow erythropoietic factors, i.e. growth differentiation factor-15 (GDF15) and twisted gastrulation protein homolog-1 (TWSG1). 44,45

Hypoxia/anaemia and hepcidin

Hypoxia/anaemia may directly induce a reduction in hepcidin release and as a consequence haemoglobin concentration was expected to positively correlate with hepcidin concentration, although this was not observed in this selected group of severely anaemic children. The degree of severity of anaemia in all children may have reduced the heterogeneity of the hepcidin response.

Hepcidin and erythroblast iron incorporation

As regulator of available utilizable iron, hepcidin was expected to be positively associated with erythroblast iron incorporation. This association was not observed. This finding differs from results in Gambian children that showed hepcidin strongly predicted red blood cell iron incorporation measured after 14 days of oral iron supplementation. Conversely Cercamondi et al observed that although hepcidin (assessed after 25 days) was associated with intestinal iron absorption, there was no association with red blood cell iron incorporation. These studies suggest that intermediate factors may interfere with iron incorporation and may explain the lack of correlation between hepcidin and erythroblast iron incorporation in our study. Furthermore, iron availability (through absorption and release from storage), and iron incorporation is a dynamic process which may be difficult to profile with cross-sectional data alone. This is further underlined by the fact that also all iron markers in this study poorly predicted erythroblast iron incorporation.
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Alternatively inflammatory factors other than hepcidin may inhibit erythroblast iron incorporation as erythrocyte iron incorporation did not correlate with hepcidin, malaria, or IL-6, but significantly correlated with CRP.

It may be argued that our study population may have been an extreme population with severe anaemia, high erythropoietin levels, and a high infection exposure, and the hypothetic model may not be applicable to populations with less severe morbidity. However, these children are very common in resource limited settings in Africa. Furthermore, the model presented in Figure 3 is the most complete model available to date. Ideally the fit of this structural equation model, represented by the root mean square area of approximation (RMSEA), would be smaller then 0.050. Our RMSEA was 0.288 which may partly be explained by missing values. Application of this model to a similar but complete data set should decrease the RMSEA and provide a stronger hypothetical model. An alternative explanation for the increased RMSEA could be a missing intermediate variable in the model, such as an unmeasured erythropoietic or inflammatory factor.

It is important to note that variation in bone marrow findings can result from an uneven distribution of iron in the bone marrow. 50 Therefore a minimum of 10 bone marrow particles per aspirate was examined to optimise sensitivity of the bone marrow examination. 51 Bone marrow assessment still has generally been considered the most reliable diagnostic test for iron status. 50–52

Clinical implications
Translated into practice, our observations raise critical questions concerning the use of iron supplements in severely anaemic children. In these children hepcidin concentrations were low, despite their highly infectious status. Normally a rise in hepcidin would be expected, which would diminish iron absorption and its availability for pathogens, such as malaria. 53 Iron supplementation in such severely anaemic children with concurrent infections could therefore enhance their infection risk, as their hepcidin response is stunted (Figure 4). Currently iron supplementation is recommended for severely anaemic children. 6 There is an urgent need for studies investigating iron uptake in severely anaemic children with malaria, or other infectious diseases, and to assess infection incidence in children with low hepcidin levels receiving iron supplementation.
Figure 4. Hypothetic model of the iron metabolism in severely anaemic children.
Severe anaemia is associated with severe infections, the associated high levels of IL-6 induce hepcidin. However in severe anaemia hypoxia stimulates the kidneys to produce erythropoietin; the erythropoietin induced erythropoietic drive will down-regulate hepatic hepcidin expression and may overrule the hepcidin stimulation of IL-6. The resulting low hepcidin values are associated with a decreased degradation of the cellular iron exporter ferroportin; in iron replete subjects or during iron supplementation this will lead to a rise in plasma iron levels. In infection endemic areas this may increase infection risk. In addition inflammatory related factors possibly inhibit erythroblast iron incorporation. CRP: c-reactive protein; cry: erythroblast; epo: erythropoietin; Fe: plasma iron; FPN: ferroportin; hep: hepcidin-25; IL-6; interleukin 6.

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References

4. WHO. Guideline for the use of iron supplements to prevent and treat iron deficiency anemia. 1998.
6. WHO. Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malarialendemic areas. 2007.
54. Punnonen K, Irijala K, Rajamaki A. Serum transferrin receptor and its ratio to serum ferritin in the diagnosis of
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