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

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Chapter seven

Conventional and novel peripheral blood iron markers compared against bone marrow in Malawian children


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Abstract

Iron deficiency is an important global child health problem. Diagnosis of iron deficiency is complicated as peripheral blood markers have not been validated against bone marrow iron, the reference standard and may have a reduced diagnostic efficiency in infection endemic areas.

In 87 healthy Malawian children (6-66 months) scheduled for elective surgery, ferritin, soluble transferrin receptor (sTfR), soluble transferrin log-ferritin index (sTfR-F), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration and two novel iron markers, hepcidin and Red blood cell Size Factor (RSf), were compared to assessment of bone marrow iron. Prevalence of bone marrow iron deficiency was 44.8%. Ferritin, sTfR, sTfR-F, hepcidin and MCV showed AUCROC >0.500 (0.797;0.691;0.801;0.673 and 0.674 respectively). Using new cut-offs, ferritin (<18ug/L, sensitivity:73.7%/specificity:77.1%) and sTfR-F (>0.8, sensitivity:72.5%/specificity:75.0%) best predicted bone marrow iron deficiency. Hepcidin (<1.4 nmol/L) was a sensitive marker (73.0%) although specificity was 54.2%. RSf poorly predicted bone marrow iron deficiency.

In this first study evaluating peripheral iron markers against bone marrow iron deficiency in healthy African children, ferritin and sTfR-F best predicted bone marrow iron status and would be preferred for surveillance and research. For clinical diagnosis of iron deficiency there is an urgent need for a more reliable bio-marker in African children.
Chapter seven | Peripheral blood iron markers compared against bone marrow

Introduction
Iron deficiency is thought to have an important impact on global child health, especially in resource limited settings \(^1\). In sub-Saharan Africa prevalence of iron deficiency in children ranges from 33 to 63\% \(^3\). This variation partly relates to demographic differences, but also to variation in choice of biomarkers and definitions of iron deficiency. Diagnosing iron deficiency using peripheral biomarkers is complicated as bone marrow iron status is considered the accepted standard even though it is invasive and limited for widespread use. Available peripheral iron markers are considered unreliable in populations with high infection pressure, \(^4\) although they have not been validated against bone marrow iron status in these settings. As a consequence different biomarkers and cut-offs, are used in epidemiological and clinical studies assessing iron status and as a result these are difficult to compare and interpret.

There is an urgent need for reliable data on the accuracy of peripheral iron markers in infection endemic populations. This study evaluates the diagnostic accuracy of commonly iron markers against bone marrow iron deficiency in healthy Malawian children. In addition, two new markers were tested; hepecidin, a recently discovered key iron regulating hormone \(^5\), and the Red cell Size Factor (RSf) an iron marker calculated from the mean cell volume (MCV) and mean reticulocyte volume (MRV) reflecting the haemoglobin content of both reticulocytes and red blood cells \(^8\), \(^9\). The diagnostic accuracy of these markers was compared to a reference bone marrow standard for iron status \(^10\), \(^12\), in order to identify the most reliable marker for iron deficiency in this high risk group of children.

Methods
Methods | Study participants and study design
Study participants were prospectively identified from children aged 6–66 months, scheduled for elective surgery at Queen Elizabeth Central Hospital and Beit Cure Orthopedic Hospital, in Blantyre, Malawi. Exclusion criteria were: blood transfusion within the previous 4 weeks, signs of infection (axillary temperature \(>37.5 \, ^\circ \text{C}\) or current infectious diagnosis, (suspected) neoplasm, known haemoglobinopathy, or a haemoglobin level below 8.0 g/dL (local guidelines for elective surgery). The recruitment procedure included a detailed history and physical examination, a venous blood sample and a bone marrow aspiration. Both samples were collected during generalized anaesthesia and prior to surgical intervention. Written informed consent was obtained from the parent or guardian of each child. The study was approved by the Ethical Committees of the College of Medicine, Malawi and of the Academic Medical Centre of Amsterdam, the Netherlands.

Ferritin, soluble transferrin receptor (sTfR), soluble transferrin log-ferritin index (sTfR-F), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) hepcidin and Red blood cell Size Factor (RSf) were assessed in peripheral blood and compared against the reference standard for iron status, bone marrow iron.

Methods | Bone marrow iron
On the same day a bone marrow aspiration was performed. Bone marrow smears were prepared, fixed with methanol and stored in a dry place at room temperature. To minimize diagnostic variation, fixed smears were all stained in one batch at the end of the study period (Hematognost Fe, Merck, Darmstadt, Germany) and graded for iron content. Histological grading classified iron status into six grades\(^13\), \(^14\). To optimize sensitivity a minimum of 10 bone marrow particles per aspirate \(^12\) were identified by two readers using a double reading...
system. Readers were unaware of patient characteristics or each others results.

Methods | Haematology
Peripheral blood samples were collected in EDTA-anticoagulant (Becton-Dickinson Vacutainer, Plymouth, UK) and assessed on the same day for haemoglobin and red blood cell indices using a Beckman Coulter AcT 5 diff Al Hematology Analyzer (Villepinte, France). MCV and mean reticulocyte volume (MRV) were assessed using a Beckman HmX Coulter counter analyzer (Durban, South Africa) for calculation of Rsf. basic characteristics of these children (n=43) were comparable to the first study population (data not shown).

Methods | Iron biomarkers
Heparin-plasma samples (Becton-Dickinson Vacutainer, Plymouth, UK) were stored in -80 °C and assessed at the end of the study period. Ferritin and sTfR were assessed using a Beckman Coulter Chemistry System Dxl880i with reagent kits from Beckman Coulter, and followed the manufacturers’ procedure. Serum hepcidin-25 measurements (the mature, active form of the peptide) were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF-MS)15. An internal standard (synthetic hepcidin-24; Peptide International Inc., Louisville, KY) was used for quantification 16. 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 15. 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.

Methods | Infection markers
C-reactive protein (CRP) was assessed using a Beckman Coulter Chemistry System Dxl880i; HIV testing was performed using two rapid tests (Determine, Abbott-Laboratories, Japan; Unigold, Trinity-Biotech, Ireland). Inflammation was defined as CRP >10 g/L (method specific reference value). Discordant results and reactive results in children less than 18 months were resolved by HIV-PCR 17. Malaria was excluded by a negative blood slide and a negative rapid test (Standard Diagnostics, Korea).

Methods | Definitions
Bone marrow iron stores deficiency was defined as grade 0 or 1 {Finch, 1982 2397 /id;Gale, 1963 22 /id}. Iron deficiency using peripheral iron markers was defined as: hepcidin <0.5 nmol/L 19; ferritin: two definitions were tested a) <12 ug/L and <30 ug/dL, the latter being recommended in populations were infections are common 20, 21; sTfR >3.3 μg/mL; and MRV’ <100 fl (method-specific reference values); MCHC <32 g/L ; MCV/77 fl/(<2 years old) and <73 fl (age 2-5 years) 2. sTfR-F was defined as [sTfR ÷ log ferritin] in which log refers to ‘base-10 log’ 22, 23. Using these cut-off values for sTfR and ferritin, a sTfR-F cut-off of >2.2 was calculated to define deficient iron stores. Rsf’s was calculated as the square root from [MCV * MRV]24; cut-off values of MCV and MRV were used for calculation of the cut-offs for Rsf (81.9 (age<2 years ) and 84.5 (age 2-5 years )). Anaemia was defined as a haemoglobin level of less than 11.0 g/dL 3.

Methods | Statistical Methods
We compared all peripheral iron markers by calculation of Receiver Operating Characteristic (ROC) curves and corresponding areas under the curve (AUCROC) 25, and by calculation of the sensitivity, specificity, positive and negative predictive values. ROC-curves demonstrate the trade-off between
sensitivity and specificity (any increase in sensitivity will be accompanied by a decrease in specificity). The closer the curve to the upper left corner of the plot, the more accurate the test. The AUC is a measure to test accuracy, with higher values indicating greater accuracy. ROC-curves were created using the dichotomous outcome of bone marrow iron status {Finch, 1982 2397 /id; Gale, 1963 22 /id} and the corresponding AUCROC was compared against the AUCºROC cut-off for an effective test (0.500)28. Differences between AUCºROC were tested using a method described by Hanley and McNeil 29. New cut-off values providing optimal sensitivity and specificity were derived from AUCºROC. Correlations between peripheral iron markers and bone marrow iron scores were assessed using the Kendall rank correlation test for ordinal data. Data were double entered in Access (Microsoft Office 2007, USA) and analyzed using SPSS 20 statistical computer packages (IBM statistics, USA). All p values reported are two-sided.

Results

Between March and October 2011 a total of 93 children were recruited. In four children the bone marrow aspiration resulted in a “dry tap”, in two children bone marrow sample quality was insufficient (no marrow fragments found). These children were excluded from analysis. From the remaining 87 children the mean age was 37 months, 64% of children were anaemic and bone marrow iron deficiency was found in 39 children (44.8 %) (Table 1). No adverse events after bone marrow aspiration or blood sampling occurred.

Apart from Rsf all iron makers were significantly correlated with bone marrow iron grading (Table 2). Hepcidin, sTfr, ferritin, sTfr-F and MCV had a greater AUCROC than 0.500 (p >0.05) (Table 2, Figure 1, colour section). The AUCROC of sTfr-F (0.801) was greater than that for hepcidin (0.673), sTfr (0.691) or MCV (0.674) (all p <0.05), although did not differ from that for ferritin (0.797, p=0.9) (Table 2). The AUCROC of ferritin was greater than that for hepcidin (p <0.05), but did not significantly differ from that for sTfr-F (p=0.9), sTfr (p=0.2) or MCV (p=0.09) (Table 2).

Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) for detection of bone marrow iron deficiency were calculated using published, as well as newly derived cut-offs based on ROC values (Table 3). Using the standard cut-offs for ferritin (<12 µg/ml and <30 µg/ml) the sensitivity and specificity of ferritin were respectively 44.7% and 89.6% (<12), and 81.6% and 37.5% (<30). Applying the new cut-off (<18 µg/L) the sensitivity and specificity of were 73.7% and 77.1% respectively. The standard cut-off for sTfr-F (>2.2 µg/ml) had a sensitivity of 51.4% and specificity of 89.6% for bone marrow iron deficiency; using the new cut-off sensitivity increased to 72.5% and specificity decreased to 75.0%. All other markers had either a sensitivity or specificity below 70% (Table 3).
Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>Demographic information</th>
<th>n</th>
<th>87</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td>42.5% (37/87)</td>
</tr>
<tr>
<td>Age in months</td>
<td></td>
<td>36.5 (17.1)</td>
</tr>
<tr>
<td>Iron status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow iron deficiency</td>
<td>44.8% (39/87)</td>
<td></td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td></td>
<td>1.10 (0.37-3.1)</td>
</tr>
<tr>
<td>RSf (fL)</td>
<td></td>
<td>83.3 (9.8)</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td></td>
<td>19 (12-36)</td>
</tr>
<tr>
<td>sTfR (µg/L)</td>
<td></td>
<td>2.2 (1.8-2.6)</td>
</tr>
<tr>
<td>sTfR-F index</td>
<td></td>
<td>1.8 (1.2-2.4)</td>
</tr>
<tr>
<td>Haematology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaemia (Hb &lt;110 g/dL)</td>
<td></td>
<td>71.3% (62/87)</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td></td>
<td>33.6 (1.4)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td></td>
<td>76.1 (10.0)</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP &gt;10 g/L.</td>
<td></td>
<td>5.7% (5/87)</td>
</tr>
<tr>
<td>Malaria parasitaemia</td>
<td></td>
<td>(0% 0/87)</td>
</tr>
<tr>
<td>HIV-infected</td>
<td></td>
<td>6.0% (5/84)</td>
</tr>
</tbody>
</table>

Bone marrow iron deficiency was defined as a bone marrow smear score of 0 or 1 (GALE et al., 1963); RSf: Red cell Size Factor; sTfR: soluble transferrin receptor; sTfR-F index: soluble transferrin-log ferritin index (Punnonen et al., 1997b); MCHC: mean cell haemoglobin concentration; MCV: mean corpus volume; CRP: C-Reactive Protein; HIV: Human Immunodeficiency Virus; Normally distributed variables are presented with means and their standard deviation; skewed variables with median values and their inter quartile range.

Table 2. Iron markers and their correlation with bone marrow iron stores and the diagnostic to identify bone marrow iron deficiency represented by their AUCroc

<table>
<thead>
<tr>
<th>Iron marker</th>
<th>correlation</th>
<th>AUCroc (95% CI)</th>
<th>AUCroc &gt; 0.500</th>
<th>AUCroc compared with (p-value):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>(p-value) hepcidin</td>
<td>ferritin</td>
<td>sTfR</td>
</tr>
<tr>
<td>sTfR-F index</td>
<td>-0.46***</td>
<td>0.801 0.706 0.895</td>
<td>&lt; 0.0001</td>
<td>0.05</td>
</tr>
<tr>
<td>Ferritin (µg/mL)</td>
<td>0.39***</td>
<td>0.797 0.696 0.897</td>
<td>&lt; 0.0001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>sTfR (µg/mL)</td>
<td>-0.29***</td>
<td>0.691 0.578 0.804</td>
<td>0.005 0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>0.28***</td>
<td>0.673 0.553 0.792</td>
<td>0.004 0.9</td>
<td>0.09</td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td>0.24**</td>
<td>0.673 0.553 0.792</td>
<td>0.005 -</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>0.20*</td>
<td>0.602 0.470 0.846</td>
<td>0.06 -</td>
<td>-</td>
</tr>
<tr>
<td>RSf (fL)</td>
<td>0.13</td>
<td>0.589 0.377 0.776</td>
<td>0.3 -</td>
<td>-</td>
</tr>
</tbody>
</table>

This table presents the Kendall rank correlation with bone marrow iron grading (*<0.05 **<0.001 ***<0.0001) and the area under the curve (AUCROC) of all iron makers in detecting iron deficiency. The difference of all markers with a AUCROC >0.500 was tested. Iron deficiency was defined as a bone marrow smear score of 0 or 1. MCHC: mean cell haemoglobin concentration; MCV: mean corpus volume; sTfR: soluble transferrin receptor; RSf: Red cell Size Factor; sTfR-F index: soluble transferrin-log ferritin index.
<table>
<thead>
<tr>
<th>Iron marker</th>
<th>cut-off*</th>
<th>Iron deficient (%)</th>
<th>Sensitivity (%)</th>
<th>95% CI</th>
<th>Specificity (%)</th>
<th>95% CI</th>
<th>PPV</th>
<th>95% CI</th>
<th>NPV</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>stTR-F index</td>
<td>&gt; 2.2</td>
<td>31.9 (29/91)</td>
<td>51.4</td>
<td>34.7</td>
<td>67.8</td>
<td>89.6</td>
<td>76.6</td>
<td>96.1</td>
<td>79.6</td>
<td>57.3</td>
</tr>
<tr>
<td></td>
<td>&gt; 1.85</td>
<td>48.4 (44/91)</td>
<td>70.2</td>
<td>52.8</td>
<td>83.6</td>
<td>75.0</td>
<td>60.1</td>
<td>85.9</td>
<td>68.4</td>
<td>51.2</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>≤ 30</td>
<td>71.7 (66/92)</td>
<td>81.6</td>
<td>65.1</td>
<td>91.7</td>
<td>37.5</td>
<td>24.3</td>
<td>52.7</td>
<td>50.8</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td>≤ 12</td>
<td>27.2 (25/92)</td>
<td>44.7</td>
<td>30.0</td>
<td>61.5</td>
<td>89.6</td>
<td>76.1</td>
<td>96.1</td>
<td>77.3</td>
<td>54.2</td>
</tr>
<tr>
<td></td>
<td>≤ 16</td>
<td>46.7 (43/92)</td>
<td>73.7</td>
<td>56.6</td>
<td>86.0</td>
<td>77.1</td>
<td>62.3</td>
<td>87.5</td>
<td>71.6</td>
<td>54.9</td>
</tr>
<tr>
<td>stTR (µg/mL)</td>
<td>&gt; 5.3</td>
<td>16.5 (15/91)</td>
<td>24.3</td>
<td>12.4</td>
<td>51.2</td>
<td>91.7</td>
<td>79.1</td>
<td>97.3</td>
<td>69.2</td>
<td>38.9</td>
</tr>
<tr>
<td></td>
<td>&gt; 2.2</td>
<td>34.9 (30/91)</td>
<td>64.9</td>
<td>47.4</td>
<td>79.3</td>
<td>58.3</td>
<td>43.3</td>
<td>72.1</td>
<td>54.5</td>
<td>39.0</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>≤ 67.0 (&lt; 2 years)</td>
<td>29.0 (27/93)</td>
<td>35.9</td>
<td>21.7</td>
<td>52.8</td>
<td>79.2</td>
<td>64.6</td>
<td>89.0</td>
<td>58.3</td>
<td>37.0</td>
</tr>
<tr>
<td></td>
<td>≤ 73.0 (≥ 2 years)</td>
<td>64.6 (60/93)</td>
<td>74.4</td>
<td>57.7</td>
<td>86.4</td>
<td>43.8</td>
<td>29.8</td>
<td>58.7</td>
<td>51.8</td>
<td>38.2</td>
</tr>
<tr>
<td></td>
<td>&lt; 73.5 (&lt; 2 years)</td>
<td>64.6 (60/93)</td>
<td>74.4</td>
<td>57.7</td>
<td>86.4</td>
<td>43.8</td>
<td>29.8</td>
<td>58.7</td>
<td>51.8</td>
<td>38.2</td>
</tr>
<tr>
<td></td>
<td>&lt; 79.8 (≥ 2 years)</td>
<td>64.6 (60/93)</td>
<td>74.4</td>
<td>57.7</td>
<td>86.4</td>
<td>43.8</td>
<td>29.8</td>
<td>58.7</td>
<td>51.8</td>
<td>38.2</td>
</tr>
<tr>
<td>Hepcidin (nmol/L)</td>
<td>≤ 0.5</td>
<td>28.9 (26/90)</td>
<td>43.2</td>
<td>27.5</td>
<td>60.4</td>
<td>83.3</td>
<td>69.2</td>
<td>92.0</td>
<td>66.6</td>
<td>44.7</td>
</tr>
<tr>
<td></td>
<td>≤ 1.4</td>
<td>58.9 (53/90)</td>
<td>73.0</td>
<td>55.6</td>
<td>85.6</td>
<td>54.2</td>
<td>39.3</td>
<td>68.4</td>
<td>55.1</td>
<td>40.3</td>
</tr>
<tr>
<td></td>
<td>&lt; 3.2</td>
<td>8.6 (8/93)</td>
<td>7.7</td>
<td>2.0</td>
<td>22.0</td>
<td>91.7</td>
<td>79.0</td>
<td>97.3</td>
<td>42.9</td>
<td>11.8</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>≤ 3.2</td>
<td>53.3 (50/93)</td>
<td>66.7</td>
<td>49.7</td>
<td>80.4</td>
<td>56.4</td>
<td>43.3</td>
<td>73.9</td>
<td>57.6</td>
<td>42.2</td>
</tr>
<tr>
<td></td>
<td>&lt; 3.3</td>
<td>53.3 (50/93)</td>
<td>66.7</td>
<td>49.7</td>
<td>80.4</td>
<td>56.4</td>
<td>43.3</td>
<td>73.9</td>
<td>57.6</td>
<td>42.2</td>
</tr>
<tr>
<td>RSF (IL)</td>
<td>≤ 81.9 (&lt; 2 years)</td>
<td>20.9 (8/43)</td>
<td>57.9</td>
<td>34.0</td>
<td>78.9</td>
<td>40.9</td>
<td>21.5</td>
<td>63.3</td>
<td>58.8</td>
<td>26.2</td>
</tr>
<tr>
<td></td>
<td>≤ 84.5 (≥ 2 years)</td>
<td>39.5 (17/43)</td>
<td>45.5</td>
<td>25.1</td>
<td>67.3</td>
<td>72.7</td>
<td>49.6</td>
<td>88.4</td>
<td>62.5</td>
<td>35.9</td>
</tr>
</tbody>
</table>

This table shows the performance of the iron markers to identify iron deficiency. The cut-off used are internationally accepted or, if not available, method specific cut-offs (grey boxes) as well as cut-offs newly derived from the ROC-curves (white boxes). Iron deficiency was defined as a bone marrow smear score of 0 or 1 (FINCH and Huebers, 1982; GALE et al., 1963). MCHC: mean cell haemoglobin concentration; MCV: mean corpus volume; stTR: soluble transferrin receptor; RSF: Red cell Size Factor; stTR-F index: soluble transferrin-log ferritin index. The iron markers are presented in order of size of AUCROC (Table 2).
Discussion

This is the first study to evaluate the diagnostic accuracy of the conventional peripheral iron markers and two new potential iron markers, hepcidin and RSf, against bone marrow iron levels in healthy African children. None of the markers had both a high sensitivity and specificity. Ferritin and sTfR-F were the most accurate markers in distinguishing children with bone marrow iron deficiency from children with iron repletion. Hepcidin, sTfR and MCV performed moderately, whereas RSf and MCHC poorly performed.

Ferritin

Ferritin is widely used and considered the most specific peripheral iron biomarker correlating with iron stores. The generally accepted cut-off for children under five years of age, is <12 µg/L. In this study this cut-off had poor sensitivity. Ferritin is also an acute phase protein that is elevated in response to inflammation. For this reason a raised cut-off of 30 µg/L is recommended for use in populations with high infection exposure. Applying this cut-off in the present study increased sensitivity (81.6 %), but specificity was low (37.5 %). These results contrast with earlier findings in severely anaemic Malawian children in which the cut-off <30 µg/L showed higher specificity than sensitivity. This difference may relate to the low prevalence of infections in the current study population (5.7 %), and suggests that a cut-off 30 µg/L is too high for use in an otherwise healthy African population.

For ferritin the cut-off with maximal accuracy in predicting iron deficient stores was <18 µg/L, which gave a sensitivity of 73.7% and specificity of 77.1%. In the two other paediatric African studies evaluating ferritin against bone marrow iron deficiency, higher cut-offs were derived (<50 µg/L) and <273 µg/L. This may be explained by the high prevalence of infection in the these studies. Notably the ferritin assay used by Fleming et al may not have been standardized, limiting comparability. In a western adult study comparing ferritin with bone marrow iron in healthy subjects, a cut-off of 12 µg/L was derived, whereas in hospitalized subjects a higher cut-off was required (30-70 µg/L).

The data from the present study suggests that ferritin is to one of the best iron biomarkers for detecting deficiency in healthy African children. Yet even in this healthy population the cut-off may need to be raised, although not to as high a value as 30 µg/L, as previously suggested.

Soluble transferrin receptor

sTfR measured in serum has been shown to be proportional to the mass of membrane-bound transferrin receptor (mTfR) which represents the marrow erythropoietic activity and iron need of the red cell precursor. Tissue in need of iron will induce a rise in mTfR and thus sTfR.

In this study the performance of sTfR to detect bone marrow iron deficiency was inferior to ferritin and sTfR-F. Using the method-specific reference value, sTfR appeared to be very specific but lacked sensitivity. This is in contrast to findings in severely anaemic children with multiple infections, suggesting that performance of sTfR may depend on the presence of anaemia or inflammation. This contrasts with previous studies promoting sTfR as the preferred iron biomarker in populations experiencing inflammation, but is consistent with other studies reporting interference of inflammation with sTfR.

Applying a newly derived cut-off (>2.2 µg/ml) did not improve diagnostic accuracy in the present study population. Other paediatric and adult studies comparing sTfR with bone marrow iron showed cut-offs for maximal accuracy varying from 2.0-2.8 µg/ml to 8.0-8.3 µg/ml. Apart from the differences in inflammatory status, different methodologies and lack of standardization of immunoassays for sTfR seriously limit comparability.
**Chapter seven | Peripheral blood iron markers compared against bone marrow**

**sTfR-log ferritin index**
As serum ferritin reflects iron stores, and sTfR is assumed to vary with the degree of tissue iron, the sTfR-log ferritin index (sTfR-F) has been suggested to be a complete estimate of the iron level of the individual 46. In this population the sTfR-F was one of the best predictors of bone marrow iron deficiency, although it was not better than ferritin alone. This differs from results from populations with a high prevalence of infections 28, 31, in which the sTfR-F index was a more accurate marker than ferritin, but is comparable to findings in African hospitalized adults whereby the AUROC of ferritin (0.94) was larger than the AUC of sTfR-F (0.84) (significance of difference not reported) 44.

The preferred cut-off for maximal accuracy is difficult to establish across studies due to the variable interference of inflammation as well as the lack of standardization of the sTfR assays are 33, 41.

In the present study the determination of sTfR, either alone or using the sTfR ratio, does not appear to have added value over the single marker ferritin for the diagnosis of iron deficiency in healthy African children, although the combination have added value in populations with high infection exposure.

**Hepcidin**
Hepcidin inhibits iron absorption in iron loaded conditions and enhances absorption in iron deficiency 37-49. In the present study hepcidin was a less accurate predictor of bone marrow iron deficiency compared to other iron biomarkers. This is the first study to compare serum hepcidin levels against bone marrow iron assessment in African children. Other studies evaluating hepcidin as a marker of iron deficiency showed better results, although comparison was with other peripheral biomarkers. Van Santen et al described hepcidin as a potential marker for differentiation of iron deficiency anaemia from anaemia of chronic disease in rheumatoid arthritic patients 50, using a reference combination of peripheral iron markers. Pasricha et al also proposed serum hepcidin as a useful marker for iron deficiency using ferritin and sTfR-index as their reference standards 51.

The sub-optimal performance of hepcidin as a marker of bone marrow iron deficiency in our study could result from other factors than iron concentration influencing hepcidin release. These factors may be stimulatory (inflammation) 47, 48, 52, 53 or suppressive (hypoxia and erythropoietic drive) 54-57, both of which probably reduce sensitivity or specificity. Anaemia, a suppressor of hepcidin, was highly prevalent in these children and although acute infection (stimulating hepcidin) was infrequent, nevertheless low grade or chronic inflammation may have influenced hepcidin production 58. Its poor performance may be also explained by the large number of the children with hepcidin values below the minimal detection limit (0.5 nmol/L). A more sensitive test for hepcidin may show an improvement of the ROC-curve characteristics. In this setting hepcidin was not an optimal peripheral marker to diagnose bone marrow iron deficiency.

However as a key regulator of iron, hepcidin may provide additional value to ferritin for evaluation of the effects and safety of iron supplementation, as shown in Gambian children in whom hepcidin strongly predicted red blood cell iron incorporation after oral iron supplementation 59. Although contrasting results have been reported 60.

**Red cell Size factor**
The reticulocyte haemoglobin concentration has been shown to be a useful screening tool for iron deficiency in young children80, 62, and it can adequately detected bone marrow iron deficiency in adults63. The reticulocyte haemoglobin concentration assessed on the ADVIA analyzer of Siemens (named CHr), on a Sysmex analyzer (named Ret-He) or Beckman analyzer (named R5f) showed equivalent results24, 64.
In this study RSF poorly predicted bone marrow iron deficiency. As the reticulocyte haemoglobin content is a sensitive marker for recent iron restricted erythropoiesis, then its performance may be limited in the present study in which children may be chronically iron deficient. Nutritional vitamin B12 deficiency, which is common in Malawi or folate deficiency, both influence the volume of the red blood cell and reticulocyte, and may interfere with RSF values. Although age could be a confounding factor, as MCV is generally higher in young children, adjustment in a multivariate logistic regression model did not change the correlation between RSF and bone marrow iron deficiency (data not shown).

The reticulocyte haemoglobin content does not appear a reliable marker to detect iron deficiency in this population of (chronically) iron deficient children.

Other iron markers
The red cell size indices MCV and MCHC, both often used when biochemical markers are not accessible, were inferior to ferritin in detecting bone marrow iron deficiency. This is consistent with current knowledge and may relate to interfering factors such as other nutritional deficiencies (vitamin B12 and folate deficiency) and/or inflammation.

Considerations and limitations

Bone marrow iron examination
Bone marrow iron smear was used as the reference for the diagnosis of deficiency of iron stores. It must be noted that variation in results can appear due to the uneven distribution of iron in the bone marrow. Nevertheless bone marrow examination has generally been considered the most reliable diagnostic test for iron status.

Methodology
For clinical purposes, the major emphasis in laboratory measurements of iron status is detecting iron deficiency rather than in estimating its severity. The cut-off for maximal accuracy to detect iron deficient stores is therefore important. However, some iron biomarkers lack standardization due to the absence of a suitable international calibrator. As a consequence results differ between methodologies, and cut-offs may be difficult to compare.

Implications
Even in this 'healthy' population of African children no single marker showed both high sensitivity and specificity. The frequent reason for poor performance of iron markers: infection cannot fully explain the limited performance of iron biomarkers in populations under high infection pressure.

Other factors including anaemia, previous infections, age, co-morbidities and the chronicity of the deficiency could influence different iron markers and this underlines the complexity of iron metabolism in children living in these settings. Depending on the purpose of diagnosing iron deficiency our data can be used to select diagnostic tests.

To estimate prevalence of iron deficiency a sensitive marker such as ferritin (cut-off <30 µg/L) could be used. Since one would like to avoid underestimation rather than overestimation, and the NPV is more important than the PPV. Yet for research purposes a high accuracy (optimal sensitivity and specificity) would be preferred to avoid classification bias (ferritin cut-off <18 µg/L).
Chapter seven | Peripheral blood iron markers compared against bone marrow

For ‘interventional purposes’ other aspects appear to be more relevant than identifying the iron deficient population, for which this study cross-sectional was not designed. For example iron supplementation programmes would be aided by a marker to predict ‘efficacy of iron supplementation’ (intestinal and erythrocyte iron uptake) and ‘safety of the intervention’, as iron supplementation may increase infection risk in certain populations. Hepcidin has been suggested to serve these purposes although further research is needed.

Conclusion

In this first study evaluating peripheral iron markers against bone marrow iron deficiency in healthy African children ferritin and sTfR-F best predicted bone marrow iron status and should be used in epidemiologic studies and for research purposes. Since none of the markers had both a high sensitivity and specificity, there is an urgent need for a more reliable peripheral iron marker in African children.

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Chapter seven | Peripheral blood iron markers compared against bone marrow

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