

1 **Hepcidin and conventional markers to detect iron deficiency in severely**  
2 **anaemic HIV-infected patients in Malawi.**

3

4 *Short title: Difficulties in detection of iron deficiency in severely anaemic HIV-*  
5 *infected patients in Malawi.*

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35 **Abstract**

36

37 **Introduction:** Iron deficiency is a treatable cause of severe anaemia in low-and-middle-  
38 income-countries (LMIC). Diagnosing it remains challenging as peripheral blood markers  
39 poorly reflect bone-marrow iron deficiency (BM-ID), especially in the context of HIV-  
40 infection.

41 **Methods:** Severe anaemic (haemoglobin  $\leq 70$ g/l) HIV-infected adults were recruited at  
42 Queen Elizabeth Central Hospital, Blantyre, Malawi. BM-ID was evaluated. Accuracy of  
43 blood markers including hepcidin alongside mean corpuscular volume, mean cellular  
44 haemoglobin concentration, serum iron, serum ferritin, soluble transferrin receptor  
45 (sTfR), sTfR -index, sTfR -ratio to detect BM-ID was valued by ROC area under the curve  
46 ( $AUC^{ROC}$ ).

47 **Results:** Seventy-three patients were enrolled and 35 (48.0%) had BM-ID. Hepcidin and  
48 MCV performed best;  $AUC^{ROC}$  of 0.593 and 0.545. Other markers performed poorly  
49 ( $ROC < 0.5$ ). The  $AUC^{ROC}$  of hepcidin in males was 0.767 (sensitivity 80%, specificity 78%)  
50 and in women 0.490 (sensitivity 60%, specificity 61%).

51 **Conclusion:** BM-ID deficiency was common in severely anaemic HIV-infected patients  
52 and is an important and potential treatable contributor to severe anaemia. Hepcidin was  
53 the best, though still suboptimal, marker of BM-ID. Hepcidin, which is directly linked to  
54 iron absorption, is a very promising marker to guide curative iron supplementation  
55 policies in severely anaemic HIV-infected patients.

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57

## 59 Introduction

60 Anaemia affects approximately a third of the world's population and substantially reduces the  
61 disability- adjusted life years worldwide (1). Iron deficiency contributes to development of  
62 anaemia and is diagnosed in more than half of all anaemic persons (2). Consequently, iron  
63 supplements remain the backbone of prevention and treatment protocols for anaemia.

64 Anaemia has an extensive list of potential causes. In sub-Saharan Africa, where this  
65 condition is most common, its aetiology is even more complex and in these setting  
66 aetiologies commonly co-occur requiring a multifactorial approach (3, 4). HIV may be the  
67 cause of anaemia by its direct effect on BM cells, but can also increase the range of  
68 aetiological factors to encompass opportunistic viral, bacterial and parasitic infections,  
69 drugs such as Zidovudine and co-trimoxazole, micronutrient deficiencies and neoplastic  
70 diseases (5, 6).

71 The exact role of iron deficiency, one of the few potentially preventable and  
72 treatable causes of anaemia, remains unclear due to its diagnostic challenges in HIV-  
73 infected patients in low resource settings (3, 4, 7). Peripheral blood markers, including  
74 erythrocyte indices, serum iron, ferritin, and soluble transferrin receptor (sTfR), have  
75 been evaluated but their accuracy is often negatively affected by inflammatory states and  
76 renal and liver conditions, which are common in both the African and HIV-infected  
77 populations (8-11). Previous studies therefore concluded that the uses of peripheral  
78 blood markers, such as ferritin, are not reliable without correction for inflammation (12).  
79 The evaluation of iron in the bone marrow is considered the 'gold standard' to diagnose  
80 iron deficiency, but bone marrow sampling is invasive and requires skilled staff for  
81 sampling and interpretation, which is challenging in low resource settings. Moreover, for  
82 large-scale use, a reliable peripheral blood marker is needed to replace bone-marrow  
83 biopsy to predict bone marrow iron deficiency (BM-ID).

84 Hepcidin is a relatively new marker, which regulates iron absorption from the  
85 gastrointestinal tract and iron release from stores, both of which are important pathways  
86 controlling the availability of iron for incorporation in the erythrocyte precursors (13).  
87 Increases of iron plasma levels stimulate the production of hepcidin, which blocks further  
88 iron absorption from the gastrointestinal tract and iron release from storage. However,  
89 hepcidin is also an acute phase protein and serum levels increases during infections (14).

90 We investigated the prevalence of BM-ID in HIV-infected Malawian adult patients  
91 with severe anaemia. We further evaluated the accuracy of peripheral blood markers, as  
92 well of hepcidin to identify BM-ID in this population.

93

94

## 95 **Methods**

96 From February 2010 to March 2011, all adults admitted to the Department of Internal  
97 Medicine of the Queen Elizabeth Central Hospital (QECH), Blantyre, Malawi with a  
98 diagnosis of severe anaemia and HIV infection were approached for informed consent  
99 and study enrolment. This study is a sub-study of the larger observational cohort study  
100 (n=199) concerning severely anaemic (haemoglobin  $\leq$  70 g/l) HIV-infected patients. Bone  
101 marrow sampling was performed if the patient consented and the patient was clinically  
102 stable. Of the 199 included patients, 73 BM (37%) samples were included in this sub-study  
103 as the BM sampling was performed and the quality of the sample taken was appropriate.

104

### 105 **Methods| Laboratory assays and blood markers**

106 Haemoglobin concentration was measured on admission using the HemoCue B-  
107 Haemoglobin analyser (HemoCue, Ängelholm, Sweden) to screen patients for eligibility.  
108 After informed consent a venous blood sample was collected and bone marrow sample  
109 taken from the iliac crest. All blood samples were analysed within 24 hours of collection  
110 or stored at  $-80^{\circ}\text{C}$ . Haemoglobin and red cell indices (MCV, MCH and MCHC) were  
111 determined using an automated haematology analyser (Beckman Coulter, Durban, South  
112 Africa). CD4-cell counts were assessed using BD FACS Count (BD Biosciences, San Jose,  
113 CA, USA). Transferrin, iron, ferritin, folate and vitamin B12 were analysed on Modular P800  
114 and Monular Analytic E170 systems (Roche Diagnostics, Switzerland). Soluble transferrin  
115 receptor (sTfR) levels were measured using ELISA (Ramco Laboratories, TX, USA).  
116 Commonly used ratios to define iron deficiency were calculated including the sTfR-index:  
117 sTfR (mg/L) divided by log ferritin (ug/L); and the 'sTfR ratio': sTfR (mg/L) x1000/ ferritin  
118 (ug/L) level (15). International accepted cut-offs were applied. For sTfR we used 2.75 mg/l  
119 and 3.6 mg/l and for the sTfR-index: 1.8 and 2.2, 2.8 respectively, as no international cut-  
120 offs have been defined, these represented the most recent consensus (16).

121 Serum hepcidin-25 measurements were performed in December 2012/January  
122 2013 (Testing lab: Hepcidinanalysis.com, Nijmegen, The Netherlands) by a combination of  
123 weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF  
124 MS) using synthetic hepcidin-24 as internal standard (17-19). Peptide spectra were  
125 generated on a Microflex LT matrix-enhanced laser desorption/ionisation TOF MS  
126 platform (Bruker Daltonics, Bremen, Germany). Hepcidin concentrations are expressed as  
127 nanomoles per litre (nmol/L). The lower limit of detection of this method was 0.5 nmol/L  
128 (18).

129

130

131 Methods| **BM-ID**

132 Aspirate samples were spread onto slides and trephine biopsies were fixed, decalcified  
133 and embedded in paraffin wax (20, 21). Bone marrow samples were sent to the  
134 Haematology- Pathology Referral Centre at the Royal Liverpool University Hospital,  
135 Liverpool UK, for analysis. Sections of the trephine blocks were stained with Perls'  
136 Prussian Blue stain to detect iron stores(21). Intracellular iron in bone marrow trephine  
137 blocks was graded using the Stuart-Smith scale, which classifies the iron content of bone  
138 marrow into six grades (0–6). For bone marrow smears, iron was graded using Gale's  
139 grading (0-4). Iron deficiency was defined as no visible or severe reduced iron particles in  
140 a few reticulum cells under high power magnification; grade 0-1 on both scales (22, 23).

141

142 Methods| **Infections**

143 HIV infection was confirmed using two point-of-care antibody tests (Unigold® and  
144 Determine®). Different types and severity of on-going infections were evaluated.  
145 Including; HIV: CD4 counts  $\leq 200$  cells/mm<sup>3</sup> and/or viral load >1000 copies/ml. Malaria:  
146 presence of malaria parasites in a thick blood film assessed by light microscopy.  
147 Tuberculosis (TB) defined as one or more of the following: a) positive sputum culture; b)  
148 chest X-ray with signs of pulmonary tuberculosis and/or; c) on-going TB treatment at time  
149 of enrolment; d) clinical diagnosis based on generalized lymphadenopathy and/or night  
150 sweats > 30 days with unknown origin; e) caseating granulomata in the bone marrow  
151 trephine. Bacteraemia was defined as blood cultures growing potential pathogen  
152 including streptococcus, enterococcus and micrococcus species, non-Typhoid Salmonella  
153 and Klebsiella pneumonia. Furthermore, viral infections including Parvo-B19,  
154 Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) were evaluated by PCR and defined  
155 as positive by viral load >100 copies/ml.

156

157 Methods| **Ethics**

158 The Research Ethics Committee of the College of Medicine, University of Malawi  
159 (P.09.09.824) and the Research Ethics Committee of Liverpool School of Tropical Medicine  
160 (research protocol 09.64) approved the study. The purpose of the study was explained to  
161 the patients in the local language (Chichewa), and written informed consent was obtained  
162 before inclusion into the study.

163

164 Methods| **Statistics**

165 The data were analysed using Stata (version 12) (STATA Corp. LP, Texas, TX, USA).  
166 Baseline characteristics were compared between BM-ID and non-deficient patients using

167 Chi-square test (dichotomous data) or t-test (continuous) or Pearson Chi-square test  
168 (continuous not normally distributed). Confounding was enhanced to evaluate hepcidin  
169 concentrations, gender, HIV disease progression, the use of ART as baseline, and TB  
170 infection (Pearson Chi-square test). The p-values reported are two-sided, and a level of  
171  $p < 0.05$  was interpreted as significant.

172 The accuracy of the different peripheral blood markers, including hepcidin, to  
173 discriminate BM-ID were evaluated by receiver operating characteristics curves  
174 (ROC)(24). Corresponding areas under the curve ( $AUC^{ROC}$ ) were created.  $AUC^{ROC}$   
175 measures the two-dimensional area underneath the ROC curve and provides a summative  
176 measure of performance across all possible classification thresholds (25). The  $AUC^{ROC}$   
177  $< 0.70$  is weighed as low diagnostic;  $AUC^{ROC}$  of  $0.70-0.90$  as moderate diagnostic and a  
178  $AUC^{ROC} \geq 0.90$ , high diagnostic accuracy (26). Sensitivity and specificity were calculated for  
179 predefined internationally accepted cut-offs (8, 15, 16, 27). For hepcidin the best cut-off  
180 value for diagnosing BM-ID were determined using ROC-curve analyses with the  
181 Youden index (maximum (sensitivity + specificity - 1))(25). As gender differences are  
182 known for hepcidin (30) hepcidin outcome was hence evaluated by gender.

183

## 184 **Results**

185

186 Of the 73 HIV-infected adults in our sub-study, a total of 45 (61.6%) had severe anaemia  
187 (Hb  $50-70$ g/dL) and 28 (38.4 %) had very severe anaemia (Hb  $< 50$ g/dL). The mean patient  
188 age was 33.7 (SD 8.7) years, and 43 (58.9%) patients were female. A CD4 count  $\leq 200$   
189 cells/mm<sup>3</sup> was seen in 31/56 (55.4%) and a viral load  $> 1000$  copies/ml was present in 57/76  
190 (75.0%) of patients. A total of 34/73 (46.6%) patients had been started on anti-retroviral ART  
191 treatment, of which most were on first line treatment at time of the study (Efavirenz,  
192 Lamivudine, Tenofovir). The most common infections in this population were  
193 tuberculosis (39/73; 53.4%) and EBV (30/45; 66.7%). All baseline characteristics are shown in  
194 table 1.

195

### 196 **Results| BMI-ID and blood markers**

197 BM-ID was seen among 35 (48.0%) of the patients, table 1. The performances of the  
198 peripheral blood markers to diagnose BM-ID are displayed in table 2. All markers  
199 displayed low diagnostic accuracy ( $AUC^{ROC} < 0.7$ ). MCV had the highest  $AUC^{ROC}$  value of  
200 the common peripheral blood markers (0.545), the sensitivity and specificity using the  
201 common cut off of 83fL were 42% and respectively 67%. The use of hepcidin to detect  
202 BM-ID resulted in an  $AUC^{ROC}$  0.593. We stratified the analysis for hepcidin according to  
203 gender; the  $AUC^{ROC}$  for men and women was 0.767 and 0.490 respectively. The optimal

204 hepcidin concentration for the detection of BM-ID was  $\leq 7$  ng/ml; sensitivity 67% &  
 205 specificity 67%. In males the optimum cut off was  $\leq 6$  ng/ml (sensitivity 80%; specificity  
 206 78%); whilst for women this was  $\leq 7$  ng/ml (sensitivity 60%; specificity 61%, figure 1). The  
 207 hepcidin concentration did not differ significantly by gender ( $p=0.831$ ), HIV disease  
 208 progression ( $p=0.819$ ), the use of ART at enrolment ( $p=0.616$ ), and TB infection ( $p=0.590$ ) in  
 209 a univariate analysis.

210  
 211 **Figure 1. Hepcidin (nmol/L) ROC curve by gender with optimal cut-off.** The best cut-off  
 212 value for diagnosing BM-ID was determined by the Youden index (maximum  
 213 (sensitivity + specificity – 1)) in the ROC-curve (25). Abbreviations: AUC<sup>ROC</sup>: area under  
 214 curve of receiver operating characteristic.

215  
 216

Characteristic	Overall	Non BM-ID	BM-ID	P-value
<b>BM-ID</b>	<b>35/73 (48.0%)</b>	<b>38/73 (52.1%)</b>	<b>35/73 (48.0%)</b>	
Age, years (mean, SD)	33.7 (8.7)	32.7 (8.6)	34.7 (8.9)	0.331
Gender (female) (%)	43/73 (58.9%)	19/38 (50.0%)	24/35 (68.6%)	0.107
<b>Haematology &amp; iron markers</b>				
Very severe anaemia (Hb $\leq$ 50g/l) (%)	28/73 (38.4%)	13/38 (34.2%)	15/35 (42.9%)	0.448
Haemoglobin (Hb)(g/l), (median, IQR)	56.0 (43.0-63.0)	58.5 (45.0-64.0)	54.0 (36.0-63.0)	0.136
MCV (fl), (median, IQR)	85.8 (79.4-98.1)	87.3 (79.6-99.0)	83.5 (79.1-94.7)	0.534
MCH (pg/cells), (mean, SD)	29.0 (5.9)	23.6 (6.1)	26.3 (5.5)	0.084
Serum iron (umol/l), (median, IQR)	5.1 (3.3-11.1)	4.7 (3.0-7.9)	5.6 (3.8-22.2)	0.053
Ferritin (ug/dL), (median, IQR)	87.2 (49.6-100.0)	87.1 (50.1-97.1)	87.9 (36.0-100.0)	0.488
sTfR receptor (mg/l), (median, IQR)	2.9 (1.6-3.7)	2.8 (1.7-3.7)	3.0 (1.2-3.9)	0.934
sTfR index (median, IQR)	1.6 (0.8-2.2)	1.5 (0.9-2.1)	1.6 (0.6-2.6)	0.901
sTfR Ratio (median, IQR)	35.1 (17.5-69.3)	33.2 (21.3-61.9)	36.6 (11.5-79.6)	0.747
Hepcidin (ng/ml) (median, IQR)	7.3 (3.3-13.3)	9.2 (4.9-13.2)	5.1 (3.1-13.7)	0.196
<b>HIV disease and treatment</b>				
ART at enrolment (%)	34/73 (46.6%)	20/38 (52.6%)	14/35 (40.0%)	0.280
CD4 count	31/56 (55.4%)	15/28 (53.6%)	14/25(56.0%)	0.859

≤ 200 cells/mm <sup>3</sup>				
Viral load >1000 copies/ml	57/76 (75.0%)	25/38 (65.8%)	30/35 (85.7%)	0.048
<b>Infection(s)</b>				
Bacteraemia <sup>3</sup>	12/73 (16.4%)	6/38 (15.8%)	6/35 (17.1%)	0.876
Malaria <sup>4</sup>	3/63 (4.7%)	2/32 (6.3%)	1/31 (3.2%)	0.573
Tuberculosis <sup>5</sup>	39/73 (53.4%)	20/38 (52.6%)	19/35 (54.3%)	0.877
Epstein-Barr virus <sup>6</sup>	30/45 (66.7%)	19/26 (73.1%)	11/19 (57.9%)	0.286
Cytomegalo virus <sup>6</sup>	18/54 (33.3%)	11/28 (39.3%)	7/26 (26.9%)	0.336
Parvo-B19 virus <sup>6</sup>	1/59 (1.7%)	0/27 (-)	1/32 (3.1%)	0.230
<b>Nutritional status</b>				
Underweight (BMI < 18.5) (%)	22/49 (44.9%)	8/24 (33.3%)	14/25 (56.0%)	0.111

217

218 **Table 1. Baseline characteristics in this population of severely anaemic HIV patients stratified**  
 219 **according to bone marrow iron deficiency (BM-ID).** Abbreviations: ART: antiretroviral  
 220 therapy. BMI: Body mass index. TB: Tuberculosis. <sup>1</sup> First line ART include combination of  
 221 Stavudine (d4T), Lamivudine (3Tc) and Nevirapine (NVP) (28). <sup>2</sup> Advanced HIV disease  
 222 including a CD4 count ≤ 200 cells/mm<sup>3</sup> and/or viral load > 1000 copies/ml. <sup>3</sup>Bacteraemia; a  
 223 blood culture with clean growing potential pathogen including streptococcus (41.7%;  
 224 5/12), enterococcus (16.7%;2/12) and non-Typhoid Salmonella (16.7%;2/12). <sup>4</sup>Malaria:  
 225 presence of malaria parasites on a thick blood film. <sup>5</sup>Tuberculosis (TB): one or more of  
 226 the following present: a) positive sputum culture, b) chest X-ray with signs of pulmonary  
 227 tuberculosis and/or c) on-going TB treatment at time of enrolment d) clinical diagnosis by  
 228 local doctor including unknown generalized lymphadenopathy and/or night sweats > 30  
 229 days with unknown origin e) caseating granulomata in the bone marrow trephine. <sup>6</sup>  
 230 Epstein-Barr, cytomegalo- and parvo-B19 virus infection are diagnosed by a virus load of  
 231 1000 copies/ml. Abbreviations: MCV; mean cellular volume, MCH; mean corpuscular  
 232 haemoglobin, s-TfR: Soluble transferrin receptor, TfR-index (sTfR(mg/L) /Log  
 233 ferritin(ug/L)), TfR Ratio (sTrR(mg/L))x1000/ferritin(ug/L)).



Potential markers	AUC <sup>ROC</sup>	95%-CI	Cut-off	Sensitivity	95%-CI	Specificity	95%-CI
MCV (fl) <sup>1</sup>	0.545	0.404-0.685	≤83	42%	25.2-58.8%	67%	51.0-83.0%
MCH (pg/cells) <sup>1</sup>	0.365	0.230-0.499	≤27	52%	35.2-69.0%	29%	13.7-44.3%
Serum iron (μmol/l) <sup>1</sup>	0.368	0.239-0.498	≤ 10	60%	43.8-76.2%	18%	5.8-30.2%
Ferritin (μg/l) <sup>1</sup>	0.441	0.293-0.588	≤30	13%	1.0-25.0%	88%	76.7-99.3%
			≤70	30%	13.6-46.4%	66%	49.6-82.4%
sTfR receptor (mg/l) <sup>3</sup>	0.522	0.378-0.667	≥1.8	71%	55.0-87.0%	29%	13.7-44.3%
			≥2.8	58%	40.6-73.1%	56%	39.3-72.7%
			≥3.6	32%	15.6-48.2%	76%	61.6-99.4%
			≥8.0	3%	0-9.0%	100%	-
sTfR index <sup>4</sup>	0.523	0.375-0.672	≥1.8	47%	29.1-64.9%	67%	46.3-79.7%
			≥2.2	27%	11.1-42.9%	75%	60.0-90.0%
			≥2.8	20%	5.7-34.3%	81%	67.4-94.6%
			≥3.5	10%	0-20.7%	97%	75.1-86.9%
sTfR Ratio <sup>4</sup>	0.508	0.359-0.656	≥100	17%	0-23.4%	91%	71.1-90.9%

236 **Table 2. Accuracy of peripheral blood markers to detect bone marrow iron deficiency (gold standard).** Abbreviations: AUC: area under curve of  
237 receiver operating characteristic (ROC), where 0.5 would be expected by chance and 1 denotes a perfect test. 95%-CI: 95% confidence interval.  
238 MCV; mean cellular volume, MCH; mean corpuscular haemoglobin, sTfR: Soluble transferrin receptor, sTfR-index (sTfR(mg/L) /Log  
239 ferritin(ug/L)),

240 sTfR Ratio (sTrR(mg/L))x1000/ferritin(ug/L)).<sup>1</sup> (29) <sup>2</sup> (11) <sup>3</sup> (29) <sup>4</sup>(15, 16)

## 241 Discussion

242

243 In this study on hepcidin and conventional markers to detect BM-ID in severely anaemic HIV-  
244 infected patients in Malawi, we found that BM-ID was present in almost half of our patients. In  
245 the first study evaluating hepcidin as a marker for BM-ID among severely anaemic HIV-infected  
246 adults in such a setting we found that hepcidin had the highest AUC<sup>ROC</sup> 0.593 amongst all  
247 peripheral markers and therefore was the best marker to use for detecting BM-ID. MCV was  
248 found to be the best conventional peripheral blood marker for BM-ID. As the MCV is  
249 commonly provided as part of routine full blood counts this marker may be of some use in  
250 resource-limited settings.

251 BM-ID was highly prevalent among our population of HIV-infected and severely  
252 anaemic patients. The prevalence of BM-ID in this study is higher than previous reports on  
253 similar populations that were published in the pre-ART era when BM-ID was reported to be  
254 18%-25% in severely anaemic HIV patients (8, 30). The increased prevalence of BM-ID may be  
255 explained by the effect of ART. Previous reports on HIV-associated anaemia before 2010  
256 included patients who were mostly ART naïve and advanced HIV disease and/or severe immune  
257 suppression were common (8). Although VL above 1000 copies/ml (75%) and CD4 counts below  
258 200 cells/ml were (55.4%) still common in the presenting population, median CD4 counts (325  
259 cells/mm<sup>3</sup>) were much higher than in the previous reports (median 67 cells/ml) (4, 8). Initiation  
260 of ART aims to stop HIV disease progression, promote immune reconstitution and reduce the  
261 risk of (opportunistic) diseases. This may also be reflected in our cohort, and has likely changed  
262 the aetiology of severe anaemia among HIV-infected patients as compared to older studies. In  
263 this group of HIV-infected patients with better immune systems the aetiology of severely  
264 anaemic may be more similar to the aetiology of non-HIV infected patients. The BM-ID  
265 prevalence of 48% is comparable to previous findings among HIV-uninfected African  
266 populations with severe anaemia, which supports this hypothesis (8). Our data need to be  
267 confirmed in the on-going 'treat all' era but they could have great impact on preventive and  
268 curative policies concerning iron supplementation in severely anaemic HIV-infected patients.  
269 Irrespective of the cause, the role of iron supplementation to prevent and treat severe anaemia  
270 appears to have gained importance.

271 Our results concerning the accuracy of peripheral blood markers to detect BM iron  
272 deficiency indicate that it is not easy to reliably detect those with deficient BM iron stores,  
273 which corroborates with previous studies (8, 9). Hepcidin, a specific hormone in metabolising  
274 iron, did perform slightly better than conventional markers, but remained far from good or  
275 even perfect. Additionally, hepcidin is a key player in the absorption of iron and thus may be  
276 used to not only select those needing iron but also may predict iron supplementation,  
277 response, safety and timing. Hepcidin as a possible marker for BM-ID, has not been evaluated

278 before in this population of severely anaemic HIV-infected adults in Africa. It is not surprising  
279 that hepcidin remains from prefect as a marker for BM-ID as we know that hepcidin levels are  
280 also affected by inflammation which is highly present among HIV-infected patients, especially  
281 living in resource-limited settings (31-33). For example, when hepcidin levels are high, the  
282 absorption of dietary iron and release of macrophage iron to serum are blocked as protection,  
283 resulting in a relative hypoferremia and an increase iron into the macrophages, which is  
284 thought to be anti-infective. Consequently during malaria or TB infection or immune deficiency  
285 with low CD4 counts, hepcidin levels are increased (32, 33). Among children in Malawi,  
286 including children with HIV, our group previously reported low hepcidin levels (34). Further,  
287 hepcidin was suggested as a possible useful marker in guiding iron therapy in severely anaemic  
288 children, as low hepcidin levels were related toward a diminished expected up regulation of  
289 hepcidin by inflammation and iron deficiency due to an increase of erythropoietin in this  
290 population (34). Our data on hepcidin and (standardized) identified cut-offs are highly likely to  
291 be relevant as there is a need for a reliable marker to define BM-ID and to start iron  
292 supplementation among HIV-infected patients in resource limited settings such as Malawi.  
293 Intervention studies, using hepcidin as a marker, should be performed to assess feasibility and  
294 effect of such an intervention.

295         Worldwide hepcidin concentrations are measured by various methods, which differ  
296 considerably in absolute hepcidin concentrations (35). Recently, secondary hepcidin reference  
297 material, that has been value assigned by a primary reference material, has become available  
298 (36). Standardization in February 2019 of a similar hepcidin assay as we used in 2012/2013 for our  
299 study, resulted in (only) 5.4 % increase of hepcidin concentrations (C. Laarakker and D.  
300 Swinkels unpublished data) (37). For this specific patient population, our study thus provides a  
301 first and rough estimate for cut off point that are universally applicable by other assays that they  
302 are standardized using this same reference material. However, for formal universal use of these  
303 cut-off points these values should be confirmed by studies that directly measure samples with a  
304 standardized hepcidin method. Additionally, hepcidin optimal cut-offs were more sensitive and  
305 lower for man in comparison to woman. A direct clarification for this effect is challenging. Age  
306 and gender differences in hepcidin concentrations are known. Within the mean age group of  
307 our study population, 30-35 years of age, (normal) hepcidin concentrations were reported to be  
308 higher on man than woman (36, 38). However reference data available is coming from Europe  
309 and no hepcidin reference levels are known for an African population. Therefore a direct  
310 comparison is challenging. At last an explanation(s) can be found in levels of infection or  
311 control of the HIV disease, which in our population was not different for woman and man (data  
312 not shown). Outcome should be formally confirmed with studies that directly measure with the  
313 standardized hepcidin method to enhance confirmed hepcidin cut-offs in this specific patient  
314 population.

315 Iron deficiency is treatable and preventable, however supplementation has been  
316 associated with an increased number of severe infections, including an increase in malaria,  
317 which, especially in an immune compromised population, may be dangerous (39, 40). Therefore  
318 a reliable diagnosis of iron deficiency is important, as supplementation will put the patient at  
319 risk. Our study underlined that the current used and known peripheral blood markers  
320 performed poorly. For several decades clinicians and researchers have evaluated peripheral  
321 markers to diagnose BM-ID in laboratory resource limiting settings like Malawi and reported  
322 poor performance which is commonly considered to be caused by inflammatory conditions  
323 which are common in African and especially in HIV infected patients (8, 9). Some of the markers  
324 tested, such as sTfR concentrations, alone or in combination with other markers, were designed  
325 to better reflect iron stores (41) irrespective of inflammation (42). These markers did not  
326 perform well in this study either. Potential explanations for the poor performances of sTfR in  
327 our population may be the lack of clear cut-offs, as suggested by other studies (8) and the fact  
328 that sTfR is also influenced by erythropoietin, which may play an (even more) important role in  
329 severe HIV-associated anaemia (32). The best performing conventional peripheral blood  
330 marker was MCV. Microcytosis is commonly used as a screening test for deficiency (27, 43);  
331 however, MCV was never found to be an accurate predictor of BM-ID (8, 9, 44). Although MCV  
332 did not have a high sensitivity or specificity and the  $AUC^{ROC}$  was of low diagnostic value, it  
333 remains of some use in this population as it was the best available common marker and it is  
334 relatively easily available as part of automated full blood counts.

335 Our study has several shortcomings. Firstly, bone marrow testing was only performed in  
336 a subset of patients, which may have introduced a sampling bias. Reasons for not taking bone  
337 marrow included a severe clinical condition of the patient or patients not consenting to this  
338 aspect of the study. However, it is one of the largest studies with BM results to date. Secondly,  
339 our study was performed in 2010 when antiretroviral treatment (ART) was provided according to  
340 the national and hospital guidelines. Accordingly, ART could only be started in the outpatient  
341 ART clinic after discharge from hospital. Currently ART is started much earlier in the course of  
342 HIV infection so our study patients are likely to have had more advance disease than current  
343 patients. Nevertheless, this is the first study combining bone marrow data with a large set of  
344 peripheral blood markers, including hepcidin, in a group of HIV-infected severely anaemic  
345 African patients. We believe our study provides important information, which is valuable for  
346 clinicians who care for HIV-infected persons with severe anaemia in Malawi and other  
347 resource-limited settings.

348

349 **Conclusion**

350

351 Bone marrow iron deficiency was present in almost half of severely anaemic HIV-infected  
352 adults. This, substantial increase compared to data from the pre-ART era, underline the  
353 potential importance of preventive and therapeutic role of iron supplementation to reduce the  
354 problem of severe anaemia in HIV-infected patients. Detection and safe treatment of BM-ID is  
355 hampered by a lack of peripheral iron markers. Hepcidin was found to be the most accurate  
356 marker and could be used to guide and predict the effect of iron supplementation. Although  
357 hepcidin evaluations are not routinely available in settings such as Malawi, our study findings  
358 are important as hepcidin could guide and predict the effect of safe iron supplementation. This  
359 is important because of the potential risk of increased infection risk due to iron  
360 supplementation, its effect and safety should be evaluated in future intervention studies.

361  
362

### 363 **Acknowledgement**

364  
365 The authors would like to thank all of the study participants, doctors, nurses and support staff  
366 of Queens Elizabeth Hospital and the Malawi-Liverpool-Wellcome centre in Blantyre for their  
367 participation and cooperation. This study was supported by the Nutricia research foundation  
368 (Project number 2017-43), The Hague, the Netherlands and the Wellcome Trust (Project number  
369 WT086559), Liverpool, United Kingdom. The funders had no role in the study design, data  
370 collection and analysis, decision to publish or preparation of the manuscript

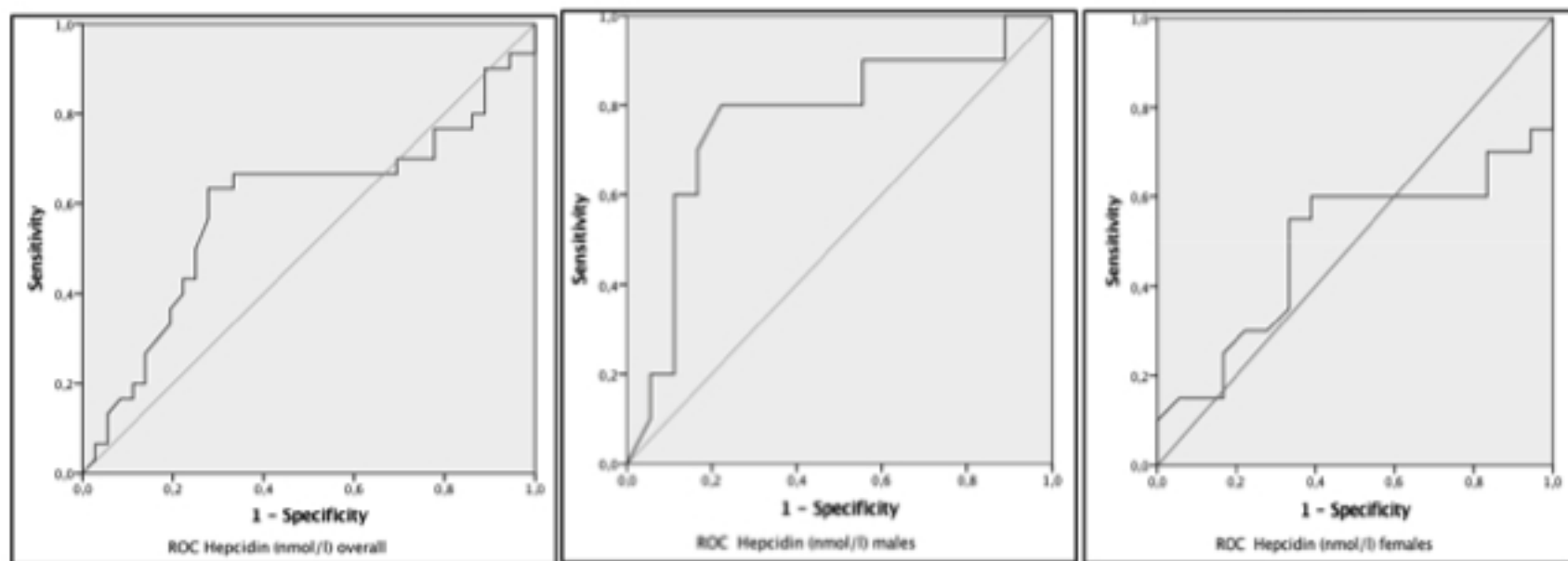
## 372 **References**

- 373 1. Kassebaum NJ, Wang H, Lopez AD, Murray CJ, Lozano R. Maternal mortality estimates -  
374 Authors' reply. *Lancet* (London, England). 2014;384(9961):2211-2.
- 375 2. Lopez A, Cacoub P, Macdougall IC, Peyrin-Biroulet L. Iron deficiency anaemia. *Lancet*  
376 (London, England). 2016;387(10021):907-16.
- 377 3. Calis JC, Phiri KS, Faragher EB, Brabin BJ, Bates I, Cuevas LE, et al. Severe anemia in  
378 Malawian children. *The New England journal of medicine*. 2008;358(9):888-99.
- 379 4. Lewis DK, Whitty CJ, Walsh AL, Epino H, Broek NR, Letsky EA, et al. Treatable factors  
380 associated with severe anaemia in adults admitted to medical wards in Blantyre, Malawi, an  
381 area of high HIV seroprevalence. *Transactions of the Royal Society of Tropical Medicine and*  
382 *Hygiene*. 2005;99(8):561-7.
- 383 5. Butensky E, Kennedy CM, Lee MM, Harmatz P, Miaskowski C. Potential mechanisms for  
384 altered iron metabolism in human immunodeficiency virus disease. *The Journal of the*  
385 *Association of Nurses in AIDS Care : JANAC*. 2004;15(6):31-45.
- 386 6. Klassen MK, Lewin-Smith M, Frankel SS, Nelson AM. Pathology of human  
387 immunodeficiency virus infection: noninfectious conditions. *Annals of diagnostic pathology*.  
388 1997;1(1):57-64.
- 389 7. Marti-Carvajal AJ, Sola I, Pena-Marti GE, Comunian-Carrasco G. Treatment for anemia in  
390 people with AIDS. *The Cochrane database of systematic reviews*. 2011(10):Cd004776.
- 391 8. Lewis DK, Whitty CJ, Epino H, Letsky EA, Mukiibi JM, van den Broek NR. Interpreting  
392 tests for iron deficiency among adults in a high HIV prevalence African setting: routine tests  
393 may lead to misdiagnosis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*.  
394 2007;101(6):613-7.
- 395 9. Jonker FA, Boele van Hensbroek M, Leenstra T, Vet RJ, Brabin BJ, Maseko N, et al.  
396 Conventional and novel peripheral blood iron markers compared against bone marrow in  
397 Malawian children. *Journal of clinical pathology*. 2014;67(8):717-23.
- 398 10. Odunukwe NN, Salako LA, Okany C, Ibrahim MM. Serum ferritin and other  
399 haematological measurements in apparently healthy adults with malaria parasitaemia in  
400 Lagos, Nigeria. *Tropical medicine & international health : TM & IH*. 2000;5(8):582-6.
- 401 11. Aguilar R, Moraleda C, Quinto L, Renom M, Mussacate L, Macete E, et al. Challenges in  
402 the diagnosis of iron deficiency in children exposed to high prevalence of infections. *PloS one*.  
403 2012;7(11):e50584.
- 404 12. Nel E, Kruger HS, Baumgartner J, Faber M, Smuts CM. Differential ferritin interpretation  
405 methods that adjust for inflammation yield discrepant iron deficiency prevalence. *Maternal &*  
406 *child nutrition*. 2015;11 Suppl 4:221-8.
- 407 13. Hugman A. Hcpidin: an important new regulator of iron homeostasis. *Clin Lab*  
408 *Haematol*. 2006;28(2):75-83.
- 409 14. D'Angelo G. Role of hepcidin in the pathophysiology and diagnosis of anemia. *Blood*  
410 *research*. 2013;48(1):10-5.
- 411 15. Skikne BS, Punnonen K, Caldron PH, Bennett MT, Rehu M, Gasior GH, et al. Improved  
412 differential diagnosis of anemia of chronic disease and iron deficiency anemia: a prospective  
413 multicenter evaluation of soluble transferrin receptor and the sTfR/log ferritin index.  
414 *American journal of hematology*. 2011;86(11):923-7.
- 415 16. Suominen P, Punnonen K, Rajamaki A, Irjala K. Evaluation of new  
416 immunoenzymometric assay for measuring soluble transferrin receptor to detect iron  
417 deficiency in anemic patients. *Clinical chemistry*. 1997;43(9):1641-6.
- 418 17. Swinkels DW, Girelli D, Laarakkers C, Kroot J, Campostrini N, Kemna EH, et al. Advances  
419 in quantitative hepcidin measurements by time-of-flight mass spectrometry. *PloS one*.  
420 2008;3(7):e2706.

- 421 18. Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, Grebenchtchikov N, Pickkers P, van Ede  
422 AE, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron  
423 metabolism disorders. *Clin Chem*. 2010;56(10):1570-9.
- 424 19. Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW. Hepcidin in human iron disorders:  
425 diagnostic implications. *Clin Chem*. 2011;57(12):1650-69.
- 426 20. Bain BJ. Bone marrow aspiration. *Journal of clinical pathology*. 2001;54(9):657-63.
- 427 21. Bain BJ. Bone marrow trephine biopsy. *Journal of clinical pathology*. 2001;54(10):737-  
428 42.
- 429 22. Gale E, Torrance J, Bothwell T. The quantitative estimation of total iron stores in human  
430 bone marrow. *The Journal of clinical investigation*. 1963;42:1076-82.
- 431 23. Finch CA. The detection of iron overload. *The New England journal of medicine*.  
432 1982;307(27):1702-4.
- 433 24. Metz CE. Basic principles of ROC analysis. *Seminars in nuclear medicine*.  
434 1978;8(4):283-98.
- 435 25. Bewick V, Cheek L, Ball J. Statistics review 13: receiver operating characteristic curves.  
436 *Critical care (London, England)*. 2004;8(6):508-12.
- 437 26. JA. S. *Signal Detection Theory and ROC Analysis in Psychology and Diagnostics*  
438 *Collected Papers: Mahwah, Erlbaum; 1996. p. 94–117.*
- 439 27. Bates I. *Practical hematology. Chapter 2: reference ranges and normal values*. 2018.
- 440 28. Ministry of Health M. *Treatment of AIDS, guidelines for the use of antiretroviral*  
441 *treatment in Malawi, edition 2010. 2010(Third edition since 2008).*
- 442 29. Rimon E, Levy S, Sapir A, Gelzer G, Peled R, Ergas D, et al. Diagnosis of iron deficiency  
443 anemia in the elderly by transferrin receptor-ferritin index. *Archives of internal medicine*.  
444 2002;162(4):445-9.
- 445 30. Kagu MB, Khalil MI, Ahmed SG. Bone marrow macrophage iron stores in patients with  
446 HIV infection and AIDS-associated Kaposi's sarcoma. *African journal of medicine and medical*  
447 *sciences*. 2007;36(2):125-8.
- 448 31. Prentice AM, Doherty CP, Abrams SA, Cox SE, Atkinson SH, Verhoef H, et al. Hepcidin is  
449 the major predictor of erythrocyte iron incorporation in anemic African children. *Blood*.  
450 2012;119(8):1922-8.
- 451 32. Wisaksana R, de Mast Q, Alisjahbana B, Jusuf H, Sudjana P, Indrati AR, et al. Inverse  
452 relationship of serum hepcidin levels with CD4 cell counts in HIV-infected patients selected  
453 from an Indonesian prospective cohort study. *PloS one*. 2013;8(11):e79904.
- 454 33. de Mast Q, Syafruddin D, Keijmel S, Riekerink TO, Deky O, Asih PB, et al. Increased  
455 serum hepcidin and alterations in blood iron parameters associated with asymptomatic *P.*  
456 *falciparum* and *P. vivax* malaria. *Haematologica*. 2010;95(7):1068-74.
- 457 34. Jonker FA, Calis JC, Phiri K, Kraaijenhagen RJ, Brabin BJ, Faragher B, et al. Low hepcidin  
458 levels in severely anemic malawian children with high incidence of infectious diseases and  
459 bone marrow iron deficiency. *PloS one*. 2013;8(12):e78964.
- 460 35. van der Vorm LN, Hendriks JC, Laarakkers CM, Klaver S, Armitage AE, Bamberg A, et al.  
461 *Toward Worldwide Hepcidin Assay Harmonization: Identification of a Commutable Secondary*  
462 *Reference Material*. *Clin Chem*. 2016;62(7):993-1001.
- 463 36. Diepeveen LE, Laarakkers CMM, Martos G, Pawlak ME, Uguz FF, Verberne K, et al.  
464 *Provisional standardization of hepcidin assays: creating a traceability chain with a primary*  
465 *reference material, candidate reference method and a commutable secondary reference*  
466 *material*. *Clinical chemistry and laboratory medicine*. 2018.
- 467 37. Reference values hepcidin: [www.hepcidineanalysis.com/provided-service/reference-](http://www.hepcidineanalysis.com/provided-service/reference-values)  
468 [values](http://www.hepcidineanalysis.com/provided-service/reference-values); 2019 [
- 469 38. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, Klaver SM, Kroot JJ, van Tienoven D, et  
470 al. Serum hepcidin: reference ranges and biochemical correlates in the general population.  
471 *Blood*. 2011;117(25):e218-25.



- 472 39. Esan MO, van Hensbroek MB, Nkhoma E, Musicha C, White SA, Ter Kuile FO, et al. Iron  
473 supplementation in HIV-infected Malawian children with anemia: a double-blind, randomized,  
474 controlled trial. *Clinical infectious diseases : an official publication of the Infectious Diseases*  
475 *Society of America*. 2013;57(11):1626-34.
- 476 40. Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A, et al. Effects of  
477 routine prophylactic supplementation with iron and folic acid on admission to hospital and  
478 mortality in preschool children in a high malaria transmission setting: community-based,  
479 randomised, placebo-controlled trial. *Lancet (London, England)*. 2006;367(9505):133-43.
- 480 41. Esan MO, Jonker FA, Hensbroek MB, Calis JC, Phiri KS. Iron deficiency in children with  
481 HIV-associated anaemia: a systematic review and meta-analysis. *Transactions of the Royal*  
482 *Society of Tropical Medicine and Hygiene*. 2012;106(10):579-87.
- 483 42. Rohner F, Namaste SM, Larson LM, Addo OY, Mei Z, Suchdev PS, et al. Adjusting soluble  
484 transferrin receptor concentrations for inflammation: Biomarkers Reflecting Inflammation  
485 and Nutritional Determinants of Anemia (BRINDA) project. *The American journal of clinical*  
486 *nutrition*. 2017;106(Suppl 1):372s-82s.
- 487 43. Volberding PA, Levine AM, Dieterich D, Mildvan D, Mitsuyasu R, Saag M. Anemia in HIV  
488 infection: clinical impact and evidence-based management strategies. *Clinical infectious*  
489 *diseases : an official publication of the Infectious Diseases Society of America*.  
490 2004;38(10):1454-63.
- 491 44. Rabindrakumar MSK, Pujitha Wickramasinghe V, Gooneratne L, Arambepola C,  
492 Senanayake H, Thoradeniya T. The role of haematological indices in predicting early iron  
493 deficiency among pregnant women in an urban area of Sri Lanka. *BMC hematology*.  
494 2018;18:37.  
495



Hepcidin	AUC <sup>ROC</sup>	95% CI	Optimal hepcidin (nmol/L) cut-off for BMI-ID	Sensitivity	95% CI	Specificity	95% CI
Overall	0.593	0.447-0.739	7.0	67%	50.2-83.8	67%	51.6-82.4
Males	0.767	0.567-0.960	6.0	80%	55.2-100.0	78%	52.9-91.1
Females	0.490	0.298-0.682	7.0	60%	38.5-81.5	61%	38.5-83.5

Figure 1