

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 ≤ 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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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°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/
118 ferritin (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).

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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 ≤ 200 cells/mm³ 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 ≤ 200
189 cells/mm³ 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 ≤ 7 ng/ml; sensitivity 67% &
 205 specificity 67%. In males the optimum cut off was ≤ 6 ng/ml (sensitivity 80%; specificity
 206 78%); whilst for women this was ≤ 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^{ROC}: area under
 214 curve of receiver operating characteristic.

215
 216

Characteristic	Overall	Non BM-ID	BM-ID	P-value
BM-ID	35/73 (48.0%)	38/73 (52.1%)	35/73 (48.0%)	
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
Haematology & iron markers				
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
HIV disease and treatment				
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 ³				
Viral load >1000 copies/ml	57/76 (75.0%)	25/38 (65.8%)	30/35 (85.7%)	0.048
Infection(s)				
Bacteraemia ³	12/73 (16.4%)	6/38 (15.8%)	6/35 (17.1%)	0.876
Malaria ⁴	3/63 (4.7%)	2/32 (6.3%)	1/31 (3.2%)	0.573
Tuberculosis ⁵	39/73 (53.4%)	20/38 (52.6%)	19/35 (54.3%)	0.877
Epstein-Barr virus ⁶	30/45 (66.7%)	19/26 (73.1%)	11/19 (57.9%)	0.286
Cytomegalo virus ⁶	18/54 (33.3%)	11/28 (39.3%)	7/26 (26.9%)	0.336
Parvo-B19 virus ⁶	1/59 (1.7%)	0/27 (-)	1/32 (3.1%)	0.230
Nutritional status				
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. ¹ First line ART include combination of

221 Stavudine (d4T), Lamivudine (3Tc) and Nevirapine (NVP) (28). ² Advanced HIV disease

222 including a CD4 count ≤ 200 cells/mm³ and/or viral load > 1000 copies/ml. ³Bacteraemia; 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). ⁴Malaria:

225 presence of malaria parasites on a thick blood film. ⁵Tuberculosis (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. ⁶

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 ^{ROC}	95%-CI	Cut-off	Sensitivity	95%-CI	Specificity	95%-CI
MCV (fl) ¹	0.545	0.404-0.685	≤83	42%	25.2-58.8%	67%	51.0-83.0%
MCH (pg/cells) ¹	0.365	0.230-0.499	≤27	52%	35.2-69.0%	29%	13.7-44.3%
Serum iron (μmol/l) ¹	0.368	0.239-0.498	≤ 10	60%	43.8-76.2%	18%	5.8-30.2%
Ferritin (μg/l) ¹	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) ³	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 ⁴	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 ⁴	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)).¹ (29) ² (11) ³ (29) ⁴(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^{ROC} 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/mm³) 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

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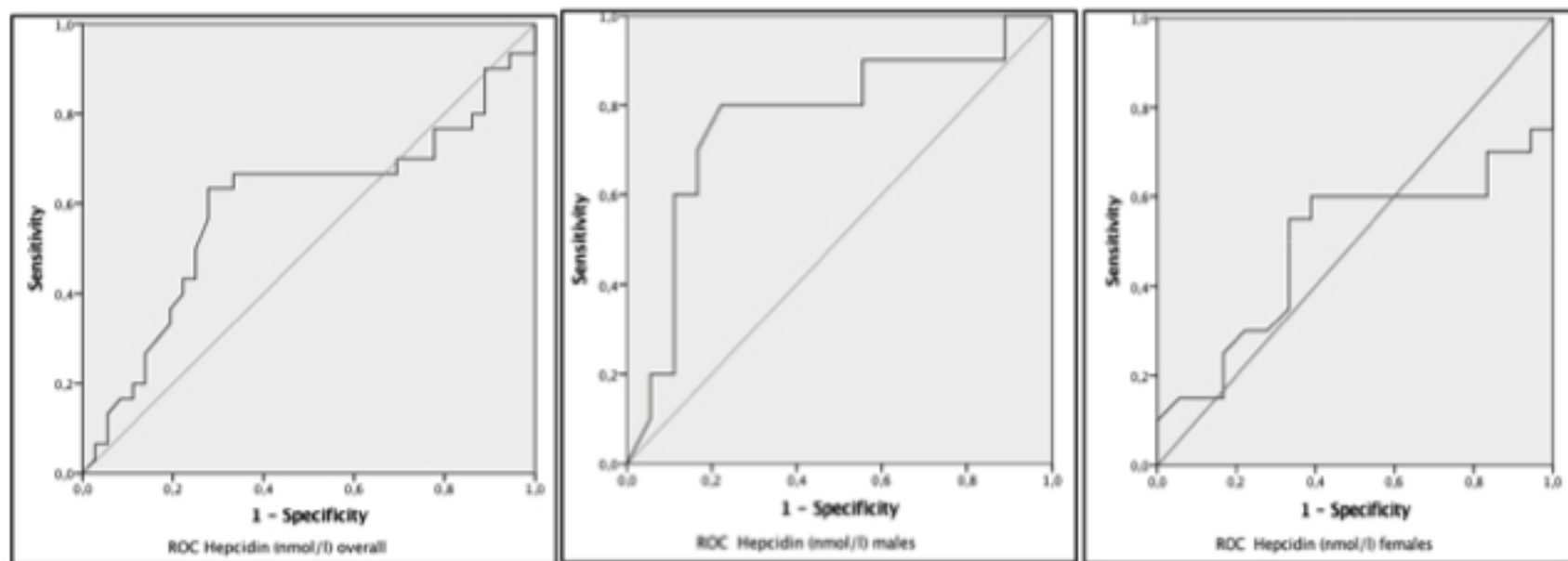
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495



Hepcidin	AUC ^{ROC}	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