Severe anaemia complicating HIV in Malawi; multiple co-existing aetiologies are associated with high mortality.

Short title: Severe anaemia complicating HIV in Malawi.

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Abstract

Background: Severe anaemia is a major cause of morbidity and mortality in HIV-infected adults living in resource-limited countries. Comprehensive data on the aetiology is lacking and needed to improve outcomes.

Methods: HIV-infected adults with severe (haemoglobin ≤70g/l) or very severe anaemia (haemoglobin ≤50 g/l) were recruited at Queen Elizabeth Central Hospital, Blantyre, Malawi. Fifteen potential causes of severe anaemia of anaemia and associations with anaemia severity and mortality were explored.

Results: 199 patients were enrolled: 42.2% had very severe anaemia and 45.7% were on ART. Over two potential causes for anaemia were present in 94% of the patients; including iron deficiency (55.3%), underweight (BMI<20: 49.7%), TB-infection (41.2%) and unsuppressed HIV-infection (viral load >1000 copies/ml) (73.9%). EBV/CMV co-infection (16.5%) was associated with very severe anaemia (OR 2.8 95% CI 1.1-6.9). Overall mortality was high (53%; 100/199) with a median time to death of 16 days. Death was associated with folate deficiency (HR 2.2; 95% CI 1.2-3.8) and end stage renal disease (HR 3.2; 95% CI 1.6-6.2).

Conclusion: Mortality among severely anaemic HIV-infected adults is strikingly high. Clinicians must be aware of the urgent need for a multifactorial approach, including starting or optimising HIV treatment; considering TB treatment, nutritional support and attention to potential renal impairment.

Introduction

Anaemia is recognized as the most common haematological complication of Human Immunodeficiency Virus (HIV) infection worldwide (1, 2). The World Health Organization (WHO) defines anaemia as a haemoglobin level below 110-120 g/l. In sub-Saharan Africa, 60% of HIV-infected adults are anaemic and 22% are severely anaemic (3, 4). Anaemia is independently associated with an increased one-year mortality in HIV infection of 8%, which rises to 55% in those with severe anaemia (5, 6).

To prevent and treat severe anaemia in HIV-infected patients, a comprehensive understanding of the aetiology and pathophysiology is essential. Severe anaemia in HIV infection has been associated with micronutrient deficiencies, infections (viral, bacterial and parasitic), medication induced (zidovudine and co-trimoxazole) and neoplastic diseases (7-11). Only a few studies have comprehensively studied the multifactorial aetiology and pathophysiology of HIV-associated severe anaemia in sub-Saharan Africa despite the high burden of HIV infection in this region (2). Commonly, studies only report on the association between HIV infection and a single cause of severe anaemia, for example iron deficiency, without considering the multiple causes of severe anaemia that may impact on an HIV-infected patient (12, 13). As a consequence, evidence to inform preventive or treatment guidelines for severe anaemia in HIV-infected patients in sub-Saharan Africa is scarce. In practice, severe anaemia management in HIV-infected patients is often still based on the same strategies used for non-HIV infected patients, including iron supplementation, malaria treatment and de-worming (2, 14). This strategy may be ineffective as the causes of HIV-associated severe anaemia may be different, and even harmful considering that (blind) iron supplementation may exacerbate infections and thus may deteriorate the patients condition (15, 16).

Better knowledge about the aetiology of severe anaemia is essential in order to develop evidence-based protocols and ultimately improve outcomes for HIV-infected adults with severe anaemia in sub-Saharan Africa. To address this knowledge gap, we performed a comprehensive observational cohort study to explore the prevalence of potential aetiologies of HIV-associated severe anaemia in Malawian in-patients and studied associations between these and the severity of the anaemia and patient outcomes.

Methods

An observational cohort study of HIV-infected patients with severe anaemia (haemoglobin ≤70 g/l) admitted to the Queen Elizabeth Central Hospital (QECH), Blantyre, Malawi between February 2010 and March 2011 was performed. All HIV-infected patients above 18 years of age with severe anaemia admitted to the general medical ward were approached and enrolled in the study if they provided informed consent. A case record form including a detailed medical history and physical examination was completed for each enrolled patient. On admission a venous blood sample was collected and a chest X-ray was performed. The patients were managed according to the hospital protocols, which included a blood transfusion if required, treatment of correctable conditions including anti-malarial medication and antibiotics. Antiretroviral treatment (ART) was provided according to the national Malawi guidelines, which stipulated that ARTs should only be initiated by specialist outpatient clinicians, so patients were not initiated on in-patient wards. At the time of the study first line ART was a combination of Stavudine, Lamivudine and Nevirapine and second line treatment was a combination of Zidovudine, Lamivudine, Tenofovir and Lopinavir/Ritonavir (17). ART was only prescribed for WHO stage 3 and 4 disease, or WHO stage 1 and 2 disease with CD4 count < 350 x 10^9/l (18). Co-trimoxazole was prescribed routinely to all HIV-infected patients on ART as Pneumocystis Jirovecii prophylaxis. For this study patients were followed up in the dedicated ART clinic after discharge. A community research nurse followed those who failed to attend their appointments up at home. Follow-up was done for a maximum of 365 days after enrolment when they attended the ART clinic for routine appointments or, if they failed to attend, by a home visit from a study nurse.

Laboratory assays

All samples were analysed within 24 hours of collection or stored at -80^{0}C for further analysis. On enrolment haemoglobin concentrations were measured on the ward using the HemoCue B-Haemoglobin analyser (HemoCue, Ängelholm, Sweden) to screen for eligibility. For patients enrolled in the study, the haemoglobin and red cell indices (MCV, MCH were determined and MCHC) using an automated analyser (Beckman Coulter, Durban, South Africa). CD4-cell counts were assessed using BD FACS Count (BD Biosciences, San Jose, CA, USA). Transferrin, iron, ferritin, folate and vitamin B12 were analysed on Modular P800 and Monular Analytic E170 systems (Roche Diagnostics, Switzerland). Soluble transferrin receptor (sTfR) levels were measured using ELISA

(Ramco Laboratories, TX, USA). Analyses for serum creatinine was by Beckman Coulter CX5 (ADVIA 2400 Siemens Healthcare Diagnostics). Renal function was measured by a estimating of Glomerular Filtration Rate (eGFR) using simplified Modification of Diet in Renal Disease (MDRD)-Study formula and the GFR was classified by Chronic Kidney Disease classification (19-21). For all tests the manufacturers' reference ranges were used; internationally accepted cut-offs were used to define deficiencies (22). Thick blood films were prepared and stained for malaria microscopy. Malaria was defined as the presence of *Plasmodium falciparum* asexual parasites in the blood films. HIV infection was confirmed using two point-of-care antibody tests (Unigold® and Determine®). A venous blood sample was inoculated into BACTEC Myco/F-Lytic culture vials and incubated in a BACTEC 9050 automated culture system (Becton Dickinson) for 56 days. Sub-culturing blood and sputum, susceptibility testing and isolate identification were performed by standard techniques(23). Possible contaminants were recorded as absence of pathogens. Sputum cultures were examined for mycobacteria using Ziehl–Nielsen staining. Whole-blood isolates were assessed for Epstein–Barr virus and cytomegalovirus infection by semi-quantitative PCR and for parvovirus B19 by real-time PCR (24). All chest X-rays were reviewed by a radiologist for signs of pulmonary tuberculosis (TB). `When TB was suspected, standardized treatment was started according to the local protocols.

Bone marrow

If the patient's clinical condition allowed and they provided consent, a bone marrow aspirate and trephine biopsy was performed. All bone marrow samples were taken from the posterior iliac crest. Samples of the aspirates were spread onto slides and trephine biopsies were fixed, decalcified and embedded in paraffin wax (25, 26). Bone marrow samples were sent to the Haematopathology Referral Centre at the Royal Liverpool University Hospital, Liverpool UK, for analysis. Sections of the trephine blocks were stained with haematoxylin and eosin and Giemsa, and also for iron with Perls stain, and for reticulin (25). All slides were examined for using a predefined format and diagnoses were allocated to the categories lymphoproliferative disease, myeloproliferative disease, myelodysplastic syndrome (MDS) and TB (27-29). The need for additional histochemical (e.g. Ziehl-Neelsen) or immunohistological (e.g. CD3, CD20) staining was determined according to the local protocol in Liverpool depending on the preliminary morphological findings.

Definitions for potential factors involved in the aetiology of severe anaemia

The following potential factors involved in the aetiology of severe anaemia were defined and evaluated; 1) Unsuppressed HIV-infection; viral load ≥1000 copies/ml. 2)TB: one or more of the following were present: a) positive sputum culture, b) chest X-ray with signs of pulmonary TB and/or c) on going TB treatment at time of enrolment d) clinical diagnosis by local doctor including unknown generalized lymphadenopathy and/or night sweats of > 30 days and of unknown origin e) caseating granulomata in the bone marrow trephine. 3) Malaria: presence of malaria parasites in a thick blood film. 4) Parvovirus B19: viral load of >1000 copies/ml. 5) Cytomegalovirus (CMV); load of >100 copies/ml. 6) Epstein-Barr virus (EBV); viral load >100 copies/ml. 7) Bacteraemia; a blood culture growing a potential pathogen. 8) Underweight (BMI ≤18.5). 9) Serum folate deficiency (≤3 ng/l). 10) Vitamin B12 deficiency (≤180 pg/ml). 11). Iron deficiency was defined as MCV≤ 83 fl (3, 22, 30). 12) Zidovudine usage. 13) Co-trimoxazole usage. 14) Bone marrow disorders; lympho-proliferative disease, myeloid-proliferative disease or MDS. 15) Renal impairment: a GFR which either indicated impaired (GFR 15–59 ml/min/1.73 m^2) or End Stage (GFR ≤15 ml/min/1.73 m^2) Renal Disease (21, 31).

Statistics

Baseline characteristics and prevalence of potential risk factors are presented as proportions or medians with IQR. Logistic regression was performed to model the association between anaemia and **potential factors involved severity of anaemia**, Results are expressed as OR with 95% CI and p-values. Variables associated with the outcome variables ($P \leq 0.10$) in the univariate analysis were included in the multivariate model in a stepwise approach. Kaplan Meier survival curves were used to assess cumulative mortality. Significant differences were investigated with a Log Rank test. Uni- and multivariate analyses were done using logistic regression and Cox regression to describe predictors of mortality. P values of ≤ 0.05 were regarded as statistically significant. All reported P values were two-sided. The data were analysed using Stata (version 12) (STATA Corp. LP, Texas, TX, USA).

Ethics

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

Results

In total, 199 patients were included in the study: 64.8% were female. The median age was 32 years (IQR 27-61 years). The median haemoglobin was 53 g/l (IQR 4.2-6.3) and 84 (42%) patients had very severe anaemia (hb≤50 g/l). A total of 91 (45.7%) patients were on ART at enrolment including 79.1% on first line ART. During the study period, an additional 41 (21%) patients started on ART treatment. Moreover 67.1% of the patients were immune suppressed with a CD4 count ≤ 200 cells/mm. Baseline characteristics of the patients are shown in table1.

	Overall
Age, years (median IQR)	32 (IQR 27-61)
Sex (female)	129/199 (64.8%)
Haematology	
Haemoglobin (Hb) (median, IQR) g/l	53 (IQR 42-63)
Severe anaemia (Hb 51-70 g/l)	115/199 (57.8%)
Very severe anaemia (Hb ≤ 50g/l)	84/199 (42.2%)
Pancytopenia[1]	42 /184 (21.2%)
Mortality	
Overall mortality (365 days)	101/199 (50.8%)
Early mortality (60 days)	81/101 (81.2%)
Days until death (median, IQR)	17.5(6-55)
HIV-disease and treatment	
CD4 (median, IQR)	175 (IQR 55-825)
CD4 ≤200 cells/mm^3	104/155 (67.1%)
Viral load ≥1000 copies/ml	136/184 (73.9%)
ART at enrolment	91/199 (45.7%)
First-line ART	72/199 (36.2%)
Second-line ART	11/199 (5.5%)
Non-specified ART	8/199 (4%)
Blood transfusions and supplemental treatment	
Blood transfusion at admission	47/199 (23.6%)
Folate supplementation at admission	57/199 (28.6%)
Iron supplementation at admission	81/199 (40.7%)
Vitamin B12 supplementation at admission	0/199 (-)

Table 1. Baseline characteristics of HIV-infected patients with severe anaemia at enrolment into study.

[1] Pancytopenia is defined as thrombocytopenia (≤150x10^9/l) and leukopenia (≤4x10^9/l) and severe anaemia (≤ 70 g/l) (22). Abbreviations: Hb: Haemoglobin, ART: antiretroviral therapy.

The prevalence of factors that were associated with severe anaemia, and factors that were co-existing in individual patients, are shown in table 2. Overall, patients had a mean of 3 (range 1-8) co-existing aetiologies of severe anaemia (figure 1).

Figure 1. Total number aetiologies for severe anaemia co-existing in each patient (n=199). Mean is 3 factors (SD 1.3), range 1-8.

An unsuppressed HIV-infection was seen among 73.9% patients. TB was the second most common infection occurring in 82 (41.2%) of the patients. In 19 (23%) of these patients TB was diagnosed on their chest X-ray. Granulomata were seen in the bone marrow trephine in 15 (18%) patients of all the 82 patients that had a diagnosis of TB. 11 (13%) patients were on TB treatment at enrolment. 69/170 (40.5%) patients had evidence of current EBV infection and 57/170 (33.5%) had evidence of current CMV infection. Co-infection with CMV and EBV was found in of 28/170 (16.5%) of the patients. Bacteraemia was diagnosed on a positive blood culture in 26 (13.1%); the most common pathogens found were E. Coli (12 patients; 42.9% and non-Typhoid Salmonella (5 patients; 19.9%). 74/148 (49.7%) patients were underweight and iron deficiency occurred in 61/180 (33.9%) of the patients. Bone marrow sampling was performed in 73 patients. Of these, 28 (38.4%) had morphological abnormalities with MDS being the most common abnormality (20 patients; 27.4%) (table 2). Renal impairment was diagnosed in 36/185 patients (19.5%) and 12 of these patients (33%) had end stage renal disease.

Comparing the different risk factors for very severe anaemia (Hb<50g/L) as compared to severe anaemia, EBV/CMV co-infection (OR 2.8 95% CI 1.1-6.9) was the only factor associated with very severe anaemia (table 2).

During the one-year follow-up period, 101 study patients (50.8%) died. The median time to death was 16 days (IQR 5-41) and 81 (80.1%) of these deaths occurred within 60 days of admission (figure 2).

Figure 2. Kaplan Meyer curve for survival probability over time (days) for adult Malawian patients with HIV infection and severe anaemia during 365 days follow-up. Abbreviation: 95% confidence interval (95%CI).

Folate deficiency and end stage renal disease were associated with mortality with Hazard Ratio 2.2 (95% CI 1.2-3.8) and Hazard Ratio 3.2 (95% CI 1.6-6.2) respectively (figure 3). Neither very severe anaemia (haemoglobin ≤50 g/l), nor the haemoglobin levels were

associated with mortality in the study patients (Hazard Ratio 0.9, 95%CI 0.6-1.4 and Hazard Ratio 1.01, 95% CI 0.9-1.2 respectively).

Figure 3. Risk factors for 365-day mortality in HIV-infected patients with severe anaemia.

Univariate and multivariate Cox regression outcome. Folate deficiency (3 ng/l); HR 2.1 95% CI 1.2-3.6 and end stage renal disease (GFR 15); HR 3.2 5% CI 1.6-6.2, were associated with overall mortality.

Abbreviations: Hb: Haemoglobin, GFR; Glomerular filtration rate, VL Viral Load.

	Overall (n=199)	Severe anaemia N=115/199 (30.8%)	Very severe anaemia N=84/199 (42.2%)	Univariate			Multivariate		
				Odds	95% -CI	P-value	Odds	95% -CI	P-value
Sex (female)	129 (64.8%)	74/115 (65.2%)	55/84 (65.4%)	1.1	0.6-1.9	0.869	-	-	-
HIV									
CD4 ≤200 cells/mm^3	104/155 (67.1%)	62/87 (71.3%)	42/68 (61.8%)	1.04	0.6-1.8	0.889	-	-	-
Viral load ≥1000 copies/ml	136/184 (73.9%)	81/104 (77.9%)	55/80 (68.8%)	0.6	0.3-1.2	0.163	0.7	0.3-1.5	0.306
On ART at enrolment	91/199 (42.7%)	52/115 (40.9%)	39/84 (45.2%)	1.1	0.6-1.8	0.865	-	-	-
Infection									
Malaria	6/167 (1%)	3/100 (3.0%)	3/67 (4.5%)	1.5	0.3-7.8	0.617	-	-	-
Tuberculosis	82/199 (41.2%)	53/115 (46.0%)	29/84 (34.5%)	0.7	0.6-0.99	0.043	0.6	0.1-2.8	0.507
Bacteraemia[1]	26/199 (13.1%)	17/115 (61.7%)	9/82 (11.0%)	0.7	0.3-1.6	0.402	-	-	-
Parvovirus B19	7/170 (4.2%)	5/99 (5.0%)	2/71 (2.8%)	0.5	0.1-2.9	0.476	-		
Cytomegalovirus (CMV)	57/170 (33.5%)	28/99 (28.3%)	29/71 (40.8%)	1.8	0.9-3.3	0.088	-	-	
Epstein-Barr virus (EBV)	69/170 (40.6%)	40/99 (40.4%)	29/71 (40.8%)	1.0	0.6-1.9	0.954	-	-	-
EBV/CMV co-infection	28/170 (16.5%)	12/99 (12.1%)	16/71 (22.5%)	2.1	0.9-4.5	0.075	2.8	1.1-6.9	0.032
Malnutrition									
Underweight	74/148 (49.7%)	40/81 (49.3%)	34/68 (50.0%)	1.0	0.5-2.0	0.940	-	-	-
Vitamin B12 deficiency	2/194 (1.0%)	1/113 (0.8%)	1/81 (1.2%)	1.4	0.09-22.7	0.813	-	-	-
Iron deficiency	61/180 (33.9%)	38/104 (36.5%)	23/76 (30.3%)	0.8	0.40-1.42	0.380	-	-	-
Folate deficiency	23/194 (11.9%)	13/113 (11.5%)	10/84 (11.9%)	1.1	0.5-2.6	0.858	-	-	-
Medication									
Co-trimoxazole	163/199 (81.9%)	99/115 (81.6%)	64/84 (76.2%)	0.5	0.3-2.3	0.076	0.5	0.2-1.2	0.099

Zidovudine	13/199 (6.5%)	5/50 (10.0%)	8/40 (20.0%)	2.3	0.7-7.5	0.187	-	-	-
Renal function									
Normal (GFR >60)	149/185(80.5%)	90/105 (85.7%)	59/80 (73.8%)	I			I		
Impaired (GFR 15-60)	24/185 (13.0%)	12/105 (11.4%)	12/80 (15.0%)	1.5	0.6-3.6	0.339	1.0	0.7-5.0	0.176
End stage (GFR ≤15)	12/185 (6.5%)	3/105 (2.9%)	9/80 (11.3%)	4.6	1.2-17.6	0.027	3.0	0.7-13.1	0.140
Bone marrow									
Bone marrow disease	28/71 (39.4%)	19/42 (45.2%)	9/29 (31.0%)	0.5	0.2-1.5	0.231	-	-	
Aetiology									
Co-existing aetiologies per patient (mean, SD)	3.3 (1.3)	3.3 (1.2)	3.2 (1.4)	0.8	0.4-1.8	0.605	-	-	-

Table 2. Distribution and multivariate analysis of co-existing factors associated with severe and very severe anaemia (Hb≤ 50 g/l) in HIV-infected adults in Malawi. [1] *A total of 28-blood cultures were positive, the most common organisms were E. coli (42.9%; 12/28) and non-Typhoid Salmonella (17.9 %; 5/28). Explanatory variables associated with the outcome variables (P > 0.10) in the univariable analysis were excluded in the multivariable model in a stepwise approach (-).* Abbreviation: GFR; Glomerular filtration rate.

8   **Discussion**

9   In this study we described the prevalence of several potential aetiologies for
10   severe and very severe anaemia in HIV-infected Malawian adults. Patients had a mean of
11   three co-existing aetiologies potentially contributing to their anaemia, the most common
12   one being unsuppressed HIV. Mortality in the study patients was extremely high, as 51%
13   of the patients died within one year and most died within 60 days of admission. Severe
14   anaemia in HIV-infected patients in a resource limited setting, such as Malawi, should
15   therefore be treated as a multi-causal critical condition with high mortality.

16   Anaemia is an independent predictor of mortality and disease progression in HIV-
17   infected patients and mortality increases with decreasing haemoglobin concentration (6,
18   32). Previous studies reported an estimated one-year mortality of 3.7% in HIV-infected
19   patients without anaemia and up to 30-55% in severe anaemia in resource limited settings
20   (5, 6). Our study results are consistent with these outcomes. Additionally we found that a
21   mean of three contemporaneous aetiologies potentially contributed to severe anaemia in
22   HIV-infected patients. This is important as it highlights the multi-causality of severe
23   anaemia in this population. Clinicians should therefore not just focus on a single cause
24   and single treatment for severe anaemia in HIV-infected patients. Different treatment
25   protocols from those used traditionally to treat anaemia (e.g. iron and folate) will be
26   needed to address these multiple co-existing aetiologies to enhance improvement of
27   mortality amongst these patients. Also, the seriousness of severe anaemia in HIV-infected
28   patients needs to be better recognized. Currently severe anaemia in HIV-infected patients
29   is classified as a stage 3 clinical condition (33). However due to the high mortality found in
30   our, and previous, studies, we recommend re-classifying severe anaemia as a marker of
31   HIV stage 4 disease.

32   Unsuppressed HIV virus was present in 79% of the patients in our study
33   population. HIV may cause anaemia directly through an inhibitory effect of the HIV-virus
34   on the erythropoietin progenitor cells in the bone marrow, or indirectly through
35   opportunistic infections causing anaemia (34). Although this potential direct/indirect risk
36   factor was very common it was not associated with the severity of the anaemia or
37   mortality in our population, explored in our multivariate analysis. This is in contrast with a
38   large cross-sectional study in Tanzania showed that the risk of developing severe anaemia
39   in HIV-infected patients increased two to three times among patients with advanced HIV
40   disease (3). We therefor postulate that controlling HIV infection by starting or switching
41   ART treatment should therefore be considered as the most important and urgent step in
42   treatment protocols for severely anaemic HIV-infected patients. After our study had been
43   completed guidelines for initiating ART changed. At the time of the study the trigger for

44 starting ART was based on a patient's CD4 count, whereas it is currently recommended
45 that ART should be started early in the course of HIV disease (17, 33). It will be important
46 for the impact of this policy change on HIV-related anaemia, and its consequences, to be
47 evaluated. Irrespective of the policy change for initiating ART, the findings of our study
48 remain very relevant because many HIV-infected patients in resource-limited settings
49 present late in the course of their disease or are unable to access reliable supplies of ART,
50 and are therefore likely to continue to have high levels of life-threatening anaemia.

51 TB has previously been associated with anaemia in HIV-infected patients (36). In
52 our study patients TB was a common co-infection (41%). This prevalence is comparable to
53 previous reports on TB prevalence (43%) among severely anaemic HIV-infected patients
54 in Africa (8, 14). The pathophysiology of TB-associated anaemia in HIV-infected patients
55 remains unclear. Bone marrow invasion by TB organisms or altered iron metabolism, as a
56 side effect of tuberculostatic drugs have been described (36). Only 13% of patients were
57 on TB treatment at enrolment. TB medication itself was not associated with the severity of
58 anaemia (data not shown). Given that we and others have found TB in nearly half of the
59 HIV-infected patients with severe anaemia, TB screening and rapid initiation of treatment
60 should therefore be a high priority in the management of these patients, especially in
61 resource limiting settings where there is a high TB prevalence.

62 Viral infections such as EBV, CMV and parvovirus B19 have been associated with
63 anaemia in HIV-infected patients (35, 37). In our study EBV and CMV were common and
64 present in 40% and 35% of patients respectively, whilst parvovirus B19 was less common
65 (4%). Although parvovirus B19 is pathophysiological linked to mild anaemia, the role in
66 the development severe anaemia in HIV infected patients has not been clearly
67 established (38). Data on co-infections are very limited in African patients (39-41) and our
68 study is the first to describe the association between co-existing CMV and EBV infections
69 and very severe anaemia (haemoglobin ≤50 g/l). Possible explanations for this association
70 include direct viral inhibition of erythropoiesis (39, 42, 43). The majority of our patients
71 with co-infection had advanced HIV disease (88.5%) but the association between EBV and
72 CMV co-infection and very severe anaemia remained significant even after correction for
73 advanced HIV disease. As CMV is a treatable infection it will be important to determine
74 the effect of ART on CMV infection and severe anaemia, as ART can reduce CMV infection
75 by improving immune status (44).

76 Malaria is unsurprisingly associated with severe anaemia in HIV-infected patients
77 in sub-Saharan Africa (2, 14). However, the contribution of malaria to anaemia in our study
78 population was small however, as only 6 patients (3.5%) had malaria parasites on
79 presentation. This is in line with data from other studies that show that the role of malaria

80   in causing anaemia, especially in HIV patients in sub-Saharan Africa (2, 14), is limited and

81   likely to have been overestimated (40, 45).

82   Malnutrition was common in our population as half of the patients' had a BMI

83   below 18.5m² and deficiencies of iron and folate were diagnosed in 33.9% and 12% of the

84   patients respectively. The prevalence of iron deficiency in our study is higher than in

85   previous studies on HIV-infected patients in sub-Saharan Africa populations that report

86   prevalence rates of iron deficiency of 18%-25% of anaemic HIV-infected patients (46, 47).

87   Interestingly, adults in sub-Saharan Africa with severe anaemia but without HIV infection,

88   have a higher prevalence of iron deficiency (59%) (45). Data from children with severe

89   anaemia in Malawi also showed a much higher overall prevalence of iron deficiency (47%)

90   than the adults in our study (40). Studies presenting this outcome are all published before

91   2010, in this time period ART was less available and all included HIV-infected patients

92   were ART naïve and sever immune suppression was highly present. In this group of HIV-

93   infected patients with better immune systems the aetiology of severely anaemic may be

94   more similar to the aetiology of non-HIV infected patients. It can be expected that the

95   prevalence of condition such as iron deficiency is also more alike the HIV-uninfected

96   groups (40). In addition to finding a high prevalence of iron deficiency, ours is the first

97   study to document on a possible association between folate deficiency and increased

98   mortality in severely anaemic HIV-infected patients. There are a limited number of studies

99   describing micronutrient supplementation, including folate, for anaemic HIV-infected

100   adults, but overall there appears to be little significant effect of supplementation on

101   reducing morbidity and mortality (37, 48). Macronutrient support using, for example,

102   fortified wheat flower, has had beneficial effects on anaemia reduction and micronutrient

103   levels in populations in sub-Saharan Africa, but has never been tested in the context of

104   severe anaemia in HIV-infected patients (49, 50). More research is therefore needed to

105   evaluate the effectiveness of both macro- and micro- nutritional support in severely

106   anaemic HIV-infected adults.

107   HIV-infected adults have an increased risk of neoplastic bone marrow diseases,

108   which can often cause anaemia (13). However, prevalence data for such conditions among

109   HIV-infected patients with anaemia, especially in resource limiting settings, are scarce.

110   Only three of our study patients had confirmed bone marrow malignancies. In contrast

111   MDS was common occurring in 27% of our patients (27, 51).

112   Renal impairment was a frequent finding among our study patients and has been

113   linked to HIV disease progression, anaemia and poor outcomes in both wealthy and

114   resource limited settings (52-55). Evaluation of renal function is an important component

115   of severe anaemia treatment protocols (30, 53) since it may affect the choice of ART (52,

116   56). It may also have implications for clinical management, for example by introducing

117   measures to prevent further deterioration or considering potential benefits of

118   erythropoietin(53).

119   Our study has several limitations. We purposely did not explore factors potentially

120   associated with anaemia that previous studies had shown were uncommon in Malawi,

121   such as haemoglobinopathies and parasitic infections (40, 57). We also did not include an

122   HIV-infected population without severe anaemia against which to compare the

123   prevalence of anaemia aetiologies and clinical outcomes. Only a sub-population of our

124   patients gave consent for bone marrow sampling. Although these were an unselected

125   group of patients it is possible that this may have introduced a hidden bias, for example

126   by excluding the patients who were particularly unwell. Nevertheless, our findings are

127   very valuable since bone marrow data from HIV-infected African patients is very scarce.

128

129   **Conclusion**

130   Our study has demonstrated that severe anaemia in HIV-infected adults in Malawi is

131   associated with multiple co-existing aetiologies and has a strikingly high mortality rate.

132   Severe anaemia in HIV-infected patients is therefore a critical indicator of mortality and

133   requires urgent and multiple interventions. Particularly important is the initiation of ART,

134   the management of infections such as TB and CMV, and optimisation of renal function.

135   Intervention studies are needed to properly define the role (and safety) of iron and folate

136   supplementation, as well as to develop and evaluate guidelines, which are feasible and

137   effective in resource-limited settings to help clinicians manage these patients more

138   effectively.

139

140

141   **Acknowledgement**

References

1.   Mocroft A, Kirk O, Barton SE, Dietrich M, Proenca R, Colebunders R, et al. Anaemia is an independent predictive marker for clinical prognosis in HIV-infected patients from across Europe. EuroSIDA study group. AIDS (London, England). 1999;13(8):943-50.

2.   Marti-Carvajal AJ, Sola I, Pena-Marti GE, Comunian-Carrasco G. Treatment for anemia in people with AIDS. Cochrane Database Syst Rev. 2011(10):Cd004776.

3.   Makubi A, Okuma J, Spiegelman D, Hawkins C, Darling AM, Jackson E, et al. Burden and Determinants of Severe Anemia among HIV-Infected Adults: Results from a Large Urban HIV Program in Tanzania, East Africa. J Int Assoc Provid AIDS Care. 2015;14(2):148-55.

4.   Petraro P, Duggan C, Spiegelman D, Hertzmark E, Makubi A, Chalamilla G, et al. Determinants of Anemia Among Human Immunodeficiency Virus-Positive Adults at Care and Treatment Clinics in Dar es Salaam, Tanzania. The American journal of tropical medicine and hygiene. 2016;94(2):384-92.

5.   Santiago-Rodriguez EJ, Mayor AM, Fernandez-Santos DM, Ruiz-Candelaria Y, Hunter-Mellado RF. Anemia in a cohort of HIV-infected Hispanics: prevalence, associated factors and impact on one-year mortality. BMC Res Notes. 2014;7:439.

6.   Johannessen A, Naman E, Ngowi BJ, Sandvik L, Matee MI, Aglen HE, et al. Predictors of mortality in HIV-infected patients starting antiretroviral therapy in a rural hospital in Tanzania. BMC infectious diseases. 2008;8:52.

7.   Shu T, Jing C, Lv Z, Xie Y, Xu J, Wu J. Hepcidin in tumor-related iron deficiency anemia and tumor-related anemia of chronic disease: pathogenic mechanisms and diagnosis. Eur J Haematol. 2015;94(1):67-73.

8.   Butensky E, Kennedy CM, Lee MM, Harmatz P, Miaskowski C. Potential mechanisms for altered iron metabolism in human immunodeficiency virus disease. J Assoc Nurses AIDS Care. 2004;15(6):31-45.

9.   Kupka R, Msamanga GI, Mugusi F, Petraro P, Hunter DJ, Fawzi WW. Iron status is an important cause of anemia in HIV-infected Tanzanian women but is not related to accelerated HIV disease progression. J Nutr. 2007;137(10):2317-23.

10.   Skikne BS, Punnonen K, Caldron PH, Bennett MT, Rehu M, Gasior GH, et al. Improved differential diagnosis of anemia of chronic disease and iron deficiency anemia: a prospective multicenter evaluation of soluble transferrin receptor and the sTfR/log ferritin index. American journal of hematology. 2011;86(11):923-7.

11.   Klassen MK, Lewin-Smith M, Frankel SS, Nelson AM. Pathology of human immunodeficiency virus infection: noninfectious conditions. Ann Diagn Pathol. 1997;1(1):57-64.

12.   Akilimali PZ, Kashala-Abotnes E, Musumari PM, Kayembe PK, Tylleskar T, Mapatano MA. Predictors of Persistent Anaemia in the First Year of Antiretroviral Therapy: A Retrospective Cohort Study from Goma, the Democratic Republic of Congo. PloS one. 2015;10(10):e0140240.

13.   Levine AM. Anemia in the setting of cancer and human immunodeficiency virus. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2003;37 Suppl 4:S304-14.

14.   Brentlinger PE, Silva WP, Vermund SH, Valverde E, Buene M, Moon TD. Practical Management of HIV-Associated Anemia in Resource-Limited Settings: Prospective Observational Evaluation of a New Mozambican Guideline. AIDS research and human retroviruses. 2016;32(1):12-25.

15.   Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A, et al. Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital

199 and mortality in preschool children in a high malaria transmission setting: community-
200 based, randomised, placebo-controlled trial. Lancet (London, England).
201 2006;367(9505):133-43.
202 16. Esan MO, van Hensbroek MB, Nkhoma E, Musicha C, White SA, Ter Kuile FO, et al.
203 Iron supplementation in HIV-infected Malawian children with anemia: a double-blind,
204 randomized, controlled trial. Clinical infectious diseases : an official publication of the
205 Infectious Diseases Society of America. 2013;57(11):1626-34.
206 17. Ministry of Health Malawi. Treatment of AIDS, guidelines for the use of
207 antiretroviral therapy in Malawi, edition 2008.
208 18. World Health Organization (WHO). Antiretroviral therapy for HIV infection in
209 adults and adolescents; recommendations for a public health approach. 2006 revision
210   2006 [Available from:
211 https://www.who.int/hiv/pub/guidelines/artadultguidelines.pdf?ua=1.
212 19. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, et al. A
213 new equation to estimate glomerular filtration rate. Annals of internal medicine.
214 2009;150(9):604-12.
215 20. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, et al. Using
216 standardized serum creatinine values in the modification of diet in renal disease study
217 equation for estimating glomerular filtration rate. Annals of internal medicine.
218 2006;145(4):247-54.
219 21. Foundation NK. K/DOQI clinical practice guidelines for chronic kidney disease
220 (2002). Retrieved 2008-06-29. 2008.
221 22. Bates I. Practical hematology. Chapter 2: reference ranges and normal values.
222 2018.
223 23. Michael L. Wislon MM, Author J. Morris, Patrick R. Muray, Larry G. Reimer, L.
224 Barth Reller, Michael Towns, Melvin P. Weinstein, Sybil A. Wellstood, W. Michael Dunne,
225 Robert C. Jerris, David F.Welch. Principles and procedures of blood cultures; approved
226 guideline (M47-A). Clinical and laboratory standards institute. 2007;27(17).
227 24. Phiri KS, Calis JC, Kachala D, Borgstein E, Waluza J, Bates I, et al. Improved
228 method for assessing iron stores in the bone marrow. J Clin Pathol. 2009;62(8):685-9.
229 25. Bain BJ. Bone marrow trephine biopsy. Journal of clinical pathology.
230 2001;54(10):737-42.
231 26. Bain BJ. Bone marrow aspiration. J Clin Pathol. 2001;54(9):657-63.
232 27. Garcia-Manero G. Myelodysplastic syndromes: 2014 update on diagnosis, risk-
233 stratification, and management. American journal of hematology. 2014;89(1):97-108.
234 28. Gale E, Torrance J, Bothwell T. The quantitative estimation of total iron stores in
235 human bone marrow. J Clin Invest. 1963;42:1076-82.
236 29. Finch CA. The detection of iron overload. N Engl J Med. 1982;307(27):1702-4.
237 30. Volberding PA, Levine AM, Dieterich D, Mildvan D, Mitsuyasu R, Saag M. Anemia
238 in HIV infection: clinical impact and evidence-based management strategies. Clinical
239 infectious diseases : an official publication of the Infectious Diseases Society of America.
240 2004;38(10):1454-63.
241 31. Gregory M. Lucas MJR, Peter G. Stock, Michael G. Shlipak, Christina M. Wyatt,
242 Samir K. Gupta, Mohamed G. Atta, Kara K. Wools-Kaloustian, Paul A. Pham, Leslie A.
243 Bruggeman, Jeffrey L. Lennox, Patricio E. Ray, Robert C. Kalayjian. Clinical Practice
244 Guideline for the Management of Chronic Kidney Disease in Patients Infected With HIV:
245 2014 Update by the HIV Medicine Association of the Infectious Diseases Society of
246 America 2014 . Published online 2014 Sep 17. doi: 10.1093/cid/ciu617

247   PMCID: PMC4271038. Clinical infectious diseases : an official publication of the
248   Infectious Diseases Society of America. 2014;59(9):e96–e138.
249   32.   O'Brien ME, Kupka R, Msamanga GI, Saathoff E, Hunter DJ, Fawzi WW. Anemia is
250   an independent predictor of mortality and immunologic progression of disease among
251   women with HIV in Tanzania. Journal of acquired immune deficiency syndromes (1999).
252   2005;40(2):219-25.
253   33.   World Health Organization (WHO). Consolidated guidelines on the use of
254   antiretroviral drugs for treating and preventing HIV infection. Recommendations for a
255   public health aproach. Second edition. 2016 [Available from:
256   https://www.who.int/hiv/pub/arv/arv-2016/en/.
257   34.   Wang Z, Goldberg MA, Scadden DT. HIV-1 suppresses erythropoietin production
258   in vitro. Experimental hematology. 1993;21(5):683-8.
259   35.   Gedefaw L, Yemane T, Sahlemariam Z, Yilma D. Anemia and risk factors in HAART
260   naive and HAART experienced HIV positive persons in south west Ethiopia: a
261   comparative study. PloS one. 2013;8(8):e72202.
262   36.   Kerkhoff AD, Meintjes G, Opie J, Vogt M, Jhilmeet N, Wood R, et al. Anaemia in
263   patients with HIV-associated TB: relative contributions of anaemia of chronic disease
264   and iron deficiency. Int J Tuberc Lung Dis. 2016;20(2):193-201.
265   37.   Siegfried N, Irlam JH, Visser ME, Rollins NN. Micronutrient supplementation in
266   pregnant women with HIV infection. Cochrane Database Syst Rev. 2012(3):Cd009755.
267   38.   Morelli P, Bestetti G, Longhi E, Parravicini C, Corbellino M, Meroni L. Persistent
268   parvovirus B19-induced anemia in an HIV-infected patient under HAART. Case report
269   and review of literature. Eur J Clin Microbiol Infect Dis. 2007;26(11):833-7.
270   39.   Bates M, Brantsaeter AB. Human cytomegalovirus (CMV) in Africa: a neglected
271   but important pathogen. J Virus Erad. 2016;2(3):136-42.
272   40.   Calis JC, Phiri KS, Faragher EB, Brabin BJ, Bates I, Cuevas LE, et al. Severe anemia
273   in Malawian children. N Engl J Med. 2008;358(9):888-99.
274   41.   Kelly MJ, Benjamin LA, Cartwright K, Ajdukiewicz KM, Cohen DB, Menyere M, et
275   al. Epstein-barr virus coinfection in cerebrospinal fluid is associated with increased
276   mortality in Malawian adults with bacterial meningitis. The Journal of infectious
277   diseases. 2012;205(1):106-10.
278   42.   Gumbo H, Chasekwa B, Church JA, Ntozini R, Mutasa K, Humphrey JH, et al.
279   Congenital and postnatal CMV and EBV acquisition in HIV-infected Zimbabwean infants.
280   PloS one. 2014;9(12):e114870.
281   43.   Olson D, Huntington MK. Co-infection with cytomegalovirus and Epstein-Barr
282   virus in mononucleosis: case report and review of literature. S D Med. 2009;62(9):349,
283   51-3.
284   44.   Mofenson LM, Brady MT, Danner SP, Dominguez KL, Hazra R, Handelsman E, et al.
285   Guidelines for the Prevention and Treatment of Opportunistic Infections among HIV-
286   exposed and HIV-infected children: recommendations from CDC, the National Institutes
287   of Health, the HIV Medicine Association of the Infectious Diseases Society of America, the
288   Pediatric Infectious Diseases Society, and the American Academy of Pediatrics. MMWR
289   Recomm Rep. 2009;58(Rr-11):1-166.
290   45.   Lewis DK, Whitty CJ, Walsh AL, Epino H, Broek NR, Letsky EA, et al. Treatable
291   factors associated with severe anaemia in adults admitted to medical wards in Blantyre,
292   Malawi, an area of high HIV seroprevalence. Transactions of the Royal Society of
293   Tropical Medicine and Hygiene. 2005;99(8):561-7.
294   46.   Lewis DK, Whitty CJ, Epino H, Letsky EA, Mukiibi JM, van den Broek NR.
295   Interpreting tests for iron deficiency among adults in a high HIV prevalence African

296   setting: routine tests may lead to misdiagnosis. Transactions of the Royal Society of
297   Tropical Medicine and Hygiene. 2007;101(6):613-7.
298   47.   Kagu MB, Khalil MI, Ahmed SG. Bone marrow macrophage iron stores in patients
299   with HIV infection and AIDS-associated Kaposi's sarcoma. African journal of medicine
300   and medical sciences. 2007;36(2):125-8.
301   48.   Semba RD, Ricketts EP, Mehta S, Netski D, Thomas D, Kirk G, et al. Effect of
302   micronutrients and iron supplementation on hemoglobin, iron status, and plasma
303   hepatitis C and HIV RNA levels in female injection drug users: a controlled clinical trial.
304   Journal of acquired immune deficiency syndromes (1999). 2007;45(3):298-303.
305   49.   Engle-Stone R, Nankap M, Ndjebayi AO, Allen LH, Shahab-Ferdows S, Hampel D, et
306   al. Iron, Zinc, Folate, and Vitamin B-12 Status Increased among Women and Children in
307   Yaounde and Douala, Cameroon, 1 Year after Introducing Fortified Wheat Flour. J Nutr.
308   2017;147(7):1426-36.
309   50.   Noor RA, Abioye AI, Ulenga N, Msham S, Kaishozi G, Gunaratna NS, et al. Large -
310   scale wheat flour folic acid fortification program increases plasma folate levels among
311   women of reproductive age in urban Tanzania. PloS one. 2017;12(8):e0182099.
312   51.   Williamson BT, Leitch HA. Higher Risk Myelodysplastic Syndromes in Patients
313   with Well-Controlled HIV Infection: Clinical Features, Treatment, and Outcome. Case Rep
314   Hematol. 2016;2016:8502641.
315   52.   Mulenga LB, Kruse G, Lakhi S, Cantrell RA, Reid SE, Zulu I, et al. Baseline renal
316   insufficiency and risk of death among HIV-infected adults on antiretroviral therapy in
317   Lusaka, Zambia. AIDS (London, England). 2008;22(14):1821-7.
318   53.   Assaram S, Magula NP, Mewa Kinoo S, Mashamba-Thompson TP. Renal
319   manifestations of HIV during the antiretroviral era in South Africa: a systematic scoping
320   review. Syst Rev. 2017;6(1):200.
321   54.   Husain NE, Ahmed MH, Almobarak AO, Noor SK, Elmadhoun WM, Awadalla H, et
322   al. HIV-Associated Nephropathy in Africa: Pathology, Clinical Presentation and Strategy
323   for Prevention. J Clin Med Res. 2018;10(1):1-8.
324   55.   Fiseha T, Tamir Z, Seid A, Demsiss W. Prevalence of anemia in renal insufficiency
325   among HIV infected patients initiating ART at a hospital in Northeast Ethiopia. BMC
326   hematology. 2017;17:1.
327   56.   Ministry of Health Malawi. 3rd Edition of the Malawi Guidelines for Clinical
328   Management of HIV in Children and Adults 2016.
329   57.   McGann PT, Williams AM, Ellis G, McElhinney KE, Romano L, Woodall J, et al.
330   Prevalence of inherited blood disorders and associations with malaria and anemia in
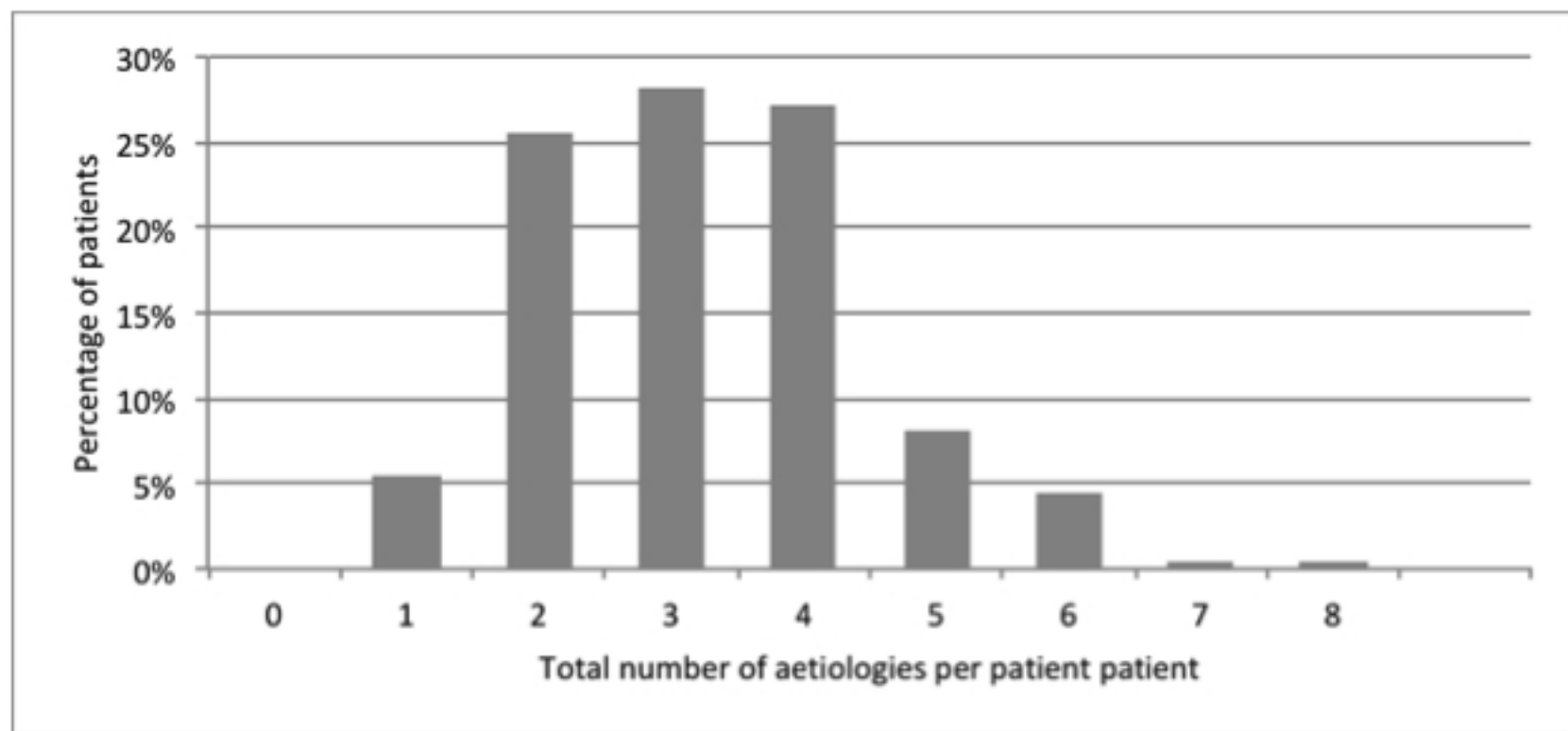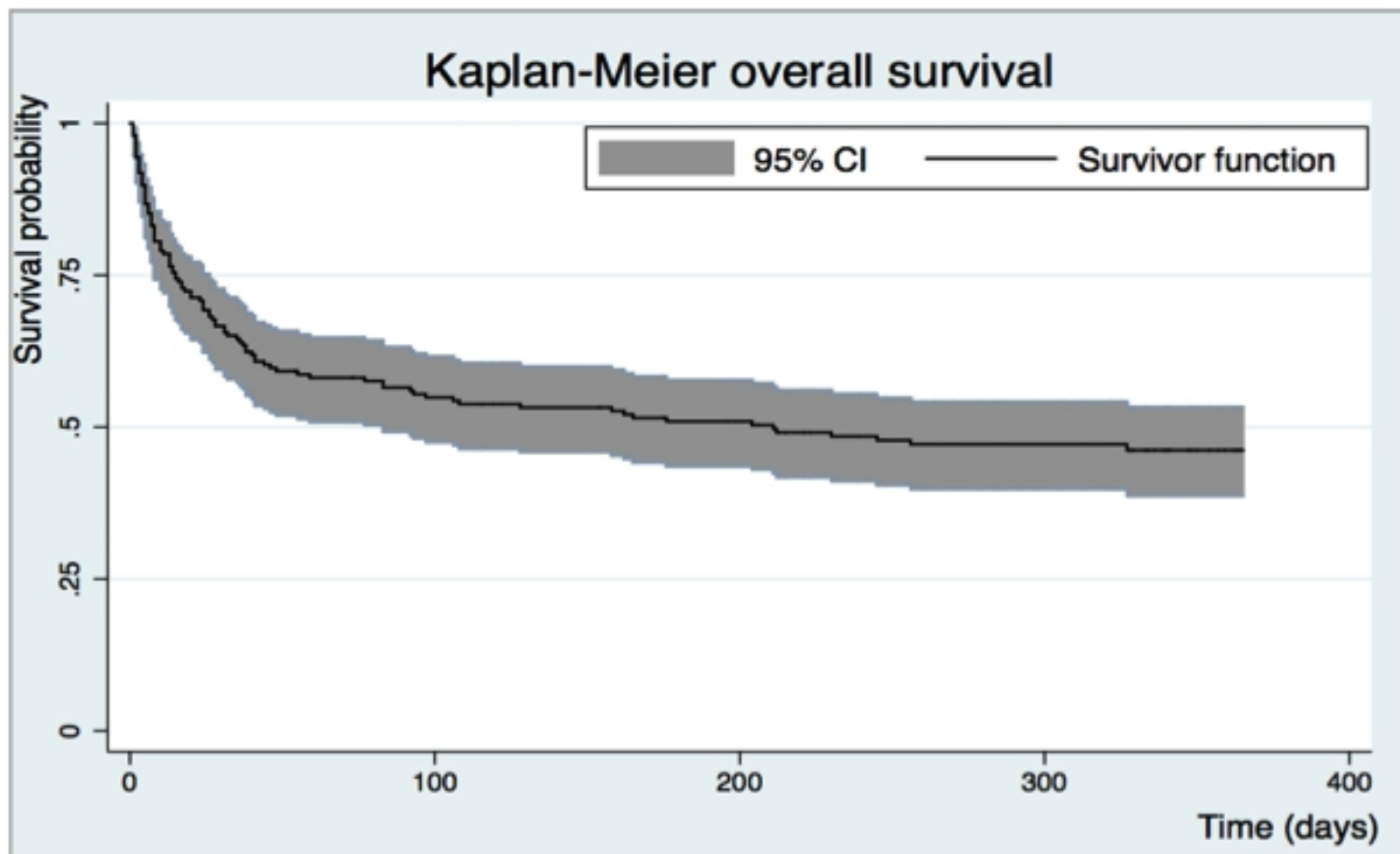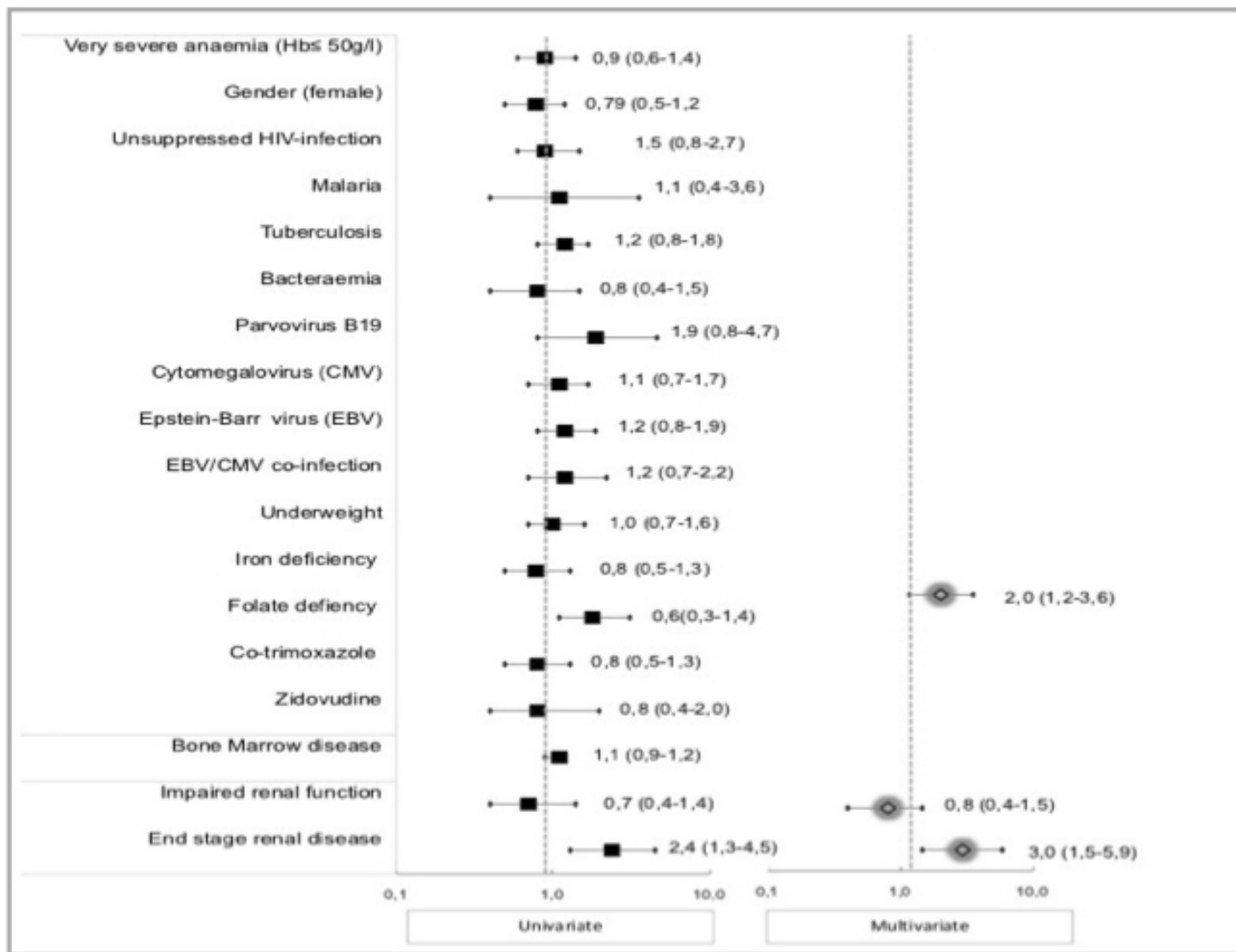331   Malawian children. Blood advances. 2018;2(21):3035-44.
332
333

Figure 1

Figure 2

	Univariate	Multivariate
Very severe anaemia (Hb≤ 50g/l)	0,9 (0,6-1,4)	
Gender (female)	0,79 (0,5-1,2	
Unsuppressed HIV-infection	1,5 (0,8-2,7)	
Malaria	1,1 (0,4-3,6)	
Tuberculosis	1,2 (0,8-1,8)	
Bacteraemia	0,8 (0,4-1,5)	
Parvovirus B19	1,9 (0,8-4,7)	
Cytomegalovirus (CMV)	1,1 (0,7-1,7)	
Epstein-Barr virus (EBV)	1,2 (0,8-1,9)	
EBV/CMV co-infection	1,2 (0,7-2,2)	
Underweight	1,0 (0,7-1,6)	
Iron deficiency	0,8 (0,5-1,3)	
Folate defiency	0,6(0,3-1,4)	2,0 (1,2-3,6)
Co-trimoxazole	0,8 (0,5-1,3)	
Zidovudine	0,8 (0,4-2,0)	
Bone Marrow disease	1,1 (0,9-1,2)	
Impaired renal function	0,7 (0,4-1,4)	0,8 (0,4-1,5)
End stage renal disease	2,4 (1,3-4,5)	3,0 (1,5-5,9)

Figure 3