Chapter 2

Severe anaemia in Malawian children

Job CJ Calis M.D., Kamija S Phiri M.D., E Brian Faragher Ph.D., Bernard J Brabin FRCPCH, Imelda Bates M.D., Luis E Cuevas M.D., Rob J de Haan Ph.D., Ajib I Phiri M.D., Pelani Malange M.Sc., Mirriam Khoka B.Sc., Paul JM Hulshof M.Sc., Lisette van Lieshout Ph.D., Marcel GHM Beld Ph.D., Yik Y Teo Ph.D., Kirk A Rockett Ph.D., Anna Richardson B.Sc., Dominic P Kwiatkowski FRCP, Malcolm E Molyneux FRCP, Michaël Boele van Hensbroek M.D.

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ABSTRACT

Background

Severe anaemia is a major cause of morbidity and mortality in African children. Current preventive and curative strategies focus on treatment of malaria and hookworm and supplementation with iron and folic acid. Yet the causes of the anaemia have been inadequately studied.

Methods

We conducted a case-control study of 381 preschool children with severe anaemia (haemoglobin concentration <5g/dL) and 757 children without severe anaemia in urban and rural settings in Malawi. Causal factors associated with severe anaemia in the past were studied. The data were examined using multivariate analysis and structural equation modelling.

Results

Bacteraemia (Adjusted Odds Ratio, AOR:5.3, 95%-Confidence Interval, 95%-CI:2.6-10.9), malaria (AOR:2.3, 95%-CI:1.6-3.3), hookworm (AOR:4.8, 95%-CI:2.0-11.8), HIV infection (AOR:2.0, 95%-CI:1.02-3.8), *G6PD*$^{-202/-376}$ (AOR:2.4, 95%-CI:1.3-4.4), and deficiency of vitamin A (AOR:2.8, 95%-CI:1.3-5.8) or vitamin B12 (AOR:2.2, 95%-CI:1.4-3.6) were associated with severe anaemia. Folate deficiency, sickle cell disease, and laboratory signs of an abnormal inflammatory response were uncommon. Iron deficiency was less prevalent in case-patients (AOR:0.37, 95%-CI:0.22-0.60) and was negatively associated with bacteraemia. Malaria was associated with severe anaemia in the urban area (seasonal transmission), but not in the rural (holoendemic) setting. Seventy-six percent of hookworm infections were found in children aged under two years.

Conclusions

In severely anemic children current recommendations promoting iron and folate-supplements and ignoring bacteraemia and vitamin B12 deficiency may not be applicable. Even in the presence of malaria parasites, additional or alternative causes of severe anaemia should be considered.

BACKGROUND

Severe anaemia (haemoglobin concentration <5g/dl) is a major cause of morbidity and mortality in sub-Saharan African children[1-4]. In different settings, 12-29% of hospitalized children are severely anemic[1-4], and in these children the in-hospital case fatality rate is 8-17%[1;3;4]. Little is known of the cause of severe anaemia in African children. Most studies have been confined to the anaemia of malaria[5,6] or other individual factors[1;2]. As a result, treatment guidelines advocated by the World Health Organization deal specifically with malaria, folate deficiency, and iron deficiency, which are widely held to be the commonest causes of severe anaemia in African children[7]. To improve our understanding of severe anaemia, we conducted a case-control study to assess causative factors in Malawian children with severe anaemia.

METHODS

Study sites

The study was conducted in Malawi at Chikwawa District Hospital in a rural area where malaria infections occur throughout the year (~170 infectious bites/person/year) and at Queen Elizabeth Central Hospital, a referral hospital in urban Blantyre, where malaria is seasonal (~1 infectious bites/person/year, T. Mzilahowa, personal communication). Predefined catchment areas were used; the urban area was confined to the city limits.

Study design

Between July 2002 and July 2004 a consecutive sample of children (382 cases) who presented at the outpatient department during working hours with a primary diagnosis of severe anaemia (defined as a haemoglobin concentration <5.0 g/dL) were recruited into a prospective case-control study. Additional inclusion criteria were: age 6-60 months and no transfusion within the previous month. For each case, a community control and a hospital control were enrolled. Community controls were recruited from apparently healthy residents living within 100-1000 meters of the case; hospital controls were recruited by selecting the first child presenting at the outpatient department on the same time of the working day following presentation of the case. Community controls and hospital controls were eligible for recruitment if their haemoglobin was $\geq$5.0 g/dL and they were aged 6-60 months, no other matching was applied. Written consent was obtained from a parent or guardian of children in all three study groups. The study was approved by the ethics committees of the College of Medicine, Malawi, and the Liverpool School of Tropical Medicine, UK.

Clinical assessment and management

On enrolment, a clinical research form, including a medical and dietary history, socio-demographic data and physical examination was completed, and samples of blood, urine, and stool were collected. In case-patients, if the clinical condition permitted, a bone marrow aspirate was obtained under local anaesthesia. Children requiring admission were treated in a study ward. All conditions were managed according to standard protocols.

Anthropometry

Nutritional Z-scores were calculated according to the WHO-growth reference curves[8] using EPI info 2000. 'Wasting' (weight-for-height), 'stunting' (height-for-age) or 'underweight' (weight-for-age) applied to children with Z-scores <-2 and were considered 'severe' if <-3.

Laboratory methods

Laboratory tests (haematology, bacteriology and parasitology) were performed within 24 hours and aliquots were stored at -80°C for later analysis. Laboratory staff were blinded to the child's study group.

Haematology:

Haemoglobin concentration was measured on site using a Hemocue system (Angelholm, Sweden). A complete blood count, including reticulocytes, was performed by Coulter counter (Coulter, Hialeah, Fla). In case-patients, bone marrow slides were stained (HaematoGnost Fe, Darmstadt, Germany) and graded for iron content[9]; these results were used to validate peripheral blood markers for iron deficiency. The soluble transferrin receptor/log ferritin-index (TfR-F Index)[10] best predicted bone marrow iron status irrespective of the presence of infection, and was used to define iron deficiency (TfR-F Index>5.6: sensitivity 70%, specificity 75%)[11].

Chemistry:

Plasma levels of C-reactive protein, haptoglobin, transferrin, iron, ferritin, folate and vitamin B12 were analyzed on a Roche p800/e170 system (Roche, Switzerland). Inflammatory cytokine profiles were measured by Cytometric Bead Array on a FACS-Calibur flow-cytometer (Becton-Dickinson, South Africa). Serum vitamin A (retinol) and soluble transferrin receptor were measured using high performance liquid chromatography[12] and enzyme linked immuno-sorbant assay (Ramco Laboratories, TX) respectively.

Parasitology:

Plasmodium falciparum asexual parasites were counted against 200 white blood cells and expressed per microliter blood. Malaria slides were read by two independent readers,

a third being used if results differed by >25%. 'Malaria' was defined by the presence of asexual *P.falciparum* parasites. 'Recent or current malaria' was defined by the presence of asexual *P.falciparum* parasites in erythrocytes or malaria pigment in monocytes or macrophages[13]. 'Hyperparasitaemia' was defined as >100,000 parasites/μL[2]. Stool samples were examined for helminths using the Kato-Katz method[14]. For hookworm, 'heavy infection' was defined as >1000 ova/g feces. A polymerase chain reaction (PCR) was used to confirm microscopy and define subspecies (*Ancylostoma duodenalis and Necator americanus*)[15]. Urine specimens were examined for *Schistosoma haematobium*, using a semi-quantitative concentration method[16].

Bacteriology:

A bone marrow or venous blood sample (1-2ml) was inoculated into BACTEC Myco/F-Lytic culture vials and incubated in a BACTEC 9050 automated system during 56 days. Sub-culturing, susceptibility testing and isolate identification were in accordance with standard techniques[17]. Cultures were checked for mycobacteria using Ziehl-Neelsen stained slides. Mixed growth or growth of micrococci, *Bacillus* species or coagulase-negative staphylococci were considered contaminants.

Virology:

Whole blood isolates[18] were assessed for Epstein-Barr virus (EBV) and cytomegalovirus (CMV) infection by semi-quantitative PCR[19] and for parvovirus by real-time PCR[20]. Infections were considered clinically important if the number of viral copies exceeded 1000c/mL blood. HIV testing was performed using two rapid tests (Determine, Abbott-Laboratories, Japan; Unigold, Trinity-Biotech, Ireland). Discordant and reactive results in children less than 18 months were resolved by PCR[21].

Genetics:

DNA was extracted using a Nucleon extraction kit (Amersham biosciences, UK) and genotyped by primer extension mass-spectrometry using MassArray (Sequenom)[22]. Sickle cell disease (homozygous HbS) and single nucleotide polymorphisms in the promoter region of *IL10* (-1117, -3585, +4949)[22] and *TNF* (-238, -308, -1031)[23] were analyzed. The term $G6PD^{-202/-376}$ is used to denote boys who were hemizygous and girls who were homozygous for both the *G6PD*202A and *G6PD*376G allele; which is strongly predictive of glucose-6-phosphate dehydrogenase deficiency[24]. The Hardy-Weinberg equilibrium was applied (cut-off: $P<0.001$) and there was no significant evidence of population stratification. We chose the allele frequency, dominant model or haplotype that was most strongly associated with severe anaemia.

Statistical Methods

The prevalence of each factor was compared individually across the three groups using the Fisher exact and chi-square tests. The combined association of characteristics

Table 1. Characteristics of the study groups.

		Case-patients	Community Controls	Hospital Controls
Total sample size		381	380	377
Aread:	Urban	205 (53.8%)	203 (53.4%)	201 (53.3%)
	Rural	176 (46.2%)	177 (46.6%)	176 (46.7%)
Sex:	Female	203 (53.3%)	191 (50.3%)	180 (47.7%)
	Male	178 (46.7%)	189 (49.7%)	197 (52.3%)
Age in months[a]	mean (s.d.)	20.4 (12.8)	25.3 (13.1)	22.5 (12.1)
Jaundice[b]		19 / 379 (5.0%)	1 / 380 (0.3%)	0 / 376 (0.0%)
Splenomegaly (>1 cm)[b]		237 / 372 (63.7%)	108 / 363 (29.8%)	86 / 349 (24.6%)
Fever (>37.5°C axillary)[c]		189 / 376 (50.3%)	41 / 375 (10.9%)	172 / 374 (46.0%)
Hemoglobin (g/dL)[b]	mean (s.d.)	3.6 (0.8)	9.9 (1.9)	9.6 (2.2)
	(not recorded)		*(7)*	*(2)*
MCV (fL)[b]	mean (s.d.)	82.9 (15.2)	75.5 (9.3)	74.2 (9.7)
	(not recorded)	*(65)*	*(58)*	*(63)*
Reticulocytes (*10^9/L)[a]	median (iqr.)	53.2 (30.2-91.7)	76.8 (46.4-114.7)	64.5 (43.0-103.2)
	(not recorded)	(115)	(96)	(98)
Admitted to hospital[d]		381 (100%)	3 (0.8%)	17 (4.5%)
Died in hospital[d]		24 (6.3%)	0	0

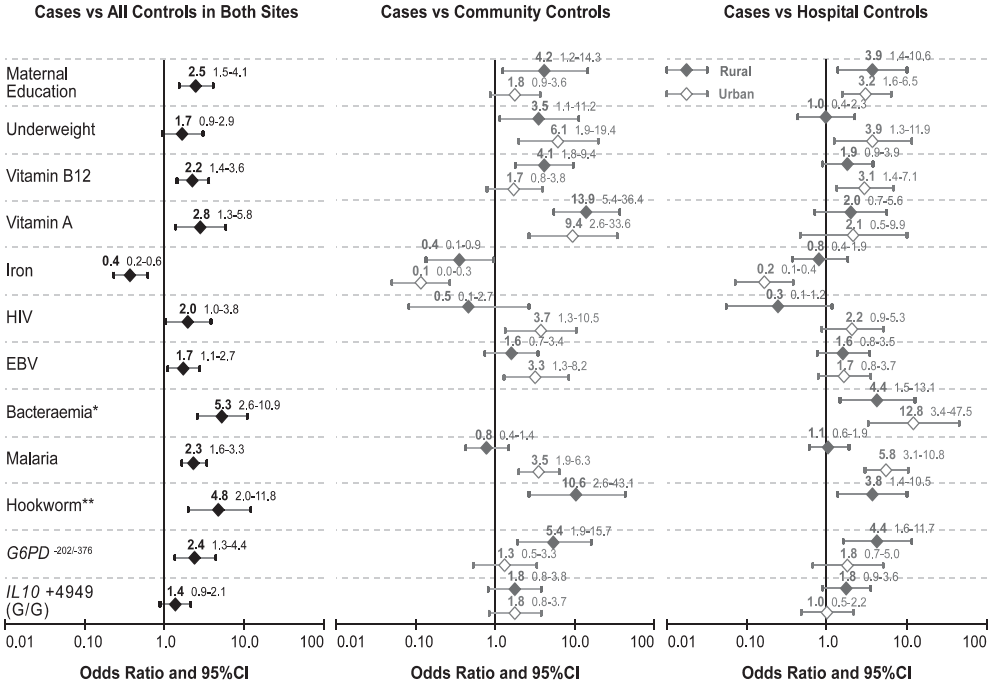
[a] Differences between all three groups statistically significant (Tukey post hoc test or Kruskal-Wallis test with Tukey multiple comparisons $p < 0.05$), [b] Community and hospital controls significantly different from case-patients (Tukey post hoc test, $p < 0.05$), [c] Community controls significantly different from case-patients and hospital controls (Tukey post hoc test, $p < 0.05$), [d] No statistical tests applied,
MCV: Mean cellular volume; s.d.: standard deviation; iqr: interquartile range; Splenomegaly: > 1cm palpable below the left costal margin in the mid axillary line

associated with the risk of severe anaemia ($P \leq 0.10$, unless uncommon) was examined using an unconditional multivariate logistic regression model correcting for potential confounding factors (age, sex, recent use of anti-malarials or haematinics, and previous transfusions). Missing observations were included in the analysis by creating 'missing value' categories. Alternative definitions for malaria, hookworm and nutritional deficiencies and status were tested. Attributable risk percentages were calculated using Adjusted Odds Ratios (AOR)[25]. The primary analysis compared all case-patients with both control groups combined. To explore the possibility that different patient characteristics were important in the two study locations, secondary analyses were performed stratified by location and with the community control and hospital control groups separated. More complex associations and alternative strategies for handling missing data (e.g. maximum likelihood imputation) were explored using structural equation modeling[26]. All reported P-values are two-sided. Data were analyzed using STATA 9 (Stata Corporation, TX), SPSS 12 and AMOS 6.0 (SPSS, IL).

RESULTS

Over a two year period, we enrolled 1141 children. Five protocol violations occurred: two hospital control children had severe anaemia and were re-designated as case-patients; one case, with a haemoglobin concentration of ≥5 g/dL, and two controls, aged less than six months, were excluded. Table 1 summarizes characteristics of the 1138 children included in the analysis. Haemoglobin levels were significantly different between the case-patients and the two controls groups, but similar between the control groups. Splenomegaly (>1cm palpable) or severe splenomegaly (≥8 cm) were more common in case-patients (P<0.001 and P=0.03, respectively). Severe splenomegaly, present in 11

Figure 1. Adjusted odds ratios and 95% confidence intervals for factors associated with severe anaemia by study group and recruitment site.



* Cultures only performed in case-patients and hospital controls. ** Hookworm was not entered in the 'urban' model because the prevalence was <5%. CI: Confidence interval; Wasting was defined as a Z-score for weight-for-height <-2[8]; Iron deficiency was defined as TfR/log(ferritin) ratio >5.6[10;11]; Concentrations of vitamin B12 <200 ng/L and vitamin A <20 µg/dL were considered deficient; HIV: Human immunodeficiency virus; EBV: Epstein-Barr virus; The rs-classification for IL10 +4949:wis rs3024500. The model was corrected for possible confounders: age, sex, recent anti-malarial treatment, recent hematinic treatment, previous transfusions and death of a parent. Owing to the high correlation between the three IL-10 polymorphisms, only one (most strongly associated to severe anemia) was included in the multivariate model. In the combined model interaction existed between malaria and site (p<0.001). The goodness-of-fit of the model was evaluated using the Hosmer and Lemeshow test (P=0.65).

Table 2. Distribution of possible aetiological and confounding factors amongst study groups by recruitment site.

	Both Sites	
	Cases n=381	**CC+HC n=757**
History		
Mother did not attend secondary school	323 / 366 (88%)	554 / 753 (74%)***
Death of a parent	25 / 284 (8.8%)	22 / 554 (4.0%)**
Recent Hematinics	85 / 376 (23%)	61 / 754 (8.1%)***
Recent Antimalarials	232 / 375 (62%)	346 / 755 (46%)***
History of Transfusion	57 / 378 (15%)	38 / 756 (5.0%)***
Malnutrition		
Wasting	52 / 330 (16%)	43 / 695 (6.2%)***
Iron deficiency	97 / 208 (47%)	288 / 415 (69%)***
Vitamin B12 deficiency	95 / 312 (30%)	94 / 603 (16%)***
Vitamin A deficiency	228 / 247 (92%)	172 / 262 (66%)***
Viral infections		
HIV	45 / 357 (13%)	41 / 682 (6.0%)***
Parvo B19	5 / 294 (1.7%)	2 / 609 (0.3%)*
EBV	89 / 269 (33%)	102 / 566 (18%)***
Bacterial infections		
Bacteremia	54 / 359 (15%)	14 / 353 (4.0%)***
Parasitic Infections		
Malaria parasites	226 / 380 (59%)	321 / 750 (43%)***
Hyperparasitemic malaria	45 / 380 (12%)	24 / 750 (3.2%)***
Recent or current malaria infection	243 / 334 (73%)	336 / 696 (48%)***
Hookworm	29 / 296 (10%)	12 / 642 (1.9%)***
Schistosoma mansoni	2 / 296 (0.7%)	8 / 643 (1.2%)
Schistosoma haematobium	4 / 307 (1.3%)	8 / 669 (1.2%)
Genetic disorders		
G6PD$^{-202/-376}$	44 / 318 (14%)	54 / 601 (9.0%)*
Sickle Cell Disease	4 / 238 (1.7%)	4 / 404 (1.0%)
IL10 -1117 (C/C+C/T vs. T/T)	196 / 324 (60%)	332 / 607 (55%)†
IL10 -3585 (A/A vs. A/T+T/T)	22 / 308 (7.1%)	25 / 575 (4.3%)†
IL10 +4949 (G/G vs. G/A+A/A)	97 / 322 (30%)	134 / 606 (22%)**
Abnormal IL-10/TNF-α ratio	4 / 276 (1.4%)	n/a

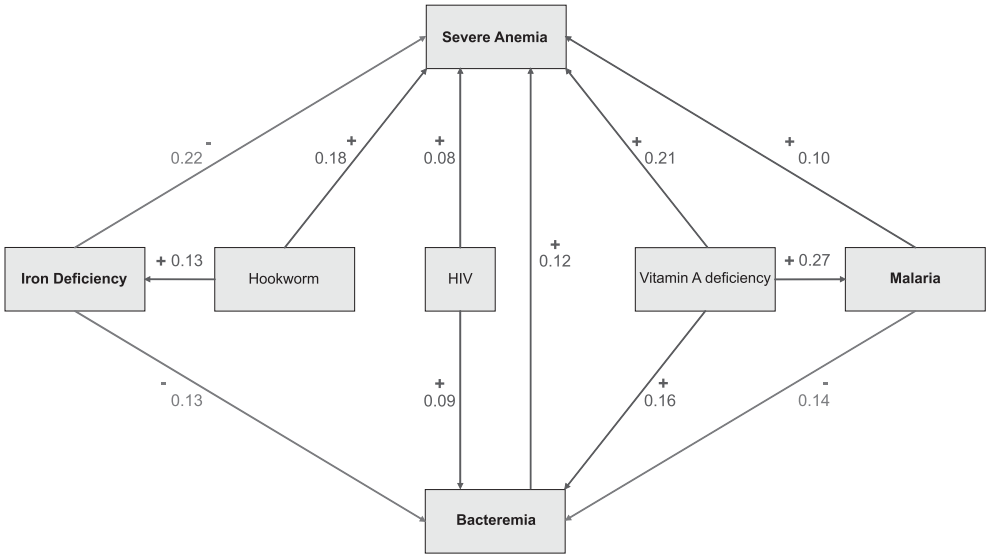
All comparisons vs. cases. †: $p \leq 0.100$ *: $p \leq 0.050$ **: $p \leq 0.010$ ***: $p \leq 0.001$

CC: Community Controls; HC: Hospital Controls; Recent use of antimalarials or hematinics applied to a period of 4 weeks prior to recruitment. HIV: Human Immunodeficiency Virus; EBV: Epstein-Barr Virus; Wasting was defined as a Z-score for weight-for-height < -2[8]. Iron deficiency was defined as TfR/log(ferritin) ratio >5.6[10;11]; Concentrations of folate <3.0 μg/L, vitamin B12 <200 ng/L and vitamin A <20 μg/dL were considered deficient; Malaria was defined as the presence of *P.falciparum* parasites, Hyperparasitemia was defined as >100,000 P.falciparum parasites per microliter blood; An IL-10/TNF- α ratio <1 was defined as abnormal[23].

Rural			Urban		
Cases n=176	**CC n=177**	**HC n=176**	**Cases n=205**	**CC n=203**	**HC n=201**
162 / 172 (94%)	154 / 176 (88%)*	143 / 176 (81%)***	161 / 194 (83%)	142 / 200 (71%)**	115 / 201 (57%)***
13 / 168 (7.7%)	5 / 163 (3.1%)†	3 / 164 (1.8%)*	12 / 116 (10%)	6 / 109 (5.5%)	8 / 118 (6.8%)
40 / 176 (23%)	7 / 177 (4.0%)***	13 / 176 (7.4%)***	45 / 200 (23%)	19 / 202 (9.4%)***	22 / 199 (11%)**
107 / 176 (61%)	79 / 177 (45%)**	90 / 176 (51%)†	125 / 199 (63%)	77 / 203 (38%)***	100 / 199 (50%)*
24 / 176 (14%)	9 / 177 (5.1%)**	13 / 176 (7.4%)†	33 / 202 (16%)	7 / 203 (3.4%)***	9 / 200 (4.5%)***
24 / 169 (14%)	9 / 174 (5.2%)**	19 / 175 (11%)	28 / 161 (17%)	6 / 182 (3.3%)***	9 / 164 (5.5%)***
71 / 101 (70%)	76 / 95 (80%)	63 / 92 (68%)	26 / 107 (24%)	76 / 113 (67%)***	73 / 115 (63%)***
46 / 142 (32%)	20 / 143 (14%)***	30 / 149 (20%)*	49 / 170 (29%)	24 / 157 (15%)**	20 / 154 (13%)***
113 / 126 (90%)	44 / 83 (53%)***	60 / 74 (81%)†	115 / 121 (95%)	32 / 60 (53%)***	36 / 45 (80%)**
7 / 176 (4.0%)	5 / 176 (2.8%)	9 / 172 (5.2%)	38 / 181 (21%)	9 / 171 (5.3%)***	18 / 163 (11%)*
2 / 143 (1.4%)	0 / 147 (0%)	1 / 146 (0.7%)	3 / 151 (2.0%)	1 / 157 (0.6%)	0 / 159 (0%)
43 / 128 (34%)	34 / 133 (26%)	24 / 127 (19%)**	46 / 141 (33%)	14 / 148 (9.5%)***	30 / 158 (19%)**
20 / 171 (12%)	not done	9 / 166 (5.4%)*	34 / 188 (18%)	not done	5 / 187 (2.7%)***
91 / 176 (52%)	93 / 175 (53%)	93 / 176 (53%)	135 / 204 (66%)	74 / 199 (37%)***	61 / 200 (31%)***
17 / 176 (10%)	3 / 175 (1.7%)**	11/176 (6.3%)	28 / 204 (14%)	3 / 199 (1.5%)***	7 / 200 (3.5%)***
113 / 169 (67%)	98 / 171 (57%)†	98/175 (56%)*	130 / 165 (79%)	83 / 180 (46%)***	57 / 170 (34%)***
27 / 154 (18%)	4 / 160 (2.5%)***	8 / 156 (5.1%)***	2 / 142 (1.4%)	0 / 164 (0%)	0 / 162 (0%)
2 / 154 (1.3%)	4 / 160 (2.5%)	4 / 156 (2.6%)	0 / 142 (0%)	0 / 164 (0%)	0 / 163 (0%)
4 / 159 (2.5%)	6 / 168 (3.6%)	1 / 162 (0.6%)	0 / 148 (0%)	0 / 171 (0%)	1 / 168 (0.6%)
21 / 152 (14%)	11 / 141 (7.8%)†	9 / 145 (6.2%)*	23 / 166 (14%)	20 / 161 (12%)	14 / 154 (9.1%)
2 / 118 (1.7%)	2 / 101 (2.0%)	0 / 86 (0%)	2 / 120 (1.7%)	0 / 106 (0%)	2 / 111 (1.8%)
98 / 155 (63%)	75 / 141 (53%)†	83 / 147 (56%)	98 / 169 (58%)	92 / 162 (57%)	82 / 157 (52%)
8 / 148 (5.4%)	6 / 134 (4.5%)	5 / 140 (3.6%)	14 / 160 (8.8%)	4 / 153 (2.6%)*	10 / 148 (6.8%)
48 / 155 (31%)	31 / 140 (22%) †	34 / 147 (23%)	49 / 167 (29%)	34 / 162 (21%) †	35 / 157 (22%)
1 / 122 (0.8%)	n/a	n/a	3 / 154 (1.9%)	n/a	n/a

The rs-classification for the genetic markers were *IL10* -1117: rs1800896; *IL10* -3585: rs1800890; *IL10* +4949: rs3024500; *TNF* -238: rs361525; *TNF* -308: rs1800629; *TNF* -1031: rs1799964. Among variables not included in the table (because they did not meet the pre-set cut of significance) are: Parental unemployment, Number of household assets, Folate deficiency, Trichuriasis, Ascariasis, CMV infection, Hemoglobin C and TNF-α alleles/genotypes (-238, -308, -1031).

Figure 2. Structural equation model for severe anemia, iron deficiency and malaria.



Exploratory model of the factors associated to severe anemia[26]. Size of the associations is indicated by numbers (standardized regression coefficients; range: -1.0/+1.0). Negative (protective) associations are indicated by red lines. This model was created containing all possible associations between the displayed variables after which all non significant arrows (p>0.05) were removed. The model furthermore contained all other variables entered in the multivariate model (omitted for clarity). The displayed variables were all adjusted for age; in addition, malaria was adjusted for previous use of anti-malarials and iron deficiency was adjusted for previous transfusions or hematinics (omitted for clarity). Replacement of severe anemia by continuous hemoglobin levels and iron deficiency (TfR/log ferritin index>5.6)[10;11] by the alternative definitions used in this paper resulted in a virtually identical model. The overall model fit was valid (RMSEA: 0.043 (0.039-0.048).

case-patients (3.0%), was not associated with thrombocytopenia or leukopenia. Jaundice was more common in cases (5%, Table 1) but was not associated with sickle cell disease, *G6PD*$^{-202/-376}$ or splenomegaly (P=1.00, 0.70 and 0.30 respectively). Twenty-four case-patients (6.3%) died during admission, nine (38%) before receiving a transfusion. We obtained 1105 (97%) peripheral blood, 1024 (90%) stool, 1042 (92%) urine, and 348 (91% of case-patients) bone marrow samples. Table 2 lists the features we investigated in the three groups and indicates P values for differences. Factors significantly associated with severe anaemia were further explored in a multivariate and structural equation model (Figure 1 and 2).

Malaria

P.falciparum was identified in 226 (59%) case-patients and 321 (43%) controls and was the predominant malaria species overall (97.5%). *P.malariae* was found in 1.6% and a mixed infection in 0.9%, equally distributed between the study groups. The attributable risk of *P.falciparum* to severe anaemia was 34% overall and 47% in the urban setting.

In the rural setting, a significant association between malaria and severe anaemia was found only in the subgroup (10%) with hyperparasitaemia (AOR: 7.1, 95% CI: 1.4-34.6, case-patients vs. community controls).

HIV

HIV infection was found in 86 children (13% of case-patients and 6% of controls). The attributable risk of HIV to severe anaemia was 6.2% overall, and 15% in the urban setting. In severely anemic children, the presence of EBV (15/30 vs. 69/226, P=0.03) or bacteraemia (11/42 vs. 38/300, P=0.02) was more common among HIV-infected children than among uninfected children, while hyperparasitaemia (2/44 vs. 42/312, P=0.09) and vitamin B12 deficiency (5/39 vs. 85/254, P=0.009) were less common.

Bacteraemia

Fifty-four (15%) case-patients and 14 (4%) controls had bacteraemia. The attributable risk of bacteraemia to severe anaemia was 12.2%. The most common pathogen was non-typhoid salmonella, which was present in 38 (70%) of the case-patients and 11 controls (79%, P=0.54) with bacteraemia. None of the specimens grew mycobacteria. Fever was absent in 37% of children with bacteraemia. In both case-patients and controls bacteraemia was less common in children with malaria than in those without (21/208 vs. 32/150, P=0.003 and 3/146 vs. 11/207, P=0.12).

Nutrition

Fifty-two (16%) case-patients and 43 (6%) controls had wasting, the attributable risk to severe anaemia being 6.2%. Severely anemic children were commonly stunted or underweight (53% and 49%), but for both conditions the unadjusted and adjusted odds ratios were similar to those for wasting (data not shown). Severe wasting occurred in 3.7% of severely anemic children. Vitamin B12 deficiency was found in 95 (30%) case-patients and 94 (16%) controls and was severe (<136 ng/L [100 pmol/L]) in 11% of case-patients and 2.8% of controls (AOR: 4.3, 95%CI: 1.9-9.9). Macrocytosis was more common in vitamin B12 deficient children than in children with normal B12 levels (P=0.02), though the sensitivity for vitamin B12 deficiency was low (18%). Severely anemic children with vitamin B12 deficiency had a history of less meals with meat than those not deficient (1.9 vs. 2.7 per month, P=0.02). Folate deficiency was not found in any child enrolled in the study. Vitamin B12 and folate levels were inversely correlated to each other among severely anemic children (Pearson correlation coefficient: -0.22, P=0.01). Vitamin A deficiency was found in 92% of case-patients and 66% of controls, and was considered severe (<10 μg/dL) in 33% of all case-patients and 15% of controls (AOR: 1.6, 95%CI: 0.91-2.8). Deficiency was associated with malaria and bacteraemia in the structural equation model. Iron deficiency was found in 47% of case-patients and 69% of controls. Further exploration indicated this finding was not affected by the definition used (Table

Table 3. Prevalence of iron deficiency in relation to the development of severe anemia using several peripheral blood markers.

	Prevalence of iron deficiency		Multivariate Analysis	
	Case-patients	Controls	Odds Ratio	95%-CI
Original definition				
TfR/Ferritin-Index	97 / 208 (47%)	288 / 415 (69%)	0.37	0.22-0.60
Alternative definitions				
CRP-containing Index	35 / 208 (17%)	212 / 415 (51%)	0.29	0.16-0.53
Microcytosis	48 / 316 (15%)	182 / 636 (29%)	0.47	0.29-0.76
Hypochromasia	137 / 314 (44%)	294 / 637 (46%)	0.61	0.41-0.91
Microcytosis & Hypochromasia	26 / 316 (8.2%)	108 / 638 (17%)	0.40	0.22-0.72

The odds ratios were obtained by replacing the original variable for iron deficiency in the multivariate analysis by the alternative definition
Iron deficiency was defined as:
Transferrin Receptor (TfR)/Ferritin-Index>5.6 [TfR (mg/L) / Log ferritin (μg/L)][10;11.]
CRP-containing index<0 [0·34+0·0043 x ferritin-(2·7 x TfR)/ferritin+0·00696 x CRP+0·05 x TfR] (all expressed in mg/L)[27].
Microcytosis: Mean Cellular Volume <67fL (<2 years old) and <73fL (2-5 years old) (WHO).
Hypochromasia: Mean Cellular Haematocrit Concentration <32g/L (WHO).
Others markers assessed but not presented since they predicted iron deficiency less well (sensitivity and/or specificity<40%) were ferritin, TfR, serum iron, serum transferrin, total iron binding capacity and transferrin saturation.

3). In the structural equation model iron deficiency was found to be negatively associated with bacteraemia (P=0.006).

Hookworm

Hookworm was the most common helminth infection. Thirty-one (76%) of the hookworm infestations occurred in children less than two years old. The attributable risk to severe anaemia in the rural site, where 95% of infections were seen, was 16%. In this site heavy infections occurred in 10% of all case-patients and 0.6% of controls (AOR: 9.4, 95%CI: 2.0-45). A PCR was performed on 36 of 41 positive samples (88%). *A.duodenale* was found in 81%, *N.americanus* in 8% and a mixed infection in 11%. Hookworm infestation was associated with iron deficiency (P=0.003, Figure 2).

Genetics

No association was found between severe anaemia and sickle cell disease or trait (P=0.40, 0.20). Jaundice was uncommon in severely anemic children with sickle cell disease or *G6PD*$^{-202/-376}$ (0% and 2% respectively). Haptoglobin levels were commonly decreased (<0.30 g/L) in *G6PD*$^{-202/-376}$ case-patients (78%). Boys accounted for 68% of children with *G6PD*$^{-202/-376}$ but after stratification *G6PD*$^{-202/-376}$ remained significantly associated with severe anaemia in both sexes (AOR-girls: 4.1, 95%CI: 1.2-13.3; boys: 2.2, 1.1-4.7).

DISCUSSION

In many African hospitals, severe anaemia is a leading cause of admissions and a major contributor to death. The cause of the anaemia has not been comprehensively investigated, but we found several important associations in this study.

Malaria is commonly considered to be a principal cause of severe anaemia in Africa[7]. In this study, *P.falciparum* parasitaemia was strongly associated with severe anaemia in the area with seasonal transmission but not in the holoendemic transmission area. However, the cumulative impact of malaria on the individual is difficult to assess in holoendemic settings where children are repetitively infected. Our findings therefore do not exclude malaria as a predisposing cause of severe anaemia in the rural area, but indicate that additional or alternative diagnoses should be considered in severely anemic children who are diagnosed with a malaria infection. In the structural equation model, malaria and bacteraemia were identified as variables modifying the association between vitamin A deficiency and severe anaemia. This is in line with earlier observations that vitamin A deficiency is associated with an increased susceptibility to infection[28]. A vitamin A supplementation trial showed a reduction in the incidence of malaria[29], though this and another study failed to show that vitamin A supplementation reduced the incidence of severe anaemia[29;30].

We found a negative association between iron deficiency and severe anaemia. The structural equation model partly explains this finding by indicating that iron deficiency was negatively associated with bacteraemia. This finding supports the hypothesis that iron deficiency protects against infection by creating an unfavourable environment for bacterial growth[31;32]. It is also in agreement with observations of increased morbidity and mortality in iron supplementation studies in areas where bacterial infections are common[32;33]. Although iron supplementation may play a role in preventing anaemia[33], supplementation following severe malaria-anaemia had no haematological benefits and resulted in an increased morbidity in Tanzanian children[34].

In rural children with severe anaemia, we found an increased prevalence and intensity of hookworm infections, *A.duodenale* being the predominant species. Three-quarters of the hookworm infested children were less than two years of age. This age group would have remained untreated according to the current guidelines[7]. Although usually hookworm is increasingly found in older children, younger children might be more vulnerable to severe haematological complications especially in the presence of heavy infections with *A.duodenale*[35].

Bacteraemia, most commonly due to non-typhoid salmonella, was strongly associated with severe anaemia. This association has been described previously[17;36;37] but is not

reflected in current management guidelines for severe anaemia in children[7]. In the structural equation model, bacteraemia was also identified as a mediating variable of the effect of HIV on severe anaemia. Although bacteraemia might not necessarily be a cause of severe anaemia, its high prevalence may justify antibiotic treatment in the standard management of severe anaemia in settings where HIV is prevalent and blood culture facilities are not available.

Although folate supplementation is recommended by WHO, deficiency was not found in our study groups. We may have underestimated its prevalence, because folate deficiency can be masked by vitamin B12 deficiency[38] and we measured plasma rather than erythrocyte folate concentrations. However, our findings concur with previous reports[39] and observations that folate supplementation in anemic children with malaria failed to raise haemoglobin concentrations[40]. Unlike folate, vitamin B12 is not recommended in the management of severe anaemia. In our population vitamin B12 deficiency was found in 30% of case-patients and was associated with severe anaemia. This is in line with findings in adults in this region[41;42] and may be explained by the lack of animal products in the diet of Malawian children.

G6PD$^{-202/-376}$ was found in 14% of case-patients and was associated with severe anaemia, while sickle cell disease was uncommon in our setting. The roles of these mutations may be different in West and Central Africa. Possible associations between IL-10 and TNF-α and severe malaria anaemia have been described[23;43], but in our study an imbalance in circulating plasma levels of IL-10 and TNF-α was uncommon.

We found that several independent yet overlapping conditions are associated with severe anaemia in Malawian children. Our findings indicate that even in the presence of malaria parasites, additional or alternative diagnoses should be considered. Current treatment recommendations promoting iron and folate-supplements and ignoring bacteraemia, vitamin B12 deficiency and, in young children, hookworm infections appear to be of limited applicability in our setting. Our findings, if confirmed in different settings, will contribute to the assessment of new therapeutic and preventive strategies for Africa.

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