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Malaria, Hookworms and Recent Fever Are Related to Anemia and Iron Status Indicators in 0- to 5-y Old Zanzibari Children and These Relationships Change with Age[1]

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ABSTRACT In Zanzibar and other tropical regions, iron deficiency, malaria and multiple helminth infections coexist. We addressed the following questions: *1)* What are the predictors of low hemoglobin in Zanzibari preschool children? *2)* Are indicators of iron status informative in this population? *3)* Does malaria modify the relation of iron indicators to hemoglobin? We used multivariate regression to analyze cross-sectional data from a community-based sample of rural Zanzibari children who were not ill (n = 490; 4–71 mo of age) in whom we assessed hemoglobin, serum ferritin (SF), erythrocyte protoporphyrin (EP), serum transferrin receptor (TfR), recent fever, malaria parasitemia and helminth fecal egg counts. Of hemoglobin values, 80% were <100 g/L and 15.5% were <70 g/L. In children <18 mo of age, 40.2% of hemoglobin values were <70 g/L. Our primary findings were as follows: *1)* In children <30 mo old, hemoglobin was associated with malaria but not hookworms, whereas in children ≥30 mo, hemoglobin was related to hookworms but not malaria. In the younger age group, male sex and recent fever also predicted lower hemoglobin. *2)* The three iron indicators were informative in this population but did not reflect only iron status. Malaria elevated SF in younger children and TfR and EP in both age groups. Fever elevated SF in older children and EP in both age groups, but not TfR. *3)* Malaria modified the relation of all three indicators to hemoglobin. The relation of SF to hemoglobin was weak overall, and absent in malaria-infected children. EP and TfR were strongly related to hemoglobin, but this relation was attenuated by malaria. J. Nutr. 130: 1724–1733, 2000.

KEY WORDS: • *iron* • *anemia* • *malaria* • *children* • *helminth infection* • *nutrition assessment*

In many tropical regions, anemia, iron deficiency, malaria and multiple helminth infections coexist and are interrelated. Sub-Saharan Africa epitomizes this situation, although similar situations exist in equatorial South America, and South and Southeast Asia. In these communities, anemia is typically prevalent and severe, especially in pregnant women and young children, and may be an important cause of mortality (Stoltzfus 1997). For example, the case fatality rate for children admitted with severe anemia to one hospital in rural Tanzania was 6.1% (Alonso Gonzalez et al. 2000).

In contexts such as this, it has been difficult to elucidate the relative contributions of iron deficiency, malaria, and helminth infections to anemia (Gillespie and Johnston 1998) for several reasons. Randomized trials of highly efficacious interventions can demonstrate causal relationships with confidence, but relatively few such trials have been conducted, and very few have reported attributable risks. Lacking these, the independent contributions of these underlying factors could be estimated from cross-sectional data, but large and wel acterized data sets are required because the underlying themselves are related, and their effects are difficult to se statistically. Furthermore, the validity of iron status ind in these contexts is uncertain (Hercberg et al. 1987, Y Dallman 1988). Thus, it has been difficult to categoriz dren as iron deficient or iron replete when they are ir with multiple parasites.

To address some of these issues, we conducted a ra ized, placebo-controlled factorial trial of iron supplemer and anthelminthic treatment of preschool children ir Pemba Island, Zanzibar, Tanzania, where malnutrition *modium falciparum* malaria and geohelminths are high demic. We assessed parasitic infections quantitatively, mothers to recall morbidity symptoms in the week assessment, and also measured hemoglobin, erythrocyte porphyrin (EP),[3] serum ferritin (SF) and serum tran receptor (TfR).

We present in this paper the relationships among pa

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[3] Abbreviations used: EP, erythrocyte protoporphyrin; SF, serum ferr transferrin receptor.

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infections, iron status indicators and erythropoiesis before intervention. These cross-sectional analyses provide evidence about the relation of anemia and malarial infection, which is not addressed by the intervention trial. The cross-sectional data may also provide a truer estimate of the risk of anemia attributable to helminth infection. Although the causal nature of the association may be established by the randomized trial, it will underestimate the magnitude because the intervention does not completely remove the infection (Albonico et al. 1999). The cross-sectional associations between infection and iron status indicators may also elucidate the extent to which these indicators relate to iron status or anemia in infected children.

We therefore used multivariate regression methods to address the following questions: *1*) Does subclinical infection with *P. falciparum*, *Ascaris lumbricoides*, *Trichuris trichiura*, hookworms or recent fever predict hemoglobin concentration in Zanzibari preschool children? *2*) Do EP, SF and TfR provide useful information about iron status in this setting? *3*) Does subclinical *P. falciparum* infection modify the relation of iron indicators to hemoglobin?

We addressed these questions previously in a cross-sectional analysis of data from Zanzibari school children, and found that malaria had little influence on SF, EP or hemoglobin concentrations, or their relations to each other (Stoltzfus et al. 1997a). However, we speculated that the answers to the same questions would be different for preschool children, whose immune response to these endemic diseases is different from that of older children. The present analysis allows us to test this hypothesis. In addition, we have added serum TfR to our series of indicators in this study.

SUBJECTS AND METHODS

Study population and sample. This study was carried out in Kengeja village, in Pemba Island, Tanzania. Pemba is the smaller and northern of the two islands that comprise Zanzibar, located just off the east coast of mainland Tanzania. Pemba is almost entirely rural, and the population survives on fishing, farming, and cultivation of cloves and seaweed for export. The staple foods are cassava and rice. Meat is eaten rarely, and large fish are relatively expensive. Small fish, legumes and vegetables are eaten more commonly. *P. falciparum* malaria is holoendemic, with year-round transmission. *P. malariae* is also present in <5% of infections (Stoltzfus et al. 1997a). A peak in malaria parasite density is observed in July, following the rainy season in April–May (M. Albonico, unpublished data). Both hookworm species, *Ancylostoma duodenale* and *Necator americanus*, are transmitted on the island (Albonico et al. 1998).

In June–July 1996, we performed a census of the entire village to ascertain the number of children, family structure and basic socioeconomic characteristics of each household. Households with preschool children were assigned a household identification number. We then created a database of all children who would be 6–59 mo of age as of September 1, 1996, according to the parent's report of the child's age. This yielded a sampling frame of 684 children. Of the 684 children invited to enter the trial, 614 completed the baseline assessment in September 1996. At the time of the baseline assessment, we verified each child's age using an official document, such as birth certificate or immunization record. Children's official ages ranged from 4 to 71 mo, and this is the age variable used for analysis.

Data from 490 children are used in these analyses because of the small amount of serum available for assays. After using drops of whole blood for a blood film, and hemoglobin and EP determinations, the remaining serum was collected and assayed for SF and TfR, in that order. Of 614 children assessed at baseline, 124 samples had insufficient serum to complete all of the analyses. Of the 490 children included in these analyses, 23 were missing helminth infection data (see below) and 3 were missing maternal report of recent fever. Thus, in some multivariate models, the sample size is reduced to 464.

This research was approved by ethical review committees of The Johns Hopkins University School of Public Health, the World Health Organization, and the Ministry of Health of Zanzibar.

Assessments. On the day before a child was scheduled for the baseline assessment, we visited the home to invite the parents to participate in the trial. If they gave their informed consent, we asked them to bring a small sample of their child's feces to the baseline clinic, and gave them a container for this purpose. Fecal samples were stained on the same day and examined within 1 h of staining by the Kato-Katz method (WHO 1994). Parents were highly cooperative with fecal assessment, and helminth egg counts were obtained for all but 23 children in this study sample (95.3%).

At the baseline clinic, any child with a temperature >38.0°C was referred for treatment and rescheduled for assessment on another date. For afebrile children, we asked the mother whether the child had experienced cough, fever, rapid or difficult breathing, liquid stools or bloody stools during the week before the assessment. These questions were asked using local terms for these symptoms, and answers were recorded as yes or no.

Blood (3 mL) was collected by venipuncture into a vacutainer tube with serum separator gel. Drops of whole blood were dispensed immediately to make a blood film, and for determination of hemoglobin using a HemoCue hemoglobinometer (HemoCue, AB, Angelhom, Sweden) and EP using a fluorometer (Aviv Biomedical, Lakewood, NJ). The remaining blood was centrifuged at 1000 × g for 20 min at room temperature and serum was collected.

Thin blood films were fixed with ethanol and stained with Giemsa, and malaria parasites were counted against leukocytes. The microscopist counted fields containing >200 leukocytes. If <10 parasites were seen, the counting continued up to 500 leukocytes. Parasite densities were calculated by assuming 8×10^9 leukocytes/L blood (Trape 1985).

Sera were stored in Pemba at −10°C for up to 3 mo, then transported in liquid nitrogen to Baltimore, MD, where they were stored at −70°C until analysis. Ferritin was assayed using a fluorescence-linked immunoassay (DELFIA System by Wallac, Gaithersburg, MD). The average CV for this assay was 3% (range: 0.2–7.0%). TfR was assayed by ELISA (Râmco, Houston, TX), with an average CV of 4% (range: 0.2–11.8%).

Statistical analysis. In initial analyses, we did not want to impose any prior expectation on the relation of the iron status indicators to each other or to hemoglobin. Therefore, we built regression models separately for hemoglobin, SF, EP and TfR without including other iron status indicators as independent variables.

Ferritin concentrations were skewed to high values; therefore they were log transformed during model fitting and then reexpressed in their natural units for reporting. Hemoglobin was normally distributed. EP and TfR were somewhat skewed to high values, but the log transformation did not make the distributions Gaussian, nor did it significantly improve the fit of the models; thus we analyzed them in their natural scales.

The following independent variables were considered: malaria parasitemia, *Ascaris* infection, *Trichuris* infection, hookworm infection, fever in the past week, sex and age. Malaria parasite densities and helminth fecal egg counts were considered both as dichotomous variables (positive vs. negative) and as categories of infection intensity. Categorical variables were modeled as dummy variables; that is, an ordinal relation was not imposed. Both main effects and interactions between variables were considered. Except for the TfR model, at least one key relation in each model was modified by age; that is, infectious disease had different effects on iron status or erythropoiesis in younger children compared with older children. This statistical interaction was consistently strongest when age was dichotomized using 30 mo as the cutoff. Therefore, we present results separately for these two age groups. Even when modeling the two age groups separately, age in months was always entered into the model and was retained if it remained significant in either age group.

We present the results from these regression models as the adjusted least-squares means and SEM estimated by the model for each level of the independent variable. These values may be interpreted as the adjusted mean value of the dependent variable for a given level of the independent variable, at the average value for all other inde-

TABLE 1

Iron status indicators, prior fever and parasitic infections in Zanzibari preschool children, by age[1]

Age	n	Hemoglobin	Transferrin receptor	Erythrocyte protoporphyrin	Serum ferritin	Fever in prior week	Malaria	Ascaris	Trichuris	Hookworms
mo		g/L	mg/L	μmol/mol heme	μg/L	% positive				
4–11	39	77 ± 16[2]	18.5 ± 9.8	249 ± 123	23.5 (10.4, 53.0)[3]	55.3	76.9	17.1	17.1	17.1
12–23	112	78 ± 15	16.7 ± 7.9	225 ± 132	24.2 (9.1, 64.5)	54.5	80.4	31.1	43.4	24.5
24–35	116	88 ± 14	14.7 ± 10.5	168 ± 90	37.4 (7.5, 91.6)	49.1	79.3	37.3	72.7	41.8
36–47	100	91 ± 11	12.4 ± 7.1	149 ± 84	34.4 (14.4, 82.0)	36.4	85.0	47.5	82.8	68.7
48–59	91	92 ± 15	12.1 ± 6.6	144 ± 83	30.6 (12.6, 74.4)	40.7	92.3	52.3	90.7	76.7
60–71	32	93 ± 15	11.4 ± 4.6	151 ± 132	23.0 (10.8, 48.9)	25.8	90.6	45.2	83.9	83.9
All ages	490	87 ± 15	14.3 ± 8.5	178 ± 111	29.9 (12.0, 74.4)	45.2	83.7	39.8	68.1	51.0
Median		88	12.2	154	30.6					
Range		36–124	2.9–67.8	14–682	1.1–458.4					

[1] For helminth infections, total n = 467. Missing observations by age group: 4, 6, 6, 1, 5, 1.
[2] Mean ± SD.
[3] Geometric mean (−1 SD, +1 SD)

pendent variables in the model (i.e., adjusted for other variables in the model). We tabulated separately the adjusted mean values for children <30 mo and ≥30 mo to present the magnitude of the effect modification by age. The significance level (P-value) of each age interaction term is given in the footnotes of Tables 2, 3 and 5.

In addition to these primary models, we tested certain prior expectations by adding iron status indicators into the hemoglobin model. We expected that SF, EP and serum TfR would act as indicators of iron status, and that anemia would be caused at least in part by iron deficiency. Thus, their association with hemoglobin would reflect to some extent their usefulness as iron status indicators. We also expected that hookworms would be associated with low hemoglobin, and that effect would be mediated through iron status. Therefore, in our regression model for hemoglobin, we expected that adding the three iron status indicators would significantly increase the fit of the model, that these iron status variables would attenuate each other within the model and would also attenuate the effect of hookworm.

To test whether malaria modified the relation of anemia to iron status indicators, we added hemoglobin and a hemoglobin × malaria interaction term to the multivariate models for SF, TfR and EP. We modeled hemoglobin as a categorical dummy variable, based on the quartiles of the overall distribution, so as not to impose a linear relation between hemoglobin and the dependent variable. We reduced the four malaria parasite density categories used previously to three categories by combining the middle two categories, because of small sample size in some categories and because the middle two categories gave similar results in the models. Thus the malaria × hemoglobin interaction term yielded 12 subgroups, with n ranging from 9 to 95. We examined these malaria × hemoglobin interaction models separately for the younger and older age groups, but present the results for the age groups combined because differences by age were either small or statistically weak ($P > 0.05$), given the available sample size. These analyses were conducted using SYSTAT statistical software (SPSS, Chicago, IL).

RESULTS

Distribution of anemia, iron status indicators and infections. Anemia was highly prevalent and severe, with 80.4% of hemoglobin concentrations <100 g/L and 15.5% of values <70 g/L. Severe anemia was strongly concentrated in children <18 mo of age, with 40.2% having values <70 g/L. Serum TfR and EP concentrations were well above normal limits, consistent with iron deficiency (**Table 1**); however, SF concentrations were not correspondingly low. Only 13.9% of SF values were <12 μg/L, and the median value was 30.6 μg/L. Hemoglobin, TfR and EP concentrations were strongly associated with age, improving with age from 4 to 35 mo, and reaching a stable but still abnormal mean value in children >36 mo of age.

Malaria parasitemia was present in >75% of children at all ages, but tended to increase in prevalence with age (P = 0.004; Table 1). Nearly half of the mothers (45.2%) reported that their child had fever in the week before the clinic visit. Fever was not strongly related to malaria parasitemia. In children ≥30 mo, there was no relation between fever and the presence of parasitemia or parasite density. However, in children <30 mo, fever was more common in children with ≥5000 parasites/μL blood (59.4% prevalence) than in children with <5000 parasites/μL blood (35.0%, P = 0.007 by chi-square test). There was no association between fever and malaria parasitemia below this threshold.

Infection with each of the three geohelminths increased steeply with age. Only 5% of children ≥48 mo were negative for all three geohelminths. By chance, the prevalence of all three geohelminths in the youngest age group was identical, representing 6 out of 35 infants infected with each geohelminth. However, these were not the same 6 infants. Of the 35 infants with fecal smears, 12 were infected with at least one helminth. Seven were infected with one, four were infected with two, and one infant was infected with all three helminths. This last-mentioned infant was an 11-mo-old girl whose hemoglobin, SF, EP and serum TfR concentrations were 68 g/L, 4.9 μg/L, 395 μmol/mol heme and 16.4 mg/L, respectively.

The three geohelminth infections were significantly associated with each other, even after adjusting for age. In *Trichuris*-infected vs. noninfected children, the age-adjusted odds ratio for hookworm was 4.7 (95% confidence interval: 2.8–7.9) and for *Ascaris* was 4.1 (2.5–6.9). Hookworm and *Ascaris* infection were less strongly associated. The age-adjusted odds ratio for *Ascaris* infection associated with hookworm infection was 1.9 (1.2–2.9).

Hemoglobin and anemia. In children <30 mo of age, sex, recent fever and malaria parasite density were strongly related to hemoglobin concentration (**Table 2**). Boys had a hemoglobin deficit of 7 g/L compared with girls. Children with recent fever had hemoglobin values that were 7 g/L lower on average than those without recent fever. The strongest predictor of

TABLE 2

Hemoglobin model used to analyze data from Zanzibari children 4–71 mo of age

Independent variable	Adjusted hemoglobin concentration (g/L)					
	Age < 30 mo[1]			Age ≥ 30 mo		
Prior fever						
No	85 ± 2[2]	(89)	$P = 0.000$	89 ± 2	(162)	$P = 0.057$
Yes	78 ± 1	(110)		86 ± 2	(103)	
Sex						
Male	78 ± 1	(100)	$P = 0.000$	88 ± 2	(120)	$P = 0.441$
Female	85 ± 1	(99)		87 ± 2	(145)	
Malaria parasites, *n/μL blood*						
0	92 ± 2	(47)	$P = 0.000$	88 ± 3	(25)	$P = 0.973$
1–999	83 ± 2	(44)		88 ± 2	(80)	
1000–4999	77 ± 2	(82)		88 ± 2	(124)	
≥5000	73 ± 3	(26)		87 ± 2	(32)	
Hookworm eggs, *n/g feces*						
0	79 ± 1	(144)	$P = 0.044$	95 ± 1	(85)	$P = 0.000$
1–1999	83 ± 2	(55)		91 ± 1	(156)	
2000–3999	—	(0)		89 ± 3	(15)	
≥4000	—	(0)		76 ± 4	(9)	
		$R^2 = 0.344$			$R^2 = 0.091$	

[1] Significance levels of variable × age-group interaction: prior fever, $P = 0.098$; sex, $P = 0.007$; malaria parasite density, $P < 0.001$; hookworm infection (positive vs. negative), $P < 0.001$.

[2] Least-squares mean ± SEM (*n*), adjusted for age and all other variables listed. *P*-values from general linear regression model as described in Subjects and Methods.

hemoglobin concentration in this age group was malaria parasite density. In the lowest and highest categories of malaria parasite density, mean hemoglobin concentration differed by 19 g/L. All hookworm infections in this age group were of low intensity. Hookworm-infected children had average hemoglobin values 4 g/L higher than children without infection.

In children ≥30 mo old, there was no sex difference in hemoglobin, and the difference with recent fever became smaller (3 g/L) and marginally significant ($P = 0.057$). Hemoglobin concentrations did not vary with malaria parasite density, but decreased strongly with increasing hookworm infection intensity. In this older age group, heavy hookworm infection intensities (>4000 eggs/g feces) were observed, but were relatively uncommon (3.5% prevalence). These heavily infected children had hemoglobin concentrations 19 g/L lower than their uninfected counterparts. In this age group, the regression model explained only 9% of the variation in hemoglobin (multiple $R^2 = 0.091$).

The different effects of malaria and hookworm infections on hemoglobin in the two age groups were also apparent when severe anemia (hemoglobin <80 g/L) was considered as the outcome (**Fig. 1**).

We added the three iron status indicators into this basic regression model to observe their influence on the coefficients for other independent variables and the overall fit of the models. EP was most strongly related to hemoglobin in both age groups ($P < 0.001$ in both models). With EP in the model, serum TfR was significant in the younger children (P

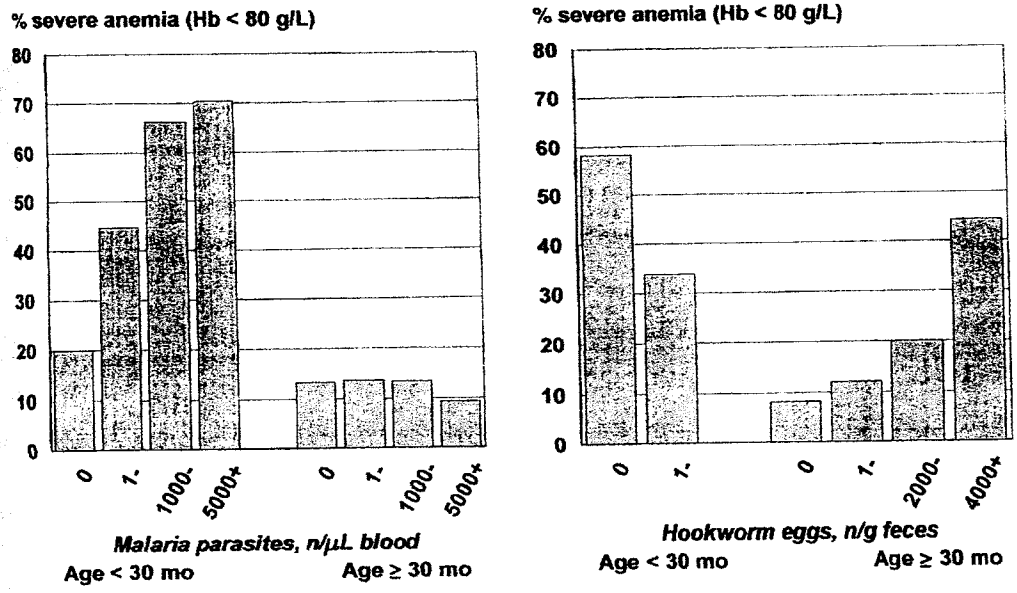

FIGURE 1 Proportion of Zanzibari children with severe anemia (hemoglobin <80 g/L) by malaria parasite density or hookworm fecal egg counts and age group. Chi-square tests for trends of association: malaria parasite density in age <30 mo, $P < 0.00001$, age ≥30 mo, $P > 0.20$. Hookworm fecal egg counts in age <30 mo, $P = 0.002$, age ≥30 mo, $P = 0.005$.

TABLE 3

Serum ferritin model used to analyze data from Zanzibari children 4–71 mo of age

Independent variable	Adjusted serum ferritin concentration (μg/L)					
	Age < 30 mo[1]			Age ≥ 30 mo		
Prior fever						
No	21.6[2]	(89)	P = 0.383	20.6	(162)	P = 0.0(
Yes	24.3	(110)		28.8	(103)	
Malaria parasites, *n/μL blood*						
0	15.5	(47)	P = 0.001	24.7	(29)	P = 0.40
1–999	19.3	(44)		21.1	(80)	
1000–4999	25.6	(82)		24.8	(124)	
≥5000	35.9	(26)		27.4	(32)	
Hookworm eggs, *n/g feces*						
0	22.3	(144)	P = 0.966	35.3	(85)	P = 0.00
1–1999	23.5	(55)		30.2	(156)	
2000–3999	—	(0)		28.3	(15)	
≥4000	—	(0)		11.7	(9)	
Trichuris eggs, *n/g feces*						
0	25.1	(110)	P = 0.240	24.9	(39)	P = 0.34
1–999	31.0	(76)		29.3	(147)	
1000–4999	30.8	(11)		26.5	(70)	
≥5000	11.5	(2)		18.3	(9)	
		R^2 = 0.118			R^2 = 0.141	

[1] Significance levels of variable × age-group interaction: prior fever, $P = 0.177$; malaria parasite density, $P = 0.089$; hookworm infection (positiv vs. negative), $P > 0.20$; trichuris infection, $P > 0.20$.

[2] Geometric least-squares mean (*n*), adjusted for age and all other variables listed. *P*-values from general linear regression model as describe in Subjects and Methods.

= 0.005), but not in the older children ($P = 0.11$). SF added the least to these hemoglobin models and did not retain any significance when EP and TfR were in the model. Addition of EP and TfR to the model greatly increased the fit in both age groups (multiple $R^2 = 0.577$ in younger children and multiple $R^2 = 0.282$ in older children).

With EP and TfR in the model for younger children, the effect of hookworm infection became small and nonsignificant. The effects of sex, fever and malaria were attenuated but remained strong. Adding EP and TfR to the model for older children attenuated but did not remove the effect of hookworm.

Serum ferritin model. In children <30 mo old, SF concentrations increased directly with malaria parasite density (**Table 3**). In children ≥30 mo old, SF was not associated with malaria parasite density, but decreased strongly with increasing intensity of hookworm infection. SF concentration was also higher in children with recent fever in this age group.

Trichuris infection intensity had a similar nonsignificant ($P = 0.20$) pattern of association with SF concentration in both age groups. Ferritin concentrations were slightly elevated in children with light infections and much lower in the few children with ≥5000 eggs/g feces. This relation was significant ($P = 0.041$) when the two age groups were combined.

Serum transferrin receptor. After adjusting for age, the only significant relation between serum TfR and infection was with malaria (**Table 4**), and this association was similar in older and younger children in the study. TfR concentration increased directly with level of malaria parasite density, but the difference was greatest between malaria-negative children and malaria-positive children (mean ± SEM: 12.0 ± 0.9 vs. 14.7 ± 0.4 mg/L, $P = 0.007$).

Erythrocyte protoporphyrin. EP to heme ratios were higher in children with recent fever and were also higher in children with higher malaria parasite densities (**Table 5**). The association with fever was of similar magnitude in younger and older children, and significant ($P = 0.003$) when the ages were combined. The increase in EP with malaria parasite densit was marginally significant in each age subgroup, and statisti cally stronger when the ages were combined ($P = 0.021$). I appears that the relation might have a different threshold ir younger vs. older children in our study. In children <30 mc old, EP values were elevated in children with any parasite present, but in children ≥30 mo old, EP values were elevatec only in children with ≥5000 parasites/μL blood.

EP was associated with hookworm infection in both age groups, but in opposite directions (Table 5). In children <30 mc old, those with light hookworm infections had lower EP values with a difference of 67 μmol/mol heme. Moderate and heavy hookworm infections were not found in this age group. In children ≥30 mo old, EP values were significantly higher in children with ≥4000 hookworm eggs/g feces compared with children with egg counts below this threshold (mean ± SEM: 155 ± 17 vs. 219 ± 30 μmol/mol heme, $P = 0.034$). The reverse association in children with light infection compared with no infection, observed in younger children, was not found in the older age group.

TABLE 4

Serum transferrin receptor model used to analyze data from Zanzibari children 4–71 mo of age

Independent variable	Adjusted transferrin receptor Concentration mg/L		
Malaria parasites, *n/μL blood*			
0	12.0 ± 0.9[1]	(80)	P = 0.036
1–999	14.2 ± 0.7	(128)	
1000–4999	14.8 ± 0.5	(223)	
≥5000	15.5 ± 1.1	(59)	
	R^2 = 0.075		

[1] Least squares mean ± SEM (*n*), adjusted for age. *P*-value from general linear regression model as described in Subjects and Methods.

TABLE 5

Erythrocyte protoporphyrin model used to analyze data from Zanzibari children 4–71 mo of age

Independent variable	Adjusted erythrocyte protoporphyrin (μmol/mol heme)					
	Age < 30 mo[1]			Age ≥ 30 mo		
Prior fever						
No	188 ± 14[2]	(89)	$P = 0.052$	157 ± 11	(162)	$P = 0.036$
Yes	221 ± 12	(110)		181 ± 12	(103)	
Malaria parasites, *n*/μL *blood*						
0	170 ± 18	(47)	$P = 0.082$	167 ± 18	(29)	$P = 0.064$
1–999	198 ± 18	(44)		147 ± 13	(80)	
1000–4999	204 ± 14	(82)		163 ± 12	(124)	
≥5000	244 ± 26	(26)		198 ± 18	(32)	
Hookworm eggs, *n*/*g feces*						
0	238 ± 10	(144)	$P = 0.001$	141 ± 10	(85)	$P = 0.044$
1–1999	171 ± 18	(55)		164 ± 8	(156)	
2000–3999	—	(0)		148 ± 23	(15)	
≥4000	—	(0)		222 ± 30	(9)	
		$R^2 = 0.148$			$R^2 = 0.074$	

[1] Significance levels of variable × age-group interaction: prior fever, $P > 0.20$; malaria parasite density, $P > 0.20$; hookworm infection (positive vs. negative), $P < 0.001$.

[2] Least squares mean ± SEM (*n*), adjusted for age and all other variables listed. *P*-values from general linear regression model as described in Subjects and Methods.

Malaria as an effect modifier. In the SF model (**Fig. 2A**), the interaction term for hemoglobin and malaria was nonsignificant ($P = 0.194$), and the model fit poorly when the hemoglobin interaction was added (multiple $R^2 = 0.082$). SF was related much more strongly to malaria parasite density than it was to hemoglobin. However, in children with a negative blood film for malaria, SF was significantly lower in the lower hemoglobin quartiles than in the upper two quartiles (adjusted geometric mean SF 14.7 vs. 27.0 μg/L, $P = 0.014$). Motivated by the strong age modification of SF predictors in Table 3, we examined further the relationship between SF and hemoglobin in children negative for malaria, by age group. The positive association between hemoglobin quartile and SF derived entirely from children <30 mo of age ($n = 50$ without malaria, $P = 0.055$), and was absent in children ≥ 30 mo of age ($n = 30$ without malaria, $P = 0.60$). The absence of a relationship in older children might be due to the strong influence of fever in this age group (see Table 3).

Adding hemoglobin quartile and the malaria interaction term to the EP model greatly improved the explanatory power of this model (multiple $R^2 = 0.331$; compare with 0.074 and 0.148 in Table 5). EP was strongly related to hemoglobin, regardless of malaria parasite density (Fig. 2B). However, this relation was strongest in children with negative blood films. Malaria modified the relation between hemoglobin and EP, and this modification was different in the lowest hemoglobin quartile than in the upper three quartiles. In children with hemoglobin <76 g/L (lowest quartile), EP values in malaria-infected children (regardless of parasite density) were much lower than those in malaria-negative children. In the upper three hemoglobin quartiles, EP values were not affected by malaria parasitemia unless it exceeded 5000 parasites/μL blood. In these three hemoglobin quartiles, EP values of children with high parasitemia were significantly elevated and rose more steeply with descending hemoglobin level [malaria (≥5000 parasites) × hemoglobin interaction term in upper three quartiles, $P = 0.125$].

In the TfR model (Fig. 2C), both hemoglobin and its interaction term with malaria were significant, and this interaction model fit the data much better (multiple $R^2 = 0.219$) than the simpler model in Table 4. In children with negative blood films, TfR concentration descended steeply with ascending hemoglobin level. In children with low malaria parasite densities, this relation remained strong, but was attenuated. In children with ≥5000 malaria parasites/μL blood, the relation was U-shaped.

The subgroup of children with hemoglobin ≥98 g/L (highest quartile) and malaria parasite density ≥5000 was small ($n = 9$); their adjusted mean TfR value plotted in Figure 2C (21.5 mg/L, range 8.1–51.3) was influenced by two very high values (case A, 48.4 mg/L, and case B, 51.3 mg/L). Case A and case B had malaria parasitemias of 5066 and 5134 parasites/μL blood, identical hemoglobin concentrations of 99 g/L, and EP values of 134 and 73 μmol/mol heme, respectively. To reduce the influence of these two points, we examined the malaria × hemoglobin interaction using the median values of TfR rather than the age-adjusted geometric mean. Using median values, the interaction was less striking, but did not disappear. Median TfR values for ascending hemoglobin quartiles were 18.1, 13.0, 9.9 and 8.6 mg/L for malaria-negative children, and 16.4, 14.8, 12.2 and 13.0 mg/L for children with ≥5000 parasites/μL blood.

DISCUSSION

These results reconfirm some current understandings and also shed new light regarding both the epidemiology of anemia in tropical sub-Saharan Africa, and the assessment of iron deficiency in this and similar situations. To interpret these data, it is important to bear in mind that these children were clinically well on the day of assessment. The fact that malaria parasite density was a significant factor in every analysis reinforces the general conclusion that, in addition to causing a large burden of death, clinical disease and disabling sequelae (Snow et al. 1999), intense transmission of *P. falciparum* infection in young children alters the metabolism of children who are not ill in remarkable ways.

Epidemiology of anemia. Our observation that malaria parasitemia is associated with lower hemoglobin concentration in young children confirms earlier observations of Spencer

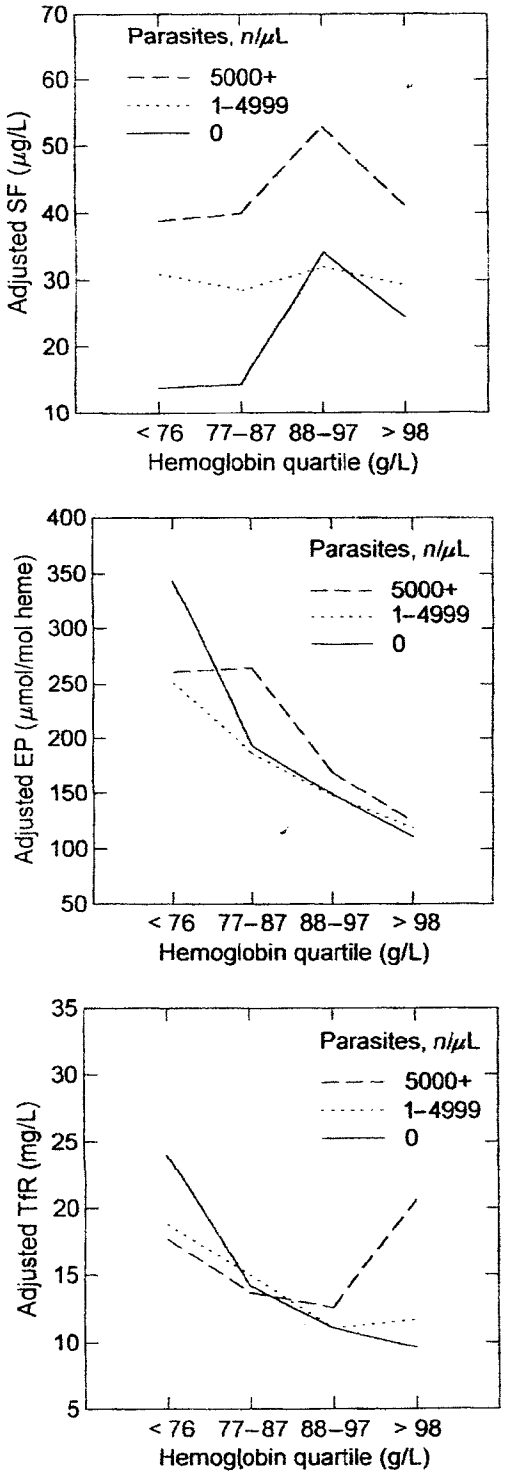

FIGURE 2 Relationships between serum ferritin (SF; *upper panel*), erythrocyte protoporphyrin (EP; *middle panel*) and transferrin receptor (TfR; *lower panel*) and hemoglobin concentration quartile in Zanzibari children, by level of malaria parasite density. Plotted values are least-squares means from multiple regression models presented in Tables 2–4, with hemoglobin and hemoglobin × malaria interaction term added. See Subjects and Methods for details. *P*-values for these terms in each model were: SF model, hemoglobin quartile $P = 0.016$, interaction $P = 0.194$; EP model, hemoglobin quartile $P < 0.001$, interaction $P = 0.038$; TfR model, hemoglobin quartile $P < 0.001$, interaction $P < 0.001$.

(1966) in Papua New Guinea, and MacGregor et al. (1966) in The Gambia, who also observed that the hemoglobin deficit associated with parasitemia decreased with age. In the Pemban children we studied, this relation was absent beyond the age of 30 mo. There was also no significant association of malarial infection and hemoglobin concentration in our previous study of Pemban school children (Stoltzfus et al. 1997a). This is consistent with the development of partial immunity to malaria that is acquired rapidly in areas of very high transmission (Snow et al. 1999).

In these young Pemban children, both hemoglobin and risk of severe anemia were strongly and directly related to the malaria parasite density. It is possible that higher parasite density increases the rate of hemolysis, resulting in a more severe anemia in children who cannot produce new red cells at an equally accelerated rate. Erythropoiesis might be limited by folate deficiency, iron deficiency, insufficient erythropoietin production or cytokine-mediated suppression of the marrow response to erythropoietin. A greater population of circulating parasites might also cause more iron to be incorporated into hemozoin complexes (Brabin 1992).

All three geohelminths were prevalent in the children studied, but only hookworm was associated with hemoglobin or severe anemia. In children ≥30 mo old, these associations were consistent with the well-described intensity-dependent relation between hookworm infection and intestinal blood loss (Roche and Layrisse 1966, Stoltzfus et al. 1996). The hemoglobin deficits associated with hookworm infection in this age group were related to iron deficiency. This was evidenced by the relation of both SF and EP to hookworm infection intensity, and the fact that hookworm's association with hemoglobin was attenuated when iron status indicators were added to the hemoglobin model.

In children <30 mo old, hookworm-infected children had significantly higher hemoglobin concentrations, less severe anemia and lower EP concentrations than uninfected children. Because these were light hookworm infections and, given the young age group, relatively recently acquired infections, it is not surprising that hookworm infection was not associated with iron-deficiency anemia (Roche and Layrisse 1966). It is surprising, however, that hookworm-infected children in this age group had significantly *better* hematologic status. Hookworm infection is transmitted through direct contact of the skin with larvae-infected soil (in contrast to *Ascaris* and *Trichuris* infections, which are transmitted orally). It is possible that young children with severe iron-deficiency anemia are less active or exploratory in their behavior (de Andraca et al. 1997) and are therefore less likely to acquire infection at a very young age.

In children <30 mo old, boys had a sizeable hemoglobin deficit (7 g/L) relative to girls. This was apparently due to poorer iron status and/or erythropoiesis in the boys, because sex did not remain an important predictor of hemoglobin in the multivariate model after EP and TfR were added. The explanation for this is not clear, and it might be due to chance. However, in our study of Zanzibari school children we also found that boys had a small hemoglobin deficit relative to girls (Stoltzfus et al. 1997b).

In the younger age group, children recovering from recent fever were more anemic. Although we adjusted for current malaria parasite density, it is possible that the effect of fever reflects past malarial illness (at least in part) because fever in the model occurred in the week before the day the blood film was made. In our previous study of school children, fever in the past week was also associated with a small hemoglobin deficit (117 vs. 115 g/L, $P = 0.005$, $n = 3298$, R. J. Stoltzfus,

unpublished data), similar to the present observation in older preschool children. We speculate that certain immunologic responses (e.g., secretion of tumor necrosis factor-α) underlie the effect of fever on erythropoiesis and that these responses change with normal child development, creating a strong age modification of fever's effect.

Serum ferritin, erythrocyte protoporphyrin and serum transferrin receptor. SF, EP and TfR are iron status indicators, but each reflects a particular physiologic process. It is important to evaluate how these assessments performed as indicators of iron status in this population, but the present cross-sectional analysis can provide only limited answers. The most informative question may not be "Were they accurate indicators of iron status?" but rather, "What do they tell us about iron metabolism and erythropoiesis in these children?"

SF is an iron storage protein that is also an acute phase protein (Bentley and Williams 1974, Cook et al. 1974, Elin et al. 1977). Both of these properties are evident in the data. The association of SF and recent fever reflects the well-known acute phase response. The strongly age-dependent relation of SF to malaria parasite density mirrors the hemoglobin-parasitemia relationship in these children. It appears that the age-dependent immune mechanisms that protect older children from the anemia of subclinical malarial infection might also remove the SF response to malaria infection. This supports our previous hypothesis that the absence of association of SF and malarial infection in Pemban school children was an age- and immunity-dependent observation (Stoltzfus et al. 1997a).

The malaria-SF relationship disappeared with age, whereas the influence of fever became stronger with age. Indeed, the strongest relationship of SF to hemoglobin was found in the subgroup of children who were malaria-free and <30 mo of age. From this, we infer that the effect of inflammation on SF does not disappear with age; rather, the effect of subclinical malarial infection on inflammation (and therefore SF as an acute phase protein) disappears with age in holoendemic populations. This is consistent with the fact that in Zanzibari school children, malarial infection was not associated with a significant elevation in SF (Stoltzfus et al. 1997a), but recent fever was associated with SF (geometric mean SF in 422 school children who reported fever in the past week, 19.8 vs. 16.9 μg/L in 2606 children who did not report recent fever, $P < 0.001$, R. J. Stoltzfus, unpublished data).

Even in the face of its behavior as an acute phase reactant, SF does carry information about iron status. This is apparent in the strong relation between hookworm infection intensity and SF concentration in the older age group. Hookworm infection, even in children as young as 2–5 y old, causes intestinal blood loss sufficient to deplete iron stores. It is possible that the nonlinear relation of *Trichuris* infection intensity and SF reflects both properties of SF. At low infection intensities, which are likely first infections in this age group, SF is elevated, possibly reflecting an inflammatory response to *Trichuris* in these nonimmune children. At high infection intensities, SF concentrations were very much lower than in the population overall, possibly reflecting intestinal iron loss (Layrisse et al. 1967).

In children with no malaria parasites, SF was significantly higher in those with hemoglobin concentrations above the median value (Fig. 2A), which again reflects ferritin's role as an iron status indicator. However, the shape of the relation was not consistent with solely iron-deficiency anemia. At hemoglobin concentrations <88 g/L, SF concentrations were low (geometric mean 14.7 μg/L) but not as low as expected for this severity of iron-deficiency anemia. This suggests that inflammatory responses in these anemic children were marginally elevating SF, or that the children were not uniformly iron deficient, despite their very low hemoglobin levels. In the upper two hemoglobin quartiles, ferritin concentrations were higher, but did not demonstrate the expected increasing values with increasing hemoglobin. Again, this suggests that ferritin's behavior as an acute phase reactant is obscuring the true relation of hemoglobin to iron stores, or that a large part of the mild-to-moderate anemia in this population is not due to iron deficiency.

EP accumulates during iron-deficient erythropoiesis. Zinc protoporphyrin IX, the form that we measured, is produced when iron is not available for the conversion of protoporphyrin to heme. When the iron supply is adequate, EP is not elevated, even after experimental phlebotomy in humans that reduced their hematocrit to 55% of its original level and increased their reticulocyte counts to 2–3 times normal (Langer et al. 1972). When healthy rabbits were phlebotomized to increase their reticulocyte counts to 7 times normal, EP values became elevated (2.6 times normal) unless the rabbits were transfused with nonviable erythrocytes to provide an additional supply of iron (Langer et al. 1972). Thus EP remains specific to iron deficiency even in the face of significant erythropoiesis.

Therefore, we interpret the inverse relation between hemoglobin and EP in this population to be strong evidence of iron-deficiency anemia. Indeed the association of EP to hemoglobin was stronger than that of serum TfR or SF (which had no association with hemoglobin). The high EP values in children with heavy hookworm infections is evidence for iron-deficient erythropoiesis in those children, as expected from their SF values and from prior knowledge of hookworm-related blood loss.

EP was higher in children with recent fever and in children with malaria parasitemia. Elevations in EP measured by the fluorometric method we used have been reported in patients hospitalized with a variety of diseases, due to substances in the plasma that fluoresce at the same wavelength as zinc protoporphyrin IX (Hastka et al. 1992). These substances can be removed by washing the red cells before measuring the EP (Hastka et al. 1992), a time-consuming process that we did not do. Although the children in our study were not acutely ill, it is possible that those with recent fever had some plasma metabolites that elevated our EP measurements.

Malaria parasitemia modified the relation of EP to hemoglobin in a complex way. At hemoglobin values ≥76 g/L, EP values were elevated only in those children with high parasite densities (who comprised 10% of the sample in that hemoglobin range). Schneider et al. (1993) also reported elevated EP, measured by the Aviv hematofluorometer, in young children from Togo with *P. falciparum* infection. They hypothesized that bile pigments, which fluoresce in this assay, are released into the plasma from parasitized red cells as they are hemolyzed. This might also explain our finding.

At hemoglobin <76 g/L, all children with circulating parasites had markedly lower EP values than malaria-negative children. Zinc protoporphyrin IX is toxic to the malaria parasite through a mechanism that appears to be identical to many antimalarial drugs (Iyer 1999, Martiney et al. 1996), and we hypothesize that children who accumulate very high levels of EP may be protected from parasitemia.

TfR is a surface receptor expressed by nearly all human cells, but 80% of the serum receptor concentration comes from erythroid cells (Huebers et al. 1990). The concentration of serum TfR is elevated by iron deficiency and by erythropoiesis (Kling et al. 1998). TfR was first proposed as a simple marker

of erythropoiesis (Kohgo et al. 1987). Serum TfR is not an acute phase protein and has therefore been proposed as a promising indicator of iron status in populations with prevalent infections (Ferguson et al. 1992), including malarial infection (Kuvibidila et al. 1995). In our data, serum TfR was the only iron status indicator that was not altered in children with recent fever.

We found that malaria-infected children had higher concentrations of serum TfR and that malaria parasitemia modified the expected relation to hemoglobin. We hypothesize that serum TfR in this population of children was reflecting mainly erythropoiesis. According to this hypothesis, children with higher malaria parasite densities experience hemolysis, which reduces the red cell mass and stimulates erythropoiesis. Thus, elevated serum TfR concentration would indicate a physiologic response to the anemia of malaria. Our observation that serum TfR was lower in severely anemic children with malaria parasitemia than in severely anemic children without malarial infection may reflect a suppression of erythropoiesis caused by malaria. This might be caused by suppressed erythropoietin secretion in the anemia of malaria (Vedovato et al. 1999) or by some malaria-induced blockade of the marrow response to erythropoietin (Yap and Stevenson 1994). In either case, the erythroid response to severe anemia would be attenuated, as we observed. Kuvibidila et al. (1995) reported normal serum TfR concentrations (mean value, 5.0 mg/L) in 17 Zairian children with symptomatic malaria, despite a mean hemoglobin concentration of 61 g/L. In the presence of such severe anemia, this lack of erythropoiesis is pathological (Beguin et al. 1993), and consistent with simultaneous hemolysis and suppressed erythropoiesis in acute malaria. In the asymptomatic severely anemic children we assessed, erythropoiesis (as reflected by serum TfR) was suppressed in malaria-infected compared with noninfected children, but was still significantly elevated (mean serum TfR, 17.5 mg/L, compared with normal values of 2.9–8.3 mg/L published using the Ramco ELISA kit (Yeung et al. 1998).

Serum TfR concentrations were also elevated in children with high malaria parasite densities but hemoglobin concentrations >98 g/L. These children were rare (9 of 490), and are remarkable for their capacity to maintain relatively high hemoglobin levels despite high parasitemia. It is possible that these children are compensating effectively for hemolysis by producing new red cells at a faster rate than their more anemic counterparts, perhaps because their cytokine response to malaria is less suppressive to the bone marrow, or because their iron or folate status is sufficient to maintain a high level of erythropoiesis. The relatively low EP values in these 9 children (see Fig. 2*B*) are consistent with a relatively permissive iron supply. In future studies, it would be informative to assess reticulocyte counts together with serum TfR in a population such as this to confirm the extent to which serum TfR is reflecting erythropoiesis (Beguin et al. 1993).

We draw the following conclusions with regard to our three research questions. *1*) Low hemoglobin was related most strongly to malaria parasite density in children <30 mo, and to hookworms in children ≥30 mo. In the younger age group, male sex and recent fever also predicted lower hemoglobin. We conclude from the strong relation of EP to hemoglobin that iron deficiency was also an important cause of low hemoglobin in this population.

2) All three indicators were informative in this population, but they did not reflect only iron status. Malaria infection significantly influenced all three indicators, and fever influenced EP and SF. Even with this reasonably large and well-characterized data set that includes multiple indicators of iron status, it would be nearly impossible to conclude which children were iron deficient or to estimate the absolute prevalence of iron deficiency in this population. The only way to determine the fraction of anemia attributable to iron deficiency in this situation is to conduct a highly supervised course of therapeutic iron supplementation. Nonetheless, each indicator we assessed provided unique information about children's hematologic status.

3) Malaria modified the relation of all three indicators to hemoglobin. This suggests that malaria not only shifts the absolute values of the indicators, but that the indicators reflect different processes in malaria-infected children than in normal children. This effect was especially large and significant in the case of TfR. The effects of subclinical infection on erythropoiesis in young children deserve further study to elucidate both the etiologies of anemia and the best methods for iron status assessment. The interpretation of serum TfR in young children experiencing intense malarial transmission is a particularly salient question.

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