Iron supplementation in anemic Zanzibari toddlers is associated with greater loss in erythrocyte iron isotope enrichment

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ABSTRACT

Background: Heavy parasitic loads increase the risk of iron (Fe) deficiency anemia, which remains prevalent globally. Where parasites are common, understanding the influence of parasitic infections on Fe incorporation and erythropoiesis in toddlers is especially important.

Objectives: The aim of this study was to identify the impacts of malarial and helminth infections on red blood cell (RBC) Fe incorporation and subsequent changes in RBC Fe isotope enrichment for 84 days postdosing in toddlers at high risk for parasitic infections.

Methods: Fe incorporation was measured in a group of Zanzibari toddlers (n = 71; 16–25 months) using a stable Fe isotopic method. At study entry, an oral stable Fe isotope was administered. Blood was collected 14 (D14) and 84 (D84) days postdosing for the assessment of Fe status indicators and RBC isotopic enrichment. Blood and stool samples were collected and screened for malaria and helminth parasites. Factors associated with changes in RBC Fe isotope enrichment were identified using regression models.

Results: Toddlers who had larger weight-for-age z-scores, lower total body Fe, and helminth infections (n = 26) exhibited higher RBC Fe incorporation. RBC Fe isotope enrichment decreased from D14 to D84 by -2.75 percentage points ($P < 0.0001$; n = 66). Greater loss in RBC Fe isotope enrichment from D14 to D84 was observed in those who received Fe supplementation, those with either helminths or both malarial and helminth infections, and in those with greater RBC Fe incorporation on D14.

Conclusions: Toddlers who received Fe supplementation exhibited significantly greater losses of RBC Fe isotope enrichment over time. We speculate this greater loss of RBC Fe enrichment is indicative of increased erythropoiesis due to the provision of Fe among anemic or helminth-infected toddlers. *Am J Clin Nutr* 2021;114:330–337.

Keywords: stable isotope, iron incorporation, toddler, malaria, helminth, red blood cell

Introduction

Iron (Fe) deficiency and iron-deficiency anemia (IDA) are particularly detrimental during the perinatal period, and low Fe stores at this time have been associated with long-term cognitive and developmental deficits (1). In populations with IDA, Fe availability is not sufficient to meet erythropoietic demands. Toddlers living in helminth- and malaria-endemic areas frequently suffer from chronic parasitemia and low-grade inflammation. These parasitic infections, coupled with low dietary Fe intakes, increase the risk of IDA in many developing countries. Mechanisms by which malaria and helminth infection impair Fe homeostasis are multifactorial. Malaria may exacerbate Fe deficiency by sequestrating Fe into malarial pigments (hemazoin) (2) and by blocking Fe recycling from senescent red blood cells (RBC). It may also shorten the RBC lifespan due to erythrophagocytosis of both parasitized and nonparasitized RBCs (3, 4). Helminth infections can lead to chronic gastrointestinal blood loss, increasing the risk of Fe deficiency and anemia (5). In addition, inflammation caused by malarial and helminth infections may further compromise Fe absorption due to increased serum hepcidin concentrations (3, 4).

Fe supplementation can be used to treat Fe deficiency and IDA, but concerns exist when providing supplemental Fe to children residing in malaria-endemic areas (6, 7), particularly if they are Fe replete (8). In malaria-endemic areas, nontargeted Fe supplementation has been associated with increased mortality of preschool children (7, 9). For this reason, the

The study was funded by a Wellcome Trust/Burroughs (grant number GR063122MF).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplemental Figure 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: AOR, adjusted odds ratio; CRP, C-reactive protein; DAE, dose-adjusted enrichment; EPO, erythropoietin; Fe, iron; Hb, hemoglobin; IDA, iron-deficiency anemia; NA, natural abundance; obs, observed abundance; RBC, red blood cell; SF, serum ferritin; sTfR, serum-soluble transferrin receptor; TBI, total body iron; D0, day 0; D14, 14 days postdosing; D84, 84 days postdosing; Δ%xs, delta percentage excess.

Received September 16, 2020. Accepted for publication February 8, 2021.

First published online April 7, 2021; doi: https://doi.org/10.1093/ajcn/nqab044.

WHO now recommends screening for Fe deficiency before Fe supplementation is initiated in malaria-endemic locations (10). In addition, Fe supplementation combined with antimalarial medication is commonly used as an effective strategy for managing postmalarial anemia (11). However, the mechanisms by which helminth and malarial infections interact to impact Fe absorption and responses to treatment are not fully understood (12). A better understanding of the differences and cumulative impact of these infections on the Fe metabolism in children is needed.

This study was undertaken on Pemba Island, Zanzibar, in 2004, when the overall prevalence of *Plasmodium falciparum* malaria was estimated to be 80%, with no distinct evidence of seasonality (13, 14). Moreover, among those aged 10–11 months, helminth and hookworm infections on Pemba Island were endemic (13). The primary outcomes of this study were RBC Fe isotope incorporation at D14 and changes in RBC Fe enrichment over an 84-day period postdosing. The current study was undertaken to *1*) identify the impacts of malarial and helminth infections on Fe incorporation into RBCs in relation to Fe status indicators; and *2*) to identify variables associated with the change in RBC Fe isotope enrichment over an 84-day period postdosing among a group of toddlers at high risk for malarial and helminth infections.

Methods

Subject recruitment and sample collection

Participants were recruited to represent a random sample of healthy toddlers between 16 and 25 months of age with no other known health conditions. Toddlers that had fevers, symptomatic malaria, sickle cell trait, or severe anemia [hemoglobin (Hb) $<$ 7 g/dL] were not recruited into the study. A total of 82 toddlers were recruited from local villages on Pemba Island in November 2004. The stable isotope dosing study was initiated in December 2004 and ended in March 2005. The study protocol was approved by the Committee of Human Research at the Johns Hopkins Bloomberg School of Public Health and the Ministry of Health, Zanzibar. Informed verbal consent was obtained from families willing to participate in the study. On the first day of the study, parents were asked about their child's diet and recent illnesses. Each toddler received a physical examination, and anthropometric z-scores were calculated using the WHO database (15). A finger prick was obtained to assess the Day 0 (D0) Hb and malaria parasitic count.

After D0 measurements were obtained, toddlers ingested a meal of porridge and remained fasted for 1.5 hours before ingesting a stable Fe isotope as ferrous sulfate. Each participant received either ^{58}Fe (0.9 mg of ^{58}Fe, along with 6.0 mg of native ferrous sulfate, to obtain a total Fe dose of 7.0 mg; $n = 37$) or ^{57}Fe (7.0 mg of ^{57}Fe; $n = 34$), based on isotope availability. Tracer doses were administered with flavored raspberry syrup containing 0.391% ascorbic acid (Humco). Toddlers remained fasted for the next 1.5 hours before ingesting a second porridge meal. At 2 weeks postdosing (D14), a 3 mL venous blood sample was obtained. Approximately 3 months postdosing (D84), an additional 5 mL of blood was collected. Following the D84 clinic visit, all participants were treated with mebendazole as previously detailed (16).

Malaria and helminths screening

On D0, D14, and D84, whole blood was screened for malaria parasites using both thick and thin blood films (17). When the D14 blood was obtained, a stool sample was collected for an analysis of the helminth burden (*Ascaris Lumbricoides*, *Trichuris Trichiura*, and hookworm) using the Kato-Katz method (18). Toddlers that had a positive blood smear for malarial parasites on D0, D14, or D84 were treated for malaria with artusonate/amodaquine.

Hematological and Fe status measures

Hb concentrations were measured using a HemoCue Analyzer. All Fe status indicators were analyzed in both D14 and D84 blood samples, except serum-soluble transferrin receptor (sTfR) which was only analyzed in the D14 samples. Any participant identified as anemic on D14 (Hb $<$ 11.0 g/dL) received liquid Fe supplementation (12.5 mg ferrous sulfate/day) (19) from D14 through D84. Serum ferritin (SF) and sTfR were measured by enzyme-linked immunosorbent assay (Ramco Laboratories, Inc). Total body iron (TBI) was calculated by the ratio of sTfR to SF [TBI (mg/kg) = -[log (sTfR$*$1000/SF) -2.8229)/0.1207] (20, 21). Erythropoietin (EPO) and C-reactive protein (CRP) were measured using an Immulite 1000 Analyzer (Siemens Healthcare Diagnostics). We defined depleted Fe stores as SF concentrations $<$12 ug/L if CRP concentrations $<$5 mg/L, or SF concentrations $<$30 ug/L if CRP concentrations $\geq$5 mg/L. Elevated sTfR was classified as a concentration $>$8.5 mg/L. Elevated CRP was defined as a concentration $>$10 mg/L. Archived D14 ($n = 42$) and D84 ($n = 38$) serum samples were used to measure hepcidin by competitive enzyme-linked immunosorbent assay (Intrinsic Life Sciences). Hepcidin values below the limit of detection (5 ng/mL) were assigned a concentration of 2.5 ng/mL.

RBC Fe enrichment and incorporation

Fe isotopic ratios were analyzed using magnetic sector thermal ionization MS (ThermoScientific, Triton TI). Mass spectrometric methods have been previously described in detail (22, 23). The delta percentage excess (Δ%xs) of each administered stable Fe isotope in RBC was determined by the observed (obs) and natural abundance (NA) isotope ratio:

$$\Delta\%\text{xs } {}^{57}\text{Fe} = \frac{{}^{57}\text{Fe}/{}^{56}\text{Fe}_{\text{obs}} - {}^{57}\text{Fe}/{}^{56}\text{Fe}_{\text{NA}}}{{}^{57}\text{Fe}/{}^{56}\text{Fe}_{\text{NA}}} \times 100 \quad (1)$$

A similar equation was used for the ^{58}Fe tracer. Natural abundance ratios used for the ^{57}Fe/^{56}Fe and ^{58}Fe/^{56}Fe ratios were 0.02317 and 0.00307, respectively.

To account for the small differences in tracer doses administered, a dose-adjusted enrichment (DAE) was calculated for each tracer based on the mean dose of each isotope administered. For those receiving the ^{57}Fe tracer, the mean dose of ^{57}Fe administered was 6.64 mg ^{57}Fe. The ^{57}Fe DAE was calculated as:

$$^{57}\text{Fe DAE} = \frac{\Delta\%\text{xs } {}^{57}\text{Fe} \times \text{Average Dose of } {}^{57}\text{Fe}}{\text{Actual Dose of } {}^{57}\text{Fe}} \quad (2)$$

A similar equation was utilized to obtain a DAE after calculating the mean dose of ^{58}Fe (0.891 mg ^{58}Fe) that would give the same enrichment as that obtained with the average ^{57}Fe

TABLE 1 General characteristics of the 71 toddlers[1]

	All toddlers, $n = 71$	Females, $n = 41$	Males, $n = 30$
Age, month	19.47 ± 2.19	19.41 ± 2.06	19.55 ± 2.39
Height,[2] cm	77.18 ± 4.23	75.91 ± 3.60	78.91 ± 4.48
Weight,[3] kg	9.78 ± 1.41	9.13 ± 0.97	10.66 ± 1.45
Weight-for-age z-scores[4]	−0.93 ± 1.06	−1.22 ± 0.93	−0.55 ± 1.13
Underweight,[5] %	15.49 [11]	21.95 [9]	6.67 [2]
Length-for-age z-scores	−1.91 ± 1.19	−2.06 ± 1.09	−1.71 ± 1.29
Stunted, %	39.43 [28]	41.46 [17]	36.67 [11]
Weight-for-length z-scores	0.00 ± 1.28	−0.26 ± 0.98	0.36 ± 1.55
Wasted, %	2.82 [2]	2.44 [1]	3.33 [1]

[1]Data are presented as either mean ± SD or % [n]. Differences between sexes were analyzed by a Student's t-test or Chi-square test.
[2]Significant difference observed between the males and females, $P < 0.005$.
[3]Significant difference observed between the males and females, $P < 0.0001$.
[4]Significant difference observed between the males and females, $P < 0.01$.
[5]Difference between males and females approached significance, $P = 0.08$.

dose administered. Throughout the manuscript, the term "RBC Fe DAE" reflects this value. In addition to the direct measurements of RBC Fe DAE, RBC Fe incorporation was calculated in D14 blood as previously reported (22, 23). The blood volume in toddlers was estimated using the Linderkamp equation (24):

$$\text{Log (Blood Volume)} = 0.6459 * \text{(Log Weight in kg)} + 0.002743 * \text{(Height in cm)} + 2.0324 \quad (3)$$

The change in RBC Fe DAE from D14 to D84 postdosing was determined by subtracting each toddler's D14 DAE from their D84 DAE. Observed decreases in RBC Fe DAE over time may occur due to increases in the size of the circulating RBC pool induced by absorption of natural isotopic Fe or mobilization of storage Fe (25).

Statistical analyses

Descriptive statistics were used to summarize characteristics of participants, using the mean ± SD or geometric mean (95% CI) for continuous variables or frequency counts with percentages for categorical variables. Student's t-tests and Wilcoxon's rank-sum tests were used to assess possible differences in concentrations of biochemical indicators between groups stratified by sex, anemia, presence of malarial or helminth infections, or presence of both parasites. A Pearson correlation or Spearman rank correlation was used to evaluate the strength of the bivariate linear association between variables. The chi-squared test of independence was used for analyzing the association between categorical variables. The OR of anemia was evaluated using logistic regression after adjustment for confounders (sex, age, and anthropometric measures). Multiple linear regression was used to estimate the association of RBC Fe incorporation and the change in RBC Fe DAE after controlling for confounding factors, such as sex and age. All analyses were performed using the JMP Pro statistical program version 13.1.0 (SAS Institute, Inc.). Significance was defined as a P value < 0.05.

Results

Characteristics of the study participants

In total, 82 women consented to allow their child to participate in the study; among this group, 71 toddlers completed the study as detailed in **Supplemental Figure 1**. Characteristics of participants are reported in **Table 1**. More female ($n = 41$) than male toddlers ($n = 30$) enrolled in the study. There were no statistically significant differences in the percentage of stunting or wasting between the males and females, but females tended to be more underweight than the males ($P < 0.08$). At enrollment on D0, 16% (11/70) of participants had a positive blood smear for malarial parasites. At D14, 37% (26/70) and 11% (8/70) of toddlers had helminths and malarial parasitemia, respectively. At D14, 6% (4/70) of toddlers were infected with both helminths and malaria. The prevalence of malaria and helminth infections did not significantly differ between males and females. On D14, 47% ($n = 33$) toddlers were anemic and were treated with Fe supplementation (Fe-supplemented group). The remaining 53% of children were nonanemic and were not supplemented with Fe (non–Fe supplemented group). Characteristics of these 2 groups of toddlers are presented in **Table 2**. The D84 blood sample was collected 84 days after tracer dosing in all but 1 toddler, in whom the blood sample was collected 83 days postdosing.

Hematological and Fe status

On D0, 56% (40/71) of toddlers were anemic. Those with elevated sTfR were nearly 5-fold more likely to be anemic (OR, 4.69; 95% CI, 1.37–13.98). Interestingly, toddlers who had helminth infections had a 40% lower risk of anemia when compared to those without helminth infections. After adjusting for sex, age, and anthropometric measurements, anemia was significantly less prevalent in helminth-infected toddlers [adjusted OR (AOR), 0.26; 95% CI, 0.07–0.97] and significantly more prevalent among those with elevated sTfR (AOR, 4.74; 95% CI, 1.01–22.15).

As expected, the mean Hb concentration increased from D14 to D84 in those who were Fe supplemented, but no significant

TABLE 2 Fe status in anemic toddlers that received Fe supplementation on D14 compared to non-anemic toddlers on D14 that were not supplemented with Fe[1]

	Time point	Fe supplemented group	Non–Fe supplemented group
Hb, g/dL	D0	10.38 ± 1.41 [33]	11.19 ± 1.42 [35][2]
	D14	9.96 ± 1.02 [33]	12.27 ± 1.00 [36][3]
	D84	11.33 ± 1.30 [32][4]	12.28 ± 1.09 [33][5]
SF, μg/L	D14	27.76 (20.54–37.51) [32]	30.98 ± 19.74 [36]
	D84	34.42 (24.29–48.78) [32][6]	19.75 (14.77–26.39) [30][2]
sTfR, mg/L	D14	9.03 (7.82–10.43) [33]	7.76 ± 2.36 [36][2]
EPO, IU/L	D14	18.67 (13.66–25.50) [31]	11.15 (8.76–14.19) [36][2]
	D84	12.14 (9.18–16.07) [30][7]	11.49 (8.78–15.05) [31]
CRP, mg/L	D14	0.27 (0.15–0.49) [31]	0.32 (0.22–0.48) [36]
	D84	0.37 (0.22–0.62) [32]	0.28 (0.13–0.58) [31]
Elevated CRP, %	D14	0	0
	D84	3.13 [1/32]	3.13 [1/32]
Serum hepcidin, ng/mL	D14	26.62 ± 21.12 [17]	14.04 (7.56–26.08) [24]
	D84	48.22 (26.10–89.08) [16][7]	23.64 (12.02–46.49) [21]
TBI, mg/kg	D14	2.56 ± 3.09 [32]	2.90 ± 2.72 [36]

[1]Data are presented as either mean ± SD [*n*] or geometric mean (95% CI) [*n*] or % [*n*]. Elevated CRP was defined as concentrations greater than 10 mg/L. Differences between toddlers who were and were not supplemented with Fe were analyzed by a Student's *t*-test or Wilcoxon's rank-sum test. Differences between D14 and D84 measurements were analyzed by a paired Student's *t*-test. Abbreviations: CRP, C-reactive protein; D, day; EPO, erythropoietin; Fe, iron; Hb, hemoglobin; SF, serum ferritin; sTfR, soluble transferrin receptor; TBI, total body iron.

[2]Significant difference observed between toddlers with and without Fe supplementation, $P < 0.05$.

[3]Significant difference observed between toddlers with and without Fe supplementation, $P < 0.005$.

[4]Significant change from D14 to D84 observed in toddlers supplemented with Fe, $P < 0.0001$.

[5]Significant difference observed between toddlers with and without Fe supplementation, $P < 0.0001$.

[6]A tendency of a significant change from D14 to D84 was observed in toddlers supplemented with Fe, $P = 0.08$.

[7]A significant change from D14 to D84 was observed in toddlers supplemented with Fe, $P < 0.05$.

change in Hb was observed in the non–Fe supplemented group ($P = 0.65$; Table 2). In addition, Fe-supplemented toddlers had a significant decrease in EPO from D14 to D84, as well as a tendency for SF concentrations to increase (Table 2). There were no significant changes in EPO or SF in the non–Fe supplemented group.

Hepcidin analyses were only conducted in a subgroup of toddlers ($n = 41$ at D14 and $n = 37$ at D84), based on the availability of serum. Hepcidin concentrations on both D14 and D84 did not significantly differ between the anemic and nonanemic participants. Hepcidin concentrations, however, did increase significantly from D14 to D84 in the Fe-supplemented group but not the non–Fe supplemented group ($P = 0.32$; Table 2). At D14, hepcidin was inversely correlated with sTfR ($P < 0.05$; r = -0.42) and positively correlated with SF ($P < 0.05$; r = 0.34). There was a lack of correlation between hepcidin and CRP on D14. At D84, hepcidin concentrations remained significantly correlated with SF concentrations ($P = 0.005$; r = 0.42) and were also positively correlated with CRP values ($P < 0.05$; r = 0.49).

Participants with malarial parasitemia on D0 had impaired Fe and hematological statuses, as evident by their significantly lower D14 TBI, higher D14 sTfR and D14 EPO concentrations, and a tendency for lower D0 Hb concentrations (**Table 3**). The Fe status of toddlers with malarial parasitemia on D14 is presented in Table 3. The Fe status of those with helminths did not differ from those without helminths at D14. For the subset of toddlers who had hepcidin measured, D14 hepcidin was significantly lower in those who had helminth infections ($P = 0.03$; $n = 42$; data not shown).

RBC Fe incorporation

Fe incorporation data are presented in **Table 4**. RBC Fe incorporation at D14 was highly variable (ranging from 1.21%–55.45%). As expected, average RBC Fe incorporation did not significantly differ between those that received the ^{58}Fe or the ^{57}Fe tracer. TBI was correlated with RBC Fe incorporation ($P = 0.009$; r = -0.32; $n = 69$), but there were no other significant associations with other Fe status indicators or with hepcidin. Infection with both parasites was associated with significantly higher RBC Fe incorporation, whereas infection with helminths only tended to be associated with higher RBC Fe incorporation ($P = 0.07$). Toddlers with malarial parasitemia did not have higher RBC Fe incorporation compared to those without this infection (Table 4). The association between Fe incorporation and parasitic infections did not appear to be driven by low Fe status, as there were no significant associations between the presence of either or both parasitic infections and any of the Fe status indicators measured. The mean D14 hepcidin tended to be higher ($P = 0.07$) in those without malaria and/or helminth infections compared to the mean observed in those with either or both of these parasitic infections (10.24 ng/mL). In the subgroup of participants with either a malarial or helminth infection, RBC Fe incorporation was negatively correlated with CRP ($P < 0.05$; r = -0.38; $n = 31$) but was not significantly

TABLE 3 Iron status as a function of malaria parasitemia[1]

	Time point	Malaria parasitemia at D0		Malaria parasitemia at D14	
		Yes	No	Yes	No
Hb, g/dL	D0	9.98 ± 1.46 [11]	11.00 ± 1.42 [58][2]	10.19 ± 1.74 [8]	10.91 ± 1.42 [61]
	D14	11.00 ± 1.00 [11]	11.17 ± 1.60 [58]	10.74 ± 2.07 [8]	11.23 ± 1.45 [62]
	D84	12.20 ± 0.91 [11]	11.70 ± 1.32 [55]	11.66 ± 0.92 [8]	11.85 ± 1.33 [58]
SF, μg/L	D14	21.5 (14.4–32.3) [11]	29.3 (23.0–37.5) [57]	41.4 (28.1–61.1) [8]	26.3 (20.9–33.2) [61][3]
	D84	20.9 (11.7–37.5) [11]	28.4 (22.1–36.6) [52]	24.7 (13.4–45.3) [8]	26.6 (20.7–34.5) [55]
sTfR, mg/L	D14	10.49 (9.12–12.06) [11]	7.77 (7.03–8.55) [58][4]	9.28 (7.27–11.85) [8]	7.97 (7.26–8.76) [62]
EPO, IU/L	D14	21.97 (13.90–34.71) [11]	13.20 (10.59–16.34) [56][4]	25.51 (13.94–46.68) [7]	13.20 (10.70–16.20) [61][3]
	D84	17.77 (11.72–26.96) [11]	10.99 (8.94–13.46) [51][4]	16.09 (8.86–29.25) [8]	11.25 (9.21–13.74) [54]
CRP, mg/L	D14	0.44 (0.20–0.97) [11]	0.28 (0.19–0.41) [56]	0.87 (0.27–2.83) [7]	0.28 (0.19–0.39) [61]
	D84	0.35 (0.11–1.17) [11]	0.32 (0.20–0.51) [53]	1.55 (0.39–6.23) [8]	0.25 (0.16–0.39) [56][3]
Elevated CRP, %	D14	0	0	0	0
	D84	9.09 [1/11]	1.89 [1/53]	12.5 [1/8]	1.79 [1/56]
Serum hepcidin, ng/mL	D14	11.88 (4.31–32.75) [9]	17.37 (10.58–28.49) [32]	24.85 (16.36–37.75) [6]	14.15 (8.61–23.18) [36]
	D84	14.95 (5.93–37.67) [9]	44.70 (27.54–72.68) [28][4]	53.25 (8.69–327.01) [6]	29.07 (18.23–46.35) [32]
TBI, mg/kg	D14	1.12 ± 2.54 [11]	3.52 {4.16} [57][4]	3.28 {2.64} [8]	3.37 {4.13} [61]

[1]Data are presented as the mean ± SD [*n*], geometric mean (95% CI) [*n*], median [IQR] [*n*], or % [*n*]. Elevated CRP was defined as concentrations greater than 10 mg/L. Differences between toddlers with and without malaria parasitemia at D0 or D14 were analyzed by a Student's *t*-test or Wilcoxon's rank-sum test. Abbreviations: CRP, C-reactive protein; D, day; EPO, erythropoietin; Hb, hemoglobin; SF, serum ferritin; sTfR, soluble transferrin receptor; TBI, total body iron.
[2]Difference between toddlers with and without malaria parasitemia at D0 approached significance, $P = 0.05$.
[3]Significant difference observed between toddlers with and without malaria parasitemia at D14, $P < 0.05$.
[4]Significant difference observed between toddlers with and without malaria parasitemia at D0, $P < 0.05$.

TABLE 4 Between-group comparison of red blood cell iron incorporation (%) at D14[1]

Groups	Iron incorporation at D14 (%)	*P*
All toddlers	11.42 (9.29–14.02) [68]	
Females	10.89 (8.44–14.05) [40]	NS
Males	12.22 (8.52–17.53) [28]	
D14 anemia	11.41 (8.54–15.25) [33]	NS
No D14 anemia	11.42 (8.41–15.52) [35]	
D14 helminths	14.46 (10.54–19.82) [26]	0.07
No D14 helminths	9.87 (7.53–12.92) [42]	
D14 malaria parasitemia	10.03 (4.44–22.68) [8]	NS
No D14 malaria parasitemia	11.61 (9.35–14.43) [60]	
Presence of parasites	14.50 (10.97–19.15) [31]	0.03
No parasites	9.17 (6.80–12.37) [36]	
D14 detectable hepcidin	8.55 (6.16–11.87) [28]	0.08
D14 undetectable hepcidin	14.76 (8.63–25.27) [12]	

[1]Data are presented as the geometric mean (95% CI) [*n*]. Comparison groups are stratified by sex; presence of anemia on D14; presence of malarial or helminth infections on D14 or parasites over the study period; or by hepcidin concentrations on D14. Hepcidin values below 5 ng/mL were defined as undetectable hepcidin. Differences were analyzed by a Student's *t*-test or Wilcoxon's rank-sum test. Abbreviations: D, days; NS, not significant.

impacted by hepcidin on D14. RBC Fe incorporation did not differ between the toddlers with and without Fe supplementation ($P = 0.5$). Significantly higher RBC Fe incorporation was evident among those with higher weight-for-age z-scores, lower TBI, and helminth infections ($P < 0.05$). Hepcidin was not significantly associated with RBC Fe incorporation, which may be due to the high variability in hepcidin, coupled with the known impact of infection on the concentrations of this biomarker.

Change in RBC Fe DAE from D14 to D84

On average, RBC Fe DAE decreased from D14 to D84 by 2.75 percentage points (from 15.94% to 13.19%; 95% CI, -3.86 to -1.65 percentage points; $P < 0.0001$; $n = 66$), but the degree of change varied more than 6-fold, from -16.41 to +12.75 percentage points. Among the 66 toddlers with data available on the change in RBC Fe DAE between D14 and D84, 12 toddlers (28%) exhibited slight increases in RBC Fe DAE from D14 to D84. Those with increases in RBC Fe DAE were equally distributed among toddlers that had received the ^{57}Fe ($n = 5$) or ^{58}Fe ($n = 7$) tracer. On average, in these 12 individuals, the mean increase observed was 1.67 ± 3.52 percentage points. After excluding the toddler with the highest increase (+12.75 percentage points), the mean RBC Fe DAE increase observed was 0.66 ± 0.49 percentage points. Possible differences in those that exhibited increases in DAE over time versus those that exhibited decreases in DAE over time were explored. Toddlers that had undetectable hepcidin on D84 ($P = 0.02$) and toddlers with lower sTfR concentrations on D14 ($P = 0.04$) were significantly more likely to have had an increase in RBC Fe DAE between D14 to D84. In addition, participants with lower RBC Fe DAE at D14 were more likely ($P = 0.04$) to experience increased RBC Fe DAE at D84. These results remained significant even when data were excluded from the toddler with the largest increase (+12.75 percentage points).

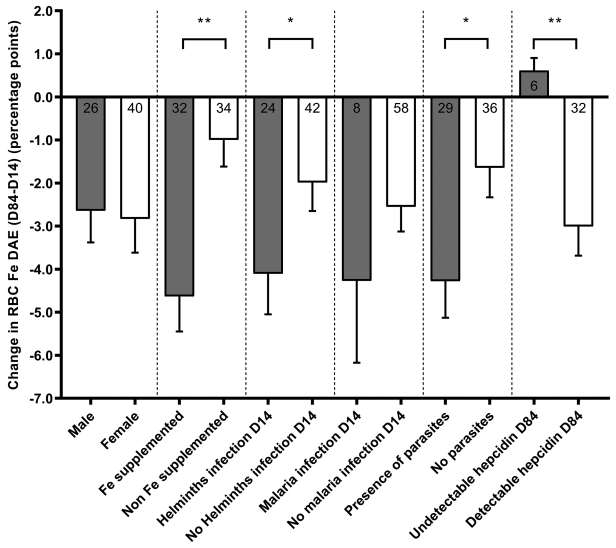

FIGURE 1 Mean change in RBC Fe DAE (D84-D14) in toddlers as a function of sex, iron status, or parasitic infections. Comparison groups are stratified by sex; iron supplementation on D14; presence of malarial or helminth infections on D14 or parasites over the study period; or by hepcidin concentrations on D84. Hepcidin values below 5 ng/mL were defined as undetectable hepcidin. The number on each bar indicates the number of toddlers in each group. Differences were analyzed between each pair of groups by Wilcoxon's rank-sum test. $^{*}P < 0.05$; $^{**}P < 0.005$. Error bars represent SEMs. Abbreviations: D, day; DAE, dose-adjusted enrichment; Fe, iron; RBC, red blood cell.

The net change in RBC Fe DAE over time was not significantly impacted by sex, age, anthropometric measurements, or malarial infections. A comparison of the change in RBC Fe DAE between different groups is presented in **Figure 1**. The change in RBC Fe DAE was significantly greater in those who were supplemented with Fe ($P = 0.001$) versus those who had not been Fe supplemented. This change in DAE in those receiving Fe supplementation appeared to be due to the addition of Fe rather than a consequence of the preexisting anemia in this group, based on the finding that toddlers with anemia at D0 did not have a significantly greater change in RBC Fe DAE compared to nonanemic toddlers at D0 (data not shown). Participants with higher hepcidin concentrations on D84 had greater reductions in RBC Fe DAE compared to those with undetectable hepcidin at D84 ($P = 0.003$; $n = 38$). RBC Fe DAE decreased to a larger extent in those found to be infected with either malaria or helminths ($P = 0.02$) across the study period. Toddlers with helminths on D14 had greater losses in RBC Fe DAE over the 70-day observation period ($P = 0.05$), but the presence of malarial infections did not have a significant impact on the change in RBC Fe DAE over time ($P = 0.19$), perhaps due to the limited sample size of those with malaria ($n = 8$). Among all hematological and Fe status indicators analyzed, Hb (either at D0 or D14) was the only biomarker that was significantly negatively associated with a change in RBC Fe DAE over time ($P < 0.005$). In addition, an increase in Hb from D14 to D84 was significantly correlated with a greater decrease in RBC Fe DAE from D14 to D84 ($P < 0.005$; $r = 0.37$).

A multiple linear regression model was constructed to identify factors significantly associated with the change in RBC Fe DAE. On D14, Fe supplementation ($\beta \pm$ SE, 0.40 ± 0.47; $P = 0.0005$), RBC Fe incorporation ($\beta \pm$ SE, 0.44 ± 0.04; $P = 0.0006$), and helminth infection ($\beta \pm$ SE, 1.36 ± 0.54; $P = 0.03$) were found to be significantly associated with a decrease in RBC Fe DAE from D14 to D84 (adjusted $R^2 = 0.38$; $n = 62$). A second model was constructed in the subgroup of toddlers with hepcidin data (adjusted $R^2 = 0.54$; $n = 36$), setting the change in RBC Fe DAE as the dependent variable and Fe incorporation and Fe status indicators as independent variables. In this smaller subpopulation, helminth infection was no longer significantly associated with a change in RBC Fe DAE, but those with detectable hepcidin at D84 exhibited a significantly greater loss in RBC Fe DAE ($\beta \pm$ SE, 0.30 ± 0.62; $P = 0.03$).

Discussion

A better understanding of the impact of parasitic infections on the Fe metabolism is needed, especially if Fe supplementation may exacerbate adverse events when given to Fe-replete children with active parasitic infections. To our knowledge, this is among the largest Fe stable isotope studies to follow RBC Fe DAE in this age group over an 84-day period. Toddlers with better weight-for-age z-scores, lower TBI, and helminth infections exhibited higher RBC Fe incorporation. A greater decrease in RBC Fe DAE postdosing was evident among those receiving Fe and among those with helminth infections.

An unexpected finding was that toddlers who had helminth infections had a nearly 40% lower risk of anemia compared to those without helminth infections. An earlier study in a larger cohort of Zanzibari children in the parent study (13) also found a lower prevalence of anemia in children (6–23 months) with helminth infections compared to children without helminth infections. In contrast, in older Zanzibari children (7–13 years), Stoltzfus et al. (26) found that hookworm infection was the strongest risk factor for severe anemia. Age differences between studies may explain these differences in part, as anemic and poorly nourished toddlers may not be actively exploring their environments and thus may have less exposure to helminth-infected soil.

Toddlers that experienced either a malarial or helminth infection across the study had significantly higher RBC Fe incorporation than those who remained free of parasites, an effect that appeared to be driven primarily by the nonsignificant increase in RBC Fe incorporation in those with helminth infections. RBC Fe incorporation did not differ between toddlers with and without malaria parasitemia, but the small number with malaria parasitemia limited our ability to address the independent effect of malaria on study outcomes. Participants in our study incorporated an average of 11.4% of ingested Fe into their RBCs. This is comparable to the 9.0% and 11.5% RBC Fe incorporation found among 48 Malawian toddlers (12–24 months) that received Fe supplements (30 mg/day) either immediately or 2 weeks after being treated for malaria (27). After 2 weeks of oral Fe supplementation in the Malawian cohort, the mean SF concentration increased by 2.5-fold and Fe incorporation decreased significantly, to 5.9% (27). This result is consistent with our finding of lower RBC Fe incorporation in those with better Fe status. The average RBC Fe incorporation in our study was slightly lower than the 18% reported in

27 Gambian toddlers (18–36 months) who were dosed with a 43% lower quantity of Fe tracer (4 mg) (21).

Among all biomarkers evaluated, the strongest determinant of RBC Fe incorporation was TBI. Our finding that a lower Fe status but not decreased inflammation explained more variability in RBC Fe incorporation is in agreement with prior data (28). It is likely that TBI better captures Fe demands by accounting for both tissue demand and storage Fe, and TBI may attenuate the impact of inflammation in populations at risk for recurrent parasitic infections. Data from 156 Ugandan children (1.5–5 years) found that subclinical inflammation (evidenced by elevated hepcidin and CRP) persisted for 12 months after antimalarial treatment (11, 29). In our cohort, hepcidin was not a significant determinant of RBC Fe incorporation. This finding differs from that observed in a population of anemic Gambian toddlers (21), where hepcidin was found to be the best predictor of RBC Fe incorporation. When toddlers are actively inflamed, hepcidin may explain more variability in RBC Fe incorporation, whereas Fe status may be a better predictor of RBC Fe incorporation in the absence of inflammation. We were unable to obtain measures of hepcidin on D0. It is likely that in this population with frequent infections, the D14 hepcidin measure may not have adequately captured the hepcidin status at the time the tracer was administered and absorbed. Our data also indicate that helminth infections are associated with higher Fe incorporation, which has also been reported in Ivorian children (30).

This study uniquely followed the change in RBC Fe isotopic enrichment over 84 days in healthy toddlers residing in a malaria- and helminth-endemic area. On average, RBC Fe DAE decreased over the 84 days, but a small subset of our cohort exhibited a slight increase in RBC Fe DAE over time. Those that exhibited increases in RBC Fe DAE were significantly more likely to have undetectable hepcidin at D84 and lower sTfR concentrations and RBC Fe DAE at D14. The slight increase in RBC Fe DAE observed draws attention to the need to further refine the timing used to evaluate RBC Fe incorporation in healthy toddlers and/or in those with parasitic infections. This methodology and the measure of RBC incorporation 2 weeks after dosing was developed from early radiotracer data in adults (31). There are no other data evaluating changes in RBC Fe enrichment over time in toddlers to determine whether our findings are unique to our cohort or whether the timing of RBC Fe incorporation is more variable in this age group. Thus, further normative data on this methodology in children are warranted.

In these toddlers, decreases in RBC Fe DAE were most pronounced in those who received Fe supplementation and exhibited significant increases in Hb postsupplementation. Greater losses in RBC Fe DAE over time did not appear to be driven by preexisting anemia, as the D14 to D84 decline in RBC Fe DAE was not significantly impacted by the presence of anemia at D0. In addition, Fe supplementation was a stronger determinant of change in RBC Fe DAE than was the presence of malaria and/or helminth parasites. Two other studies in toddlers followed RBC Fe enrichment over an extended period. Fomon et al. (32) studied 35 Caucasian infants (5.6 months) and found the change in RBC Fe enrichment over 13 months was significantly correlated with Fe absorption, which is similar to our finding of greater decreases in RBC Fe DAE among toddlers with higher RBC Fe incorporation. More recently, Speich et al. (25) dosed a group of African toddlers (14–20 months) and allowed isotopic equilibration to occur for an 8–11-month period postdosing before evaluating the impact of Fe supplementation on RBC tracer enrichment. Our study cannot be directly compared to the Speich study since we did not allow sufficient time for the tracer to equilibrate with body Fe pools, but there are some similarities in findings. Consistent with their study, we found that hepcidin was not significantly correlated with RBC Fe incorporation, and our observed decreases in RBC Fe enrichment were positively correlated with Fe absorption, increases in Hb, and Fe supplementation. Speich et al. (25) uniquely found that although Fe supplementation increased the absolute amount of Fe absorbed, the additional Fe absorbed did not appear to be retained.

Our study contributes to the data available on factors that impact RBC Fe incorporation and changes in RBC Fe DAE over time in toddlers residing in a malaria- and helminth-endemic area, but there are several limitations. The inflammatory status was evaluated using CRP, but this may not be a robust inflammatory marker in those with chronic infections (33–35). We could not differentiate a new onset of malarial infection from a relapse of an existing infection. Although antimalarial/antihelminths medications were provided, the recurrence of new infections was difficult to prevent in a parasite-endemic context. Our classification of infection as involving either malaria or helminths likely increased our power to identify the effects of these 2 parasitic infections, but we could not fully evaluate the impact of each individual infection, due in particular to the lower number of toddlers with malaria.

In summary, RBC Fe incorporation was best predicted by weight-for-age z-scores and TBI in these Zanzibari toddlers. RBC Fe DAE decreased significantly across an interval of time that approaches the typical RBC lifespan in this age group (36). Toddlers who received Fe and incorporated more Fe into RBCs exhibited significantly greater losses in RBC Fe DAE postdosing. Future longer-term labeling studies may provide greater insight into whole-body Fe homeostasis and adaptations to parasitic loads or Fe supplementation in these vulnerable age groups.

The authors' responsibilities were as follows – WK: analyzed and interpreted the data and wrote the manuscript; KOO: designed the research, performed experiments, analyzed and interpreted the data, wrote the manuscript, and had primary responsibility for the final content; NB, MR, FV, RJS: were responsible for the clinical implementation of the studies and assisted with the design of the research, analysis and interpretation of the data, and preparation of the manuscript; and all authors: read and approved the final manuscript. The authors report no conflicts of interest.

Data Availability

Data described in the manuscript, code book, and analytic code will not be made available because of the pediatric composition of the patient population and the confidential nature of the data collected.

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