Iron supplementation does not affect copper and zinc absorption in breastfed infants

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ABSTRACT
Background: Iron supplements are commonly recommended for infants but were suggested to inhibit zinc and copper absorption.
Objective: The objective of this study was to investigate potential effects of iron supplementation, infant age, and mineral status on zinc and copper absorption in infants at 6 and 9 mo of age.
Design: Twenty-five healthy breastfed term infants were recruited from a larger randomized iron supplementation trial. Six of these infants received iron supplements (1 mg · kg⁻¹ · d⁻¹) from 4 to 9 mo, 8 were supplemented from 6 to 9 mo, and 11 received placebo only. Zinc and copper absorption was measured at 6 and 9 mo of age, using orally administered ⁷⁷⁰Zn and ⁶⁵⁰Cu and fecal monitoring of recovered stable isotopes.
Results: Mean (±SD) zinc absorption was 51.9 ± 17.9%, and mean copper absorption was 79.0 ± 13.5%. No significant difference was observed in zinc or copper absorption between 6 and 9 mo of age. When combining all measurements, no significant effect of prior iron supplementation was observed on zinc or copper absorption. No significant correlation was observed between plasma zinc and zinc absorption or between plasma copper and copper absorption. No significant correlation was observed between erythrocyte copper-zinc-dependent superoxide dismutase activity and copper absorption.
Conclusions: The study does not support the contention that iron supplements inhibit the absorption of zinc or copper in healthy breastfed infants at 6–9 mo of age. In addition, we did not find any age-related changes in zinc or copper absorption between 6 and 9 mo of age. Am J Clin Nutr 2009;89:185–90.

INTRODUCTION
Zinc and copper are essential nutrients. Zinc is important for gene expression, signal transduction, apoptosis, cellular proliferation, differentiation, and growth (1). Zinc deficiency is common in infants and young children in developing countries and leads to stunted growth, increased risk of infection, and possibly poor neurodevelopment (2). Zinc homeostasis is maintained by regulation of absorption and endogenous excretion into the gastrointestinal tract. Fractional absorption of zinc in term infants is 20–60%, but it is higher from breast milk (50–60% absorption) than from formula (3). Homeostatic regulation of zinc absorption is present early in postnatal mammalian life (4), but there is probably a limit to the extent by which regulation of zinc homeostasis can compensate for poor intake and bioavailability of zinc.

Copper is an important component of several enzymes in the electron transport chain and the antioxidant system (5, 6). Symptomatic copper deficiency with anemia, neutropenia, and osteoporosis is a rare condition, which is described predominantly in low-birth-weight infants (4, 7). However, little is known about the prevalence and possible health effect of marginal copper deficiency (8). Regulation of copper homeostasis is believed to involve changes in intestinal absorption and biliary excretion (9). Fractional copper absorption is usually 50–80% and is modified by dietary promoters and inhibitors as well as by copper status (9, 10). Relative retention of copper appears to be higher from breast milk than from copper-supplemented infant formula: 74% compared with 52% in term infants according to one balance study (11).

To date, there have been few studies on copper absorption in infants. Iron requirements are high in late infancy, and iron supplements (usually 1 mg · kg⁻¹ · d⁻¹) are therefore recommended if infants do not receive sufficient amounts of iron-rich complementary foods (12). However, iron supplementation was suggested to have negative effects on absorption of both zinc and copper (4). Zinc deficiency often coexists with iron deficiency in young children in developing countries, and combined iron and zinc supplementation is therefore often recommended (13). However, these 2 minerals were suggested to compete for absorptive pathways, resulting in a negative effect of zinc supplementation on iron status and vice versa. Clinical supplementation trials have yielded conflicting data on such possible interaction effects (12, 14).

Several interactions are known between iron and copper metabolism (15). We have previously found that a higher concentration of iron in infant formula was associated with lower serum copper (16) and that iron supplementation of infants reduced copper- and zinc-dependent superoxide dismutase (CuZn-SOD) activity, suggesting a negative effect on copper status (17). However, these effects may be due to interactions beyond the absorption step, because another study showed that iron fortification did not influence copper absorption in infants (18).

Thus, it is still not clear whether iron supplements inhibit zinc and copper absorption in infants. Furthermore, little is known

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2 Supported by the Swedish Research Council for Environment, Agricultural Sciences, and Spatial Planning; the Västerbotten County Council; the Oskar Foundation; and the Jerring Foundation.

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Received August 27, 2008. Accepted for publication October 6, 2008. First published online December 3, 2008; doi: 10.3945/ajcn.2008.26887.
about the developmental changes in zinc and copper absorption during infancy and about the relation between zinc and copper status and the absorption of these minerals in infants. The aims of this study were to investigate potential effects of iron supplementation, infant age, and mineral status on zinc and copper absorption in breastfed infants at 6 and 9 mo of age.

**SUBJECTS AND METHODS**

Twenty-five term (≥37 wk gestation) Swedish infants of normal birth weight (>2500 g) were randomly chosen at 4 mo of age from a larger cohort of infants participating in an iron supplementation trial (11). Mother-infant pairs were eligible if the mother intended to breastfeed exclusively for 6 mo and at least partially for 9 mo and if the infant had no chronic illnesses. A total of 121 infants were included in the iron supplementation trial; they were randomly assigned to receive 1) iron supplements from 4 to 9 mo of age, 2) placebo from 4 to 6 mo of age and iron supplements from 6 to 9 mo of age, or 3) placebo from 4 to 9 mo of age. From each of these 3 groups, 12–14 families were asked to participate in the current study; a total of 25 families agreed (6 in group 1, 8 in group 2, and 11 in group 3). The study protocol was approved by the Ethics Committee, Faculty of Medicine, Umeå University. All participating families gave their written informed consent.

To maximize the power of the study, the power analysis was based on measurement rather than on individual and time point. Assuming a 75% success rate of stool collections and a dropout rate of 25% between 6 and 9 mo of age and a similar distribution of infants from the intervention groups, an initial group size of 25 infants would result in 32 measurements of which 20 would be performed in infants with prior iron supplementation. With the use of means and SDs from previous studies, this group size would enable us to identify differences in copper and zinc absorption of 14–18% units with a power of 80% and a significance level of 0.05.

**Iron supplements and diet**

The iron supplement was a liquid formulation of ferrous sulfate (Fer-In-Sol; Mead Johnson, Evansville, IN). The placebo solution was prepared on the basis of the original recipe for Fer-In-Sol but with ferrous sulfate omitted and food coloring added to give the placebo an appearance similar to that of the iron supplement. The investigators and the parents were blinded to the intervention. The supplement was given in a dose corresponding to 1 mg elemental Fe · kg⁻¹ · d⁻¹; the volume was adjusted monthly according to the infant’s weight. The supplement or placebo was given by the mother each morning, just before or after breastfeeding and ≥1 h before or after the infant consumed any other food. The infants were almost exclusively breastfed until ≥6 mo of age. When the infants were 4–6 mo of age, the mothers were discouraged from giving any other foods or fluids except for so-called taste portions (≤1 tablespoon/d) of commercial baby foods (strained fruit or vegetables) that contained little or no iron and were provided by the investigators. When the infants were between 6 and 9 mo of age, the mothers continued breastfeeding, and parents gave complementary foods at their own discretion; no attempt was made by the investigators to influence the choice of foods or the extent of breastfeeding (19).

**Stable isotopes and test meals**

Stable ⁷⁰Zn and ⁶⁵Cu isotopes were obtained as metallic zinc and copper (Trace Sciences International Corp, Richmond Hill, Canada). Metal pieces were weighed, dissolved in a minimal amount of concentrated nitric acid, heated to 300°C until dry, resuspended in 0.1 mol/L HCl, and pH adjusted to 5.6. The obtained zinc chloride and cuprous chloride solutions were sterile filtered, diluted with sodium chloride to 70.4 μg ⁷⁰Zn/mL and 52.1 μg ⁶⁵Cu/mL, respectively, divided into aliquots and stored under nitrogen at 4°C until used.

Zinc and copper absorption was estimated twice in each subject by giving identical test meals at 2 wk before 6 mo of age (x: 168 d; range: 164–174 d) and 2 wk before 9 mo of age (x: 259 d; range: 253–264 d). For simplicity, these ages are referred to as 6 and 9 mo, respectively.

Milking from each mother was collected ~2 wk before the test meal with the use of a manual or electric breast pump; milk was stored at −20°C in sterile containers until used. For each test meal, a portion of milk (76–150 mL) obtained from the infant’s mother was thawed in a preweighed plastic feeding bottle and mixed with 93.2 μg ⁷⁰Zn and 40.8 μg ⁶⁵Cu. Because an iron absorption study was performed simultaneously, 150 μg ⁵⁷Fe was also added. The bottle was then slowly rotated at 4°C overnight to allow for equilibration. The test meal was given in the morning during a home visit by one of the investigators (MD) and a research nurse. The labeled milk was heated in a water bath to ~37°C before it was fed to the infant. To accurately assess the dose given, all vials were weighed before and after feeding, and all small losses of milk (from spitting up or spilling) were determined by absorbing the losses into preweighed napkins; the corresponding stable isotope content was subtracted from the original total dose. No iron supplement or placebo was given on the day of the test meal. No food other than human milk was given from 6 h before until 6 h after the test meal.

**Stool collection and preparation**

All stools were collected for a minimum period of 78 h after the test meal (until the first stool after 78 h). To ensure the quality of sampling and to avoid losses, parents were carefully trained in how to collect the fecal samples from their infants. For each stool, parents documented date, time, and any collection problems. Ileostomy bags were used for stool collection (Naturess; Convatec, Bromma, Sweden) and stored at −20°C. For each collection period, collection bags were semithawed, feces was transferred to a glass mixer (Moulinex Q50; Groupe SEB, Upplands Väsby, Sweden), and bags were rinsed 3 times with ultrapure water. Ultrapure water (~5 times fecal volume) was added, stool was homogenized in the mixer, and 4-mL samples were transferred to glass vials and stored at −20°C untilashing. The mixer vial was washed with detergent, rinsed once in 10% nitric acid, and rinsed 3 times in ultrapure water before each homogenization. Samples were microwave digested as previously described (20).

**Other data collection**

Blood samples were obtained at 6 and 9 mo of age and analyzed for plasma zinc, plasma copper, hemoglobin, and plasma ferritin as previously described (19, 21). CuZn-SOD in erythrocytes was
measured as previously described (17). Body weight was measured at 6 and 9 mo of age with a Seca model 835 digital infant scale (Seca Corporation, Hamburg, Germany). Intake of complementary food (defined as all food, fluid, or solid, except human milk) was recorded by two 5-d food diaries at 8 and 9 mo of age, respectively. Intakes of specific nutrients were calculated with the use of the Food Composition Tables from the Swedish National Food Administration combined with information from Swedish baby food manufacturers as previously described (22). Nutrient intake at 9 mo was calculated as the average of the 8- and 9-mo recordings.

Analyses and calculations

Fecal samples (2 mL of homogenized fecal sample) were microwave digested with the use of concentrated nitric acid as previously described (20). Digested solutions were heated to incipient dryness on hot plate and diluted with deionized water to a desired volume of 12 mL. An aliquot of 0.15 mL of digested solution was then diluted to a total of 3 mL in 1% ultrapure nitric acid for copper and zinc concentration and ratio analyses with the use of a high-resolution inductively coupled plasma mass spectrometry (ThermoFisher Scientific, Waltham, MA). A high-mass resolution of ~4000 was applied to eliminate interferences during analysis. Germanium was selected as the internal standard for matrix correction. Analytic precision for copper and zinc concentrations was <1.5%. Analytic precision for zinc and copper was <0.7% and <0.5%, respectively.

The determination of the stable isotopes in the fecal samples was based on the ratios of $^{70}$Zn to $^{66}$Zn and of $^{65}$Cu to $^{63}$Cu, respectively, and the amount of total zinc and copper in each fecal sample. The equations used for calculation of the amounts of stable isotope in the fecal samples were according to tracer:tracee methods. The natural abundance was assumed to be 0.00624 for $^{70}$Zn and 0.31 for $^{65}$Cu (20). Fractional absorption was calculated as the difference between the amount of tracer given in the test meal and the cumulative fecal excretion of $^{70}$Zn and $^{65}$Cu, in excess of natural abundance, during the stool collection period.

For the statistical analyses, SPSS 14.0 (SPSS Inc, Chicago, IL) was used. Because plasma ferritin was not normally distributed, it was log transformed and presented as geometric means and SDs. Analysis of variance was used for comparison between groups. In addition, the paired $t$ test was used for comparison of absorption data between 6 and 9 mo of age. Analysis of covariance (ANCOVA) was used for multivariate analysis. Linear regression was used for correlation analyses.

RESULTS

Twenty-five infants were recruited into the study. Within the larger study cohort ($n = 121$), no significant differences were observed between participants and nonparticipants with regard to infant sex, birth weight or current weight, hemoglobin, or ferritin at study entry. Infants received 1 of 3 interventions according to the allocation in the previously published randomized, blinded trial (19): 6 infants received early iron supplements (1 mg · kg$^{-1}$ · d$^{-1}$) from 4 to 9 mo of age, 8 infants received placebo from 4 to 6 mo of age and late iron supplements from 6 to 9 mo of age, whereas 11 infants received placebo from 4 to 9 mo of age. We examined all 25 infants at 6 mo of age, and 18 of them were reexamined at 9 mo of age. The reason for declining participation in the second examination (7 families) was the demanding study procedure. No significant difference was observed between those who declined the second measurement and those who did not with regard to intervention group, infant sex, birth weight or current weight, hemoglobin, or ferritin at study entry.

Only measurements with complete stool collections were included in the final analysis. This was obtained in 34 of 43 cases (18 at 6 mo of age and 16 at 9 mo of age). Those infants from which at least one complete stool collection was available were included in the analysis ($n = 20$; 8 placebo, 5 early iron, 7 late iron). In 14 of them, complete stool collections were obtained at both 6 and 9 mo of age.

Of the 20 included infants, 11 (55%) were boys. Mean (±SD) birth weight was 3550 ± 372 g. At 6 mo of age, mean weight was 7600 ± 790 g, hemoglobin concentration was 118.6 ± 5.6 g/L, and plasma ferritin was 58 ± 0.4 μg/L. Iron intake [mean ± SD (range)] from complementary food was 0.53 ± 0.50 mg · kg$^{-1}$ · d$^{-1}$ (0.08–2.33 mg · kg$^{-1}$ · d$^{-1}$). Infants with the lowest iron intake from complementary food were still virtually exclusively breastfed at 9 mo of age, whereas infants with the highest intake consumed significant amounts of iron-fortified complementary foods, such as fruit drinks and cereals.

Mean [±SD (range)] plasma zinc concentration was 0.71 ± 0.14 mg/L (0.45–1.07 mg/L) with no significant difference between 6 and 9 mo of age ($P = 0.99$). Mean plasma copper concentration was 1.03 ± 0.20 mg/L (0.60–1.54 mg/L) with no significant difference between 6 and 9 mo of age ($P = 0.40$).

No significant difference was observed in zinc or copper absorption between the 3 intervention groups at 6 or 9 mo of age (Table 1). Furthermore, no significant difference was observed between infants with or without prior iron supplementation (Table 1). Combining the measurements at 6 and 9 mo of age, no difference was observed in zinc or copper absorption between infants who had received prior iron supplementation ($n = 13$ or placebo ($n = 21$) for 2–5 mo (53.0% compared with 51.3%, $P = 0.79$, and 80.6% compared with 77.9%, $P = 0.58$, respectively). To exclude any effect of differences between individuals or infant age in the analysis of all combined measurements, we repeated these analyses while adjusting statistically for individual (study ID) and infant age (6 or 9 mo) by including these variables in the ANCOVA model. No interaction was observed between individual and intervention (iron supplementation), and iron supplementation still had no significant effect on zinc absorption ($P = 0.99$) or copper absorption ($P = 0.36$) in the adjusted models. Furthermore, no significant correlation was observed between total iron intake (supplements + complementary food) and zinc or copper absorption at 9 mo of age. Because no significant effect of iron supplementation was observed on zinc or copper absorption, all intervention groups were combined in the remaining analyses.

Including all 34 absorption measurements, mean fractional zinc absorption was 51.9 ± 17.9% (8.0–82.1%) and copper absorption was 79.0 ± 13.5% (39.2–97.4%). No significant difference was observed in zinc absorption between 6 and 9 mo of age (48.5% compared with 55.8%; $P = 0.24$). In addition, no significant difference was observed in copper absorption between 6 and 9 mo of age (81.3% compared with 76.3%; $P = 0.28$). These differences were also not significant when analyzing paired samples.
DISCUSSION

We did not observe a significant effect of long-term iron supplementation on zinc or copper absorption in infants. Most studies of the interaction between 2 trace elements, eg, iron-zinc and iron-copper, were designed to assess the direct interaction—ie, does the addition of one element adversely affect the absorption of another element from a meal. This is of interest when elucidating mechanisms of interactions, usually whether 2 elements share an absorptive pathway. Such an interaction may, however, be temporary because of homeostatic mechanisms regulating the metabolism in the body. From a population view it is more relevant to study whether long-term supplementation with a trace element will affect the absorption of other trace elements when such interactions have been shown or suggested.

Zinc and copper absorption at 6 and 9 mo of age in infants with or without previous iron supplementation

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<sup>1</sup> Intervention group receiving iron supplements (1 mg · kg<sup>−1</sup> · d<sup>−1</sup>) from 4 to 9 mo of age.
<sup>2</sup> Intervention group receiving placebo from 4 to 6 mo of age and iron supplements (1 mg · kg<sup>−1</sup> · d<sup>−1</sup>) from 6 to 9 mo of age.
<sup>3</sup> Infants who had received iron supplementation before the absorption measurement.
<sup>4</sup> Infants who had not received iron supplementation before the absorption measurement.
<sup>5</sup> Mean ± SD; n in brackets (all such values).
<sup>6</sup> Measurements at 6 and 9 mo of age combined.

(n = 14). A borderline positive correlation was observed between zinc absorption and log plasma ferritin (r = 0.27, P = 0.087; r = 0.32, P = 0.050 when adjusting for age), but no significant correlation was observed between copper absorption and log plasma ferritin (r = 0.23, P = 0.14). No significant correlation was observed between plasma zinc and zinc absorption (P = 0.34; Figure 1). Similarly, no significant correlation was observed between plasma copper and copper absorption (P = 0.32; Figure 2). CuZn-SOD activity in erythrocytes was measured at 9 mo of age, and successful measurements were obtained in 12 of the 18 infants (7 from the placebo group, 2 from the early iron group, and 3 from the late iron group). No significant correlation was observed between CuZn-SOD activity and copper absorption (P = 0.87).

Among those infants who were investigated at both time points, a significant positive correlation was observed between plasma ferritin and change in zinc absorption from 6 to 9 mo of age, adjusting for baseline absorption—ie, infants with a high plasma ferritin at 6 mo of age had a tendency to increase zinc absorption from 6 to 9 mo of age. No significant similar correlation was observed for ferritin and copper absorption.

In this double-blind, randomized, controlled study on infants given iron supplements or placebo, we had the opportunity to evaluate whether iron supplements given to infants for 2 or 5 mo affected zinc or copper absorption. The results for iron status and hematometry, growth and morbidity, and iron absorption were published previously (19, 22, 23).

We found that mean fractional zinc absorption was 52% in these healthy breastfed infants, which is similar to previous results in breastfed infants (50–60%) (3, 24, 25) and higher than that in previous reports on formula-fed infants (3, 26). Zinc absorption was not correlated to plasma zinc concentrations in this cohort, which may reflect an absence of regulatory effect of plasma zinc, that plasma zinc poorly reflects zinc status or that the range in zinc status was relatively narrow in this population. Even though serum or plasma zinc is the most commonly used biomarker, it is not a sensitive marker of marginal zinc deficiency (27).

It is known that supplemental iron can interfere with the absorption of zinc when given together in an oral dose (28). The
Infants (50–80%) (9, 10). In another study from our group, using breastfed infants was 79%, which is similar to previous studies in young children (23).

The mechanism behind this acute effect of iron on zinc absorption is not yet known. The main zinc transporters regulating zinc uptake and transport across the enterocyte are as follows: Zip4, which imports zinc across the apical membrane; ZnT1, which exports zinc across the basolateral membrane; and ZnT5, which appears to be a bidirectional zinc transporter in the enterocyte (4, 29). Little is known about the effect of other cations on these transporters, but at least one zinc transporter (Zip14) was shown to also transport iron (30). In the present study, however, we explored whether long-term iron supplementation affects the absorption of zinc. We found that iron supplementation had no effect on fractional zinc absorption in infants. Similarly, in a previous study on human adults (28), it was reported that 2 wk of iron supplementation had no effect on zinc absorption, strongly suggesting that iron status has no effect on zinc absorption in infants or adults.

Interaction effects between iron and serum zinc in clinical supplementation trials recently have been reviewed (14). In 9 of 10 reviewed trials of iron-only supplementation in young children, there was no effect of iron supplementation on serum zinc. In 4 reviewed trials, the addition of iron to zinc supplements had no adverse effect on serum zinc. In one trial (13), combined iron and zinc supplementation had less positive effect on growth than did zinc supplementation alone. In a more recent study, the addition of iron to a zinc supplement reduced the positive effect of zinc on serum zinc and growth in breastfed infants (31). This negative interaction with respect to growth was suggested to be due to an iron-induced inhibition of zinc absorption, which is not consistent with the results of the current study but may instead be due to a negative effect of iron on growth in iron-replete young children (23).

We found that mean copper absorption in these healthy breastfed infants was 79%, which is similar to previous studies in infants (50–80%) (9, 10). In another study from our group, using similar methods, copper absorption in infants was ~80% at 1–3 mo of life (20). Similar to this study, there was no effect of age, suggesting that copper absorption does not change much during the first 9 mo of age. In adults, copper absorption in subjects fed a copper-adequate diet appeared to be lower (36%) (32); however, these adults were fed solid diets, whereas the infants received breast milk. In the current study we found no correlation between copper absorption and markers of copper status (plasma copper and CuZn-SOD). This supports the results from the previous study in which copper absorption was independent of copper supplementation at a concentration of 80 μg·kg⁻¹·d⁻¹ (20). However, it is possible that copper absorption may be down-regulated if the infant were exposed to even higher doses of copper. In a study on infant rhesus monkeys that were given copper in infant formula at a high concentration (6000 μg·kg⁻¹·d⁻¹), copper absorption was shown to be lower but became normal when copper intake was normalized (33). Note that markers of copper status were shown to reflect low copper status, but they are not useful for detecting excess copper exposure (9). In our study, however, there is no reason to believe that the infants had been exposed to high copper because copper concentrations in the water were reported to be low in this area (16).

The main copper transporter regulating copper uptake into the enterocyte is Ctr1 (4, 15, 34). This copper transporter is specific for copper, and iron is therefore not expected to interfere with copper absorption, which is similar to what we found. However, there have been some reports on isolated epithelial cells, suggesting that the main intestinal iron transporter DMT1 (divalent metal ion transporter) also can transport copper across the apical membrane (15, 35); thus, iron could potentially interfere with copper absorption. However, because copper is absorbed in the cuprous (+1) form and not in the cupric (+II) form (36), and because DMT1 transports only divalent cations, this suggests that DMT1 is not a significant transporter for copper, which is consistent with our finding of no effect of iron supplementation on copper absorption. In a recent study iron supplementation of infants reduced CuZn-SOD activity in erythrocytes, suggesting a negative effect on copper status (17). However, that effect may be due to interactions beyond the absorption step, because another study showed that iron fortification did not influence copper absorption in infants (18). We speculate that the potential effect of iron supplements on copper status may not simply be an inhibition of copper absorption.

Incomplete fecal collection is a possible source of error in the current study. However, each stool was documented, and cases were excluded when fecal collections were not complete. The collection period was >78 h, which is even longer than in our previous study (20). Furthermore, even if incomplete stool collection cannot be completely excluded, this risk would be the same for iron-supplemented and unsupplemented infants so it would not affect the main result of the study. The small number of infants is a limitation of the study. Because we included only healthy breastfed infants, the results cannot necessarily be generalized to malnourished or formula-fed infants.

In conclusion, the current study does not support that iron supplementation (1 mg·kg⁻¹·d⁻¹) inhibits the absorption of zinc and copper in healthy breastfed infants at 6–9 mo of age. In addition, we did not find any age-related changes in zinc and copper absorption between 6 and 9 mo of age. Further clinical studies are needed to investigate whether these conclusions are also valid in other populations.

We thank research nurse Margareta Bäckman for assistance with test meal administration and blood sampling, Carina Lagerqvist and Yvonne Andersson for helping with the preparation of fecal samples, Penni Hicks for performing laboratory analyses, Ian Griffin for help with isotope calculations, and Hans Stenlund for assistance with the statistical analyses.
The authors' responsibilities were as follows—MD, OH, SAA, and BL: designed the study; MD: performed data collection and did most of the data analysis and writing of the manuscript; ZC: developed the method for the inductively coupled plasma mass spectrometry measurement of copper and zinc concentrations; and BL, OH, SAA, ZC, and BL: participated in the data analysis and writing of the manuscript. None of the authors had a financial or personal interest in any of the organizations or companies supporting this research.

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