Dose-response trial of prophylactic zinc supplements, with or without copper, in young Ecuadorian children at risk of zinc deficiency1–3

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ABSTRACT

Background: Multiple studies have shown the benefits of zinc supplementation among young children in high-risk populations. However, the optimal dose and safe upper level of zinc have not been determined.

Objectives: The objectives of this study were to measure the effects of different doses of supplemental zinc on the plasma zinc concentration, morbidity, and growth of young children; to detect any adverse effects of 10 mg supplemental Zn on markers of copper or iron status; and to determine whether any adverse effects are alleviated by providing copper with zinc.

Design: This randomized, double-masked, community-based intervention trial was conducted in 631 Ecuadorian children who were 12–30 mo old at baseline and who had initial length-for-age z scores ≤−1.3. Children received 1 of 5 daily supplements for 6 mo: 3, 7, or 10 mg Zn as zinc sulfate, 10 mg Zn + 0.5 mg Cu as copper sulfate, or placebo.

Results: The change in plasma zinc concentration from baseline was positively related to the zinc dose (P < 0.001). Zinc supplementation, including doses as low as 3 mg/d, reduced the incidence of diarrhea by 21–42% (P < 0.01). There were no other significant group-wise differences.

Conclusions: Zinc supplementation with a dose as low as 3 mg/d increased plasma zinc concentrations and reduced diarrhea incidence in the study population. There were no observed adverse effects of 10 mg Zn/d on indicators of copper or iron status. The current tolerable upper level of zinc recommended by the Institute of Medicine should be reassessed for young children. Am J Clin Nutr 2008;87:723–33.

KEY WORDS Zinc, copper, dose response, tolerable upper level, diarrhea, lipoprotein concentration, iron, growth response

INTRODUCTION

The beneficial effects of zinc supplementation include reductions in the incidence and prevalence of diarrhea (1, 2), the incidence of pneumonia (1, 2), and the rates of mortality (3–8) among young children in low-income countries. Moreover, zinc supplementation increases the growth of stunted children (9). However, the dose of supplemental zinc required to achieve these beneficial outcomes, while avoiding potential adverse effects of excessive zinc intake, remains unknown. Previously, the recommended dietary allowance of zinc for preschool children was set at 10 mg/d by national and international organizations (10, 11), but the United States Food and Nutrition Board–Institute of Medicine, the International Zinc Nutrition Consultative Group (IZiNCG), and the World Health Organization (WHO) now recommend a lower recommended dietary allowance of 3 mg Zn/d for 1–3-y-old children (12–14). These expert groups also recommended tolerable upper levels (ULs) of zinc intake ranging from 7 to 23 mg/d to avoid the possible adverse effects of excessive intakes of zinc on copper and iron status and lipoprotein concentrations, which are the first expected signs of excessive zinc intake.

Previously reported supplementation trials in young children that lasted ≥2 mo evaluated a single daily dose of zinc ranging from 5 to 20 mg, and most provided ≥9 mg Zn (2, 9); 51% of 1–3-y-old children in the United States consume more dietary zinc than the recommended UL (15). However, possible adverse effects of this level of intake have not been systematically explored. Thus, additional information is needed with respect to the UL for this age group.

The objectives of the present study were to determine the lowest daily dose of supplemental zinc that effectively increased the plasma zinc concentrations, reduced the morbidity, and increased the growth of young Ecuadorian children presumed to be at increased risk of zinc deficiency, on the basis of their relatively low length-for-age z score (LAZ), more than did placebo; to determine whether there are adverse effects of 10 mg Zn/d on markers of copper and iron status and lipoprotein concentrations; and to determine whether any observed adverse effects could be prevented by providing 0.5 mg Cu/d in combination with 10 mg Zn/d. The first objective was assessed among children who were given 1 of 5 supplements containing 3, 7, or 10 mg Zn/d, 10 mg Zn + 0.5 mg Cu/d, or placebo; the second and third objectives were assessed among a subset of the children who consumed 10 mg Zn/d, 10 mg Zn + 0.5 mg Cu, or placebo. To maximize the
likelihood of detecting functional responses to zinc supplementation (13, 16), we selected children with an initial LAZ $<-1.3$ as compared with 1978 reference data from the WHO and the National Center for Health Statistics (NCHS)(17).

**SUBJECTS AND METHODS**

**Study design and sites**

The present study was designed as a community-based, randomized, double-masked intervention trial. It was conducted in 3 sites in Ecuador that were selected in consultation with the Ecuadorian Ministry of Health: El Carmen, a small town in the coastal plains, and the communities surrounding it; Latacunga, a medium-sized town in the Andean highlands, and several surrounding rural communities; and 2 shantytowns in the hills adjacent to the capital city of Quito, also in the Andean highlands. Community surveys, enrollment, and follow-up were conducted between November 2001 and April 2005.

**Subjects**

Children 9–29 mo old were identified by house-to-house surveys and during well-child checkups at local health centers. To avoid the loss of potentially eligible children due to changes in LAZ during the screening period, the screening LAZ cutoff was set at $<-1.25$ as compared with the WHO/NCHS international reference data (17). All of these children were given iron sulfate supplements to provide $\approx 2.5 \text{ mg Fe} \cdot \text{kg body wt}^{-1} \cdot \text{d}^{-1}$ (18) for 1 mo, unless they had recently received iron supplementation from the local health center. The intent was to treat any existing iron deficiency before the study to avoid possible interactions between iron and zinc supplements during the study (19–21). After 1 mo of iron supplementation, potentially eligible children were invited to participate in the pretrial screening measurement of blood hemoglobin concentrations conducted with the use of the HemoCue Analyzer (HemoCue AB, Angelholm, Sweden). If the child’s hemoglobin was $\geq 10.5 \text{ g/dL}$ [adjusted for altitude (22)], he or she was invited to attend a further screening appointment to determine eligibility for the 6-mo intervention trial. Those children with lower hemoglobin concentrations were provided an additional month of iron supplementation, after which they were reassessed for eligibility for the full study. If anemia persisted beyond 3 mo of iron treatment, the child was excluded from the trial and referred for more detailed medical assessment and care. Final eligibility criteria for the intervention trial included LAZ $<-1.3$ for children 12–20 mo old and $<-1.5$ for children 21–29 mo old, assessed by comparison with the WHO/NCHS international reference data (17); hemoglobin $\geq 10.5 \text{ g/dL}$, adjusted for altitude (22); and the absence of chronic disease or congenital defects that restrict normal growth. The cutoff of $-1.5$ LAZ was selected because of meta-analyses indicating that growth response to zinc occurs among populations with LAZ $<-1.5$ (9). This cutoff was relaxed among children 12–20 mo old, because their LAZ may have still been declining over that age range.

A flow chart shows the numbers of children involved in each step of the initial survey, prescreening examinations, and intervention trial and indicates the reasons why children did not continue at each stage (Figure 1). Ultimately, 631 children were included in the trial.

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**FIGURE 1.** Flow sheet of participants in screening survey and 6-mo intervention trial in Ecuadorian children 12–36 mo old. Entry criteria into the full study were defined as length-for-age $z$ score (LAZ), which was assessed by comparison with international reference data from the World Health Organization and the National Center for Health Statistics (16)—ie, $<-1.3$ for children 12–20 mo old and $<-1.5$ for children 21–29 mo old—and hemoglobin $\geq 10.5 \text{ g/dL}$ after adjustment for altitude (22). These children did not report to the neighborhood screening site for anthropometric screening, and no reasons for nonattendance were provided. Reasons for ineligibility: LAZ $>-1.25$, 74%; over age, 26%; reasons for loss to follow-up: moved from area or not at home at follow-up, 92%; refused hemoglobin assessment, 8%. Reasons for nonenrollment: refused study design or written consent or did not attend the final eligibility screening (no reason given), 52%; LAZ not $<-1.3$ or $<-1.5$ according to age group at time of enrollment, 41%; refused blood draw, 7%.

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Written informed consent was obtained from the parents of each child before his or her enrollment in the study; if a child’s parents were unable to read, write, or both, oral informed consent was obtained. Permission to conduct this study and approval of informed consent documentation were obtained from the National Institutes of Health–approved Human Subjects Committees of the University of California, Davis, and the Corporación Ecuatoriana de Biotecnología (Quito, Ecuador).

Interventions

Group assignment and supplements

On entering the study, participants were stratified by age (12–20 or 21–30 mo old) and sex and then randomly assigned to a study group. Children in each of the study groups consumed 1 of 5 daily supplements containing 3, 7, or 10 mg Zn as zinc sulfate, 10 mg Zn + 0.5 mg Cu as copper sulfate, or placebo per 5 mL of flavored syrup. The randomization lists for each of these strata were generated independently by using a fixed block randomization procedure.

Supplements were prepared by the Pharmacology Laboratory at the Universidad Central del Ecuador. Mineral concentrations were confirmed by independent laboratory analyses before distribution. Bottles of the assigned supplement were left in each child’s home and exchanged twice monthly. Caregivers were encouraged to provide the supplement once daily upon waking the child or between morning meals. Fieldworkers visited the children’s homes 3–5 times/wk to record supplement consumption as full or partial and to ascertain whether consumption was observed by the fieldworker or reported by the caregiver. Data were also collected on the consumption of any other nutritional supplements.

Socioeconomic status indicators

The family’s socioeconomic status (SES) was assessed by interview and observations of housing quality at the time of entry into the study. SES data included possessions (eg, stove, oven, refrigerator, television, radio, animals, bicycle, automobile, or family-owned shop or roving sales cart), housing characteristics (eg, building materials, type of water source, hygienic services, cooking facilities, availability of electricity, and home ownership), and parental characteristics (educational level and occupation).

Treatments for morbidity

Fieldworkers provided oral rehydration solution for diarrhea and acetaminophen for fever. Children were referred to the study doctor for symptoms of fever (≥38 °C axillary or ear canal temperature), elevated respiratory rate (≥40 respirations/min), diarrhea (≥3 semi-liquid or watery stools/24 h), chronic diarrhea (≥14 consecutive days of diarrhea), or blood in the stool or whenever a referral was deemed necessary by the fieldworker or was requested by the caregiver. The doctor’s evaluation, treatment, and, when necessary, referral were provided gratis, either in the child’s home or at a centralized clinic, according to previously defined illness categories and treatment algorithms.

Outcomes

Biochemistry

The children’s venous blood was drawn in the morning, after either an overnight fast or a delay of ≥2 h since the last reported meal. This procedure was completed at a central location at months 0, 3, and 6 of the intervention. Biochemical markers were measured in plasma, serum, whole blood, and red blood cells (RBCs). Zinc status was assessed among all 5 study groups by using the plasma zinc concentration. Copper status was assessed by analyzing the plasma copper concentration, serum ceruloplasmin activity, and activity of erythrocyte zinc-copper superoxide dismutase (SOD)/mg hemoglobin. Iron status was assessed with whole blood hemoglobin and serum ferritin concentrations. Serum total and HDL-cholesterol concentrations were measured to assess lipoprotein responses to the intervention. Serum C-reactive protein (CRP) concentrations were analyzed to control for possible effects of an acute phase response on plasma zinc and copper concentrations and serum ceruloplasmin and ferritin concentrations.

To permit baseline comparisons, initial analyses of plasma zinc and copper concentrations, whole blood hemoglobin, and serum CRP concentrations were conducted on all samples available from participating children. However, final values were assessed only on a randomly selected subset of paired samples, as described later in reference to sample size estimations. Other markers of copper and iron status and lipoprotein concentrations were analyzed only in a subset of the 3 study groups for which possible adverse events were assessed—namely, the placebo, 10 mg Zn/d, and 10 mg Zn + 0.5 mg Cu/d study groups.

Blood was drawn by pediatric phlebotomists into Sarstedt monovettes (Sarstedt AG & Co, Nümbrecht, Germany). Blood for plasma was obtained from trace element–free tubes containing lithium heparin anticoagulant (no. 01.1604.400, with needles, and no. 01.1602.01; Sarstedt AG) and serum from separator tubes (no. 01.1602.01; Sarstedt). RBC pellets were obtained from the lithium heparin–treated blood after 3 saline solution washes. Blood samples for plasma were centrifuged within 30 min, and those for serum were centrifuged within 45–60 min. All other blood processing occurred within 2 h of sample collection. All processed samples were stored in coolers until they were transferred to a −20 °C freezer within 6 h of collection. All other sample transportation was on dry ice. Baseline and follow-up analyses for each biochemical marker of a particular child were completed within the same analytic run.

Plasma zinc and copper concentrations were measured by using an inductively coupled plasma–optical emission spectrometer (ICP-OES; Varian Australia Pty Ltd, Palo Alto, CA); with a zinc standard (#S4400-1000681; CPI International, Santa Rosa, CA) and a bovine liver standard (#SRM 1577b; National Institute of Standards and Technology, Boulder, CO). Serum CRP concentrations were analyzed by using radial immunodiffusion (GT044.3; The Binding Site Limited, Birmingham, United Kingdom). Serum ceruloplasmin activity was measured in response to o-dianisidine dihydrochloride (23, 24) and in relation to human ceruloplasmin control (#C4519; Sigma-Aldrich Corporation, St Louis, MO).

Erythrocyte SOD activity was measured according to the method of Peskin and Winterbourn (25) in relation to RBC hemoglobin concentration (Drabkin’s solution and standards no. 0320–650; Stanbio Laboratory, Boerne, TX) and standardized to human erythrocyte SOD control (#S9636; Sigma-Aldrich Corporation). A fixed volume of the stored, triple-washed RBC pellet was lysed in cold double-deionized water. After being mixed by vortex and after centrifugation (2500 × g, 10 min, 4 °C), a fixed volume of this lysate was mixed with a 37.5%
chloroform–62.5% ethanol solution, mixed by vortex, and cen-
trifuged (2500 × g, 10 min, 4 °C) to differentiate zinc-copper SOD from manganese or iron SOD. This aqueous supernatant was stored at −80 °C until analysis, as described above. RBC and whole blood hemoglobin concentrations were measured by using Drabkin’s solution. Serum ferritin concentration was measured by using immunoradiometric assay (Coat-A-Count Ferritin IRMA no. IKFE and standards; Diagnostic Products Corporation, Los Angeles, CA). Serum total and HDL-cholesterol concentra-
tions were analyzed colorimetrically (manual kits no. CH201 and CH2673 and standards; Randox Laboratories Ltd, Crumlin, United Kingdom).

**Morbidity questionnaire**

Morbidity events were recorded during home visits 3–5 times/wk by using a systematic, symptom-based questionnaire and observation of the child. Elicited information included the child’s general health status, appetite, number and consistency of stools, and symptoms of cough, fever, nasal discharge, vomiting, and earache or discharge from the ear during each day since the previous visit. Body temperature was measured when fever, cough, or diarrhea was reported and once monthly on a nonillness day. Respiratory rates were measured for 1 min in duplicate by the fieldworker when fever, cough, or diarrhea was reported. The doctor repeated these assessments when the child was referred for illness. Diarrhea was defined as number of new episodes (separated by ≥3 days) of ≥3 semi-liquid or liquid stools, and symptoms of cough, fever, nasal discharge, vomiting, and earache or discharge from the ear during each day since the previous visit. Body temperature was measured when fever, cough, or diarrhea was reported and estimated measurements differed by 3 mm between the 2 participating anthropometrists. The 2 closest measurements were averaged. Length measurements were repeated when the av-
eraged measurements differed by ≥3 mm between the 2 participate-
ing anthropometrists. The 2 closest measurements were aver-
ergated. For statistical analysis, weight and length measures were converted to nutritional status indexes of LAZ, weight-for-age (WAZ), and weight-for-length (WLZ) z scores as compared with the 2006 WHO child growth standards (27).

**Sample size estimation and statistical analyses**

The sample size was designed to detect a difference among groups of 0.5 SD units in anthropometry at 80% power, which resulted in a planned sample size of 97 children per group (plus 25 children to account for possible attrition), or 122 children per intervention group. To detect a group-wise difference of 0.7 SD units with 80% power in change in the biochemical indicators, a sample size of 50/group was selected for analyses that included all 5 study groups, and a sample size of 40/group was selected for analysis of just 3 study groups. For these latter analyses, a subset of paired specimens was randomly selected from the 472 subjects who had sufficient quantities of both baseline and final samples of blood to allow the conduct of all required analyses. Although study compliance markers were assessed, primary analyses were completed on an intention-to-treat basis.

Statistical analyses were completed by using SAS for WINDOWS software (version 9.1; SAS Institute Inc, Cary, NC). Logarithmic transformations were required to normalize distrib-
utions for concentrations of zinc, ferritin, ceruloplasmin, eryth-
rocyte SOD, and HDL and total cholesterol and for the presence of purulent nasal discharge. The incidence of diarrhea and the presence of low appetite, cough, fever, and vomiting were normalized by square-root transformations. Suitable transforma-
tions were not found for full or partial supplement consumption or for rates of earache or discharge, elevated respiratory rate, and acute upper or lower respiratory infection, and thus these vari-
ables were analyzed by using nonparametric statistics. Factor analyses were conducted to aggregate SES characteristics into 3 main SES factors, which generally corresponded to material possessions, housing quality, and parental characteristics. Possession factors were given a value of 1 if present and of 0 if not present. Parental and housing characteristics were graded from worst to best as 0 to a maximum value, depending on the possible number of categories. Each of these graded values was trans-
formed into a fraction between 0 and 1 by dividing the actual value into the maximal value for each characteristic. The value of each SES factor is a summation of these transformed values and the 0–1 values of the SES characteristics included in each respective SES factor. Thus, the maximal values for material pos-
sessions, housing quality, and parental characteristics are 7, 6, and 4, respectively.

Group-wise differences at baseline were compared by using analysis of variance (ANOVA) for continuous variables and a chi-square test for categorical variables. All group-wise postint-
ervention changes were compared by using analysis of covari-
ance (ANCOVA) for continuous variables after control for rel-
levant baseline variables. Baseline variables used as covariates included site, age, sex, SES factors, baseline values of the re-
spective dependent variables, elevation of the respective CRP (in the case of biochemical outcomes) (yes or no), and baseline anthropometric z scores. Possible interactions between selected covariates and treatment group were tested by ANCOVA, and all significant covariates were retained. Because no suitable trans-
formation was found, supplement consumption was analyzed by using the Kruskall-Wallis test, with no covariates, and the non-
normally distributed morbidity variables mentioned above were analyzed by using ANCOVA based on ranks. Morbidity analyses were weighted by the number of days of observation and were restricted to children for whom ≥30 d of data were available (>80% of all children). However, including all available data did not affect the results. When overall results were statistically significant, group means were compared with the use of the Tukey-Kramer test after control for significant covariates. To test for nonlinearity of the response of plasma zinc concentrations to supplemental zinc, the supplemental zinc dose was included as a continuous variable, supplemental copper was included as a catego-
erical variable, and both linear and higher-order polynomial terms were tested in the model.
RESULTS

Enrollment, attrition, and baseline characteristics

Of the 938 surveyed children who were eligible for the full intervention study, 631 were enrolled (Figure 1). Of these 631 children, 531 (84%) completed the 6-mo intervention with at least the final anthropometry assessment. Of the 100 enrolled children who were lost to follow-up during the intervention period, 55 moved out of the study area, 11 refused to continue supplement consumption, 25 refused blood draws, and 9 withdrew consent without a specified reason. There were no significant differences in rates of attrition by treatment group (Table 1) nor any significant differences between the baseline characteristics of the children who left the study prematurely and those of children who completed the full 6-mo intervention.

No significant group-wise differences were found at baseline by age, sex, SES markers, anthropometric measurements, or biochemical status (Table 1, and Tables 2, 3, and 4). Fifty-three percent of children were male, and the mean ± SD age at entrance into the full intervention trial was 20.9 ± 5.4 mo. On average, mothers and fathers had completed 4.3 ± 1.9 and 4.7 ± 1.9 y of education, respectively. Electricity and propane cooking stoves were available in most households (99%), but there was greater diversity in other material possessions and home quality indicators, such as building materials, water source, and latrine facilities. The overall geometric mean plasma zinc concentration at baseline was 70.8 μg/dL (95% CI: 69.6, 72.1 μg/dL), and 31.8% of children had values < 65 μg/dL. The mean plasma copper concentration was 136.4 μg/dL; <1% of children had values < 80 μg/dL. Mean baseline whole blood hemoglobin was 11.7 ± 1.1 g/dL after adjustment for altitude (22), and the geometric mean serum ferritin concentration among the 3 groups measured was 27.7 ng/mL (95% CI: 24.7, 31.0 ng/mL). Although children were treated for iron deficiency anemia before beginning the study and although they had hemoglobin concentrations ≥ 10.5 g/dL, by HemoCue analyses, when status was confirmed by using venous blood samples, 18.0% of children began the study with adjusted hemoglobin concentrations < 11.0 g/dL, and 12.8% had ferritin values < 11.2 ng/mL. The baseline geometric mean serum HDL-cholesterol concentration of the children was 40.1 mg/dL (95% CI: 37.3, 43.0 mg/dL), and their total cholesterol concentration was 106.9 mg/dL (95% CI: 102.4, 111.6 mg/dL). The mean baseline LAZ was −2.3 ± 0.6, and 60% of children were stunted (LAZ < −2). The mean baseline WAZ was −1.2 ± 0.8, and 15% of children had WAZ < −2. The mean baseline WLZ was −0.1 ± 0.9, and <3% of children had WLZ < −2.

Several differences were found in the children’s baseline characteristics by study site. Children in El Carmen began the study with the lowest values for WAZ and WLZ, plasma zinc and copper concentrations, and all 3 SES factors. Children in the Latacunga area tended to be the oldest, and children in the Quito area tended to be the youngest and most stunted. However, children were assigned to treatment groups within each study site, and thus, despite these site-specific differences, there were no significant differences in any initial biochemical or anthropometric indicators by treatment group (Tables 1–4).

Adherence to supplement consumption

Supplement consumption was observed by fieldworkers on 34% of the possible days of administration. The entire supplement was consumed on 95% of the observed days and was reported to be consumed on 91% of the remaining days. According to both observed and reported data, the entire supplement dose was consumed on 91.5% of observed days and at least half of the dose was consumed on another 3% of observed days. The 7 children with

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>3 mg Zn/d</th>
<th>7 mg Zn/d</th>
<th>10 mg Zn/d</th>
<th>10 mg Zn + 0.5 mg Cu/d</th>
<th>P²</th>
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<td>Subjects (n)</td>
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<td>124</td>
<td>126</td>
<td>126</td>
<td>128</td>
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<tr>
<td>Age (mo)</td>
<td>21.2 ± 5.3¹</td>
<td>20.9 ± 5.1</td>
<td>20.8 ± 5.2</td>
<td>20.6 ± 5.6</td>
<td>21.2 ± 5.5</td>
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<td>Male (%)</td>
<td>52.8</td>
<td>53.2</td>
<td>53.2</td>
<td>53.2</td>
<td>53.1</td>
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<tr>
<td>SES factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Possession</td>
<td>3.3 ± 1.5</td>
<td>3.3 ± 1.5</td>
<td>3.3 ± 1.7</td>
<td>3.4 ± 1.6</td>
<td>3.3 ± 1.4</td>
<td>0.95²</td>
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<td>Parental</td>
<td>1.5 ± 0.6</td>
<td>1.5 ± 0.6</td>
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<td>Housing</td>
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<td>4.1 ± 1.0</td>
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<td>4.1 ± 1.1</td>
<td>4.1 ± 1.0</td>
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<td>Full supplement consumption (%)</td>
<td>92.4 ± 10.8</td>
<td>92.3 ± 12.7</td>
<td>90.9 ± 14.8</td>
<td>92.3 ± 12.5</td>
<td>89.5 ± 15.0</td>
<td>0.52²</td>
</tr>
<tr>
<td>Partial supplement consumption</td>
<td>2.9 ± 6.6</td>
<td>2.2 ± 9.3</td>
<td>3.9 ± 12.4</td>
<td>2.7 ± 10.4</td>
<td>4.9 ± 12.2</td>
<td>0.15²</td>
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<tr>
<td>Attrition before 6 mo (%) of children</td>
<td>9.5</td>
<td>12.9</td>
<td>13.5</td>
<td>7.1</td>
<td>13.3</td>
<td>0.04²</td>
</tr>
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¹ SES, socioeconomic status.
² P values are group-wise.
³ ± SD (all such values).
⁴ ANOVA.
⁵ Chi-square test.
⁶ Includes ownership of household appliances, animals, and vehicles.
⁷ Includes the parents’ years of education and vocation.
⁸ Includes the materials from which the home was constructed, water source, and type of latrine or toilet facilities and whether the house was rented, owned, or borrowed.
⁹ Kruskall-Wallis test.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>3 mg Zn/d</th>
<th>7 mg Zn/d</th>
<th>10 mg Zn/d</th>
<th>10 mg Zn + 0.5 mg Cu/d</th>
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<td>Maximum number of subjects</td>
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<td>Baseline (n)</td>
<td>116</td>
<td>113</td>
<td>117</td>
<td>116</td>
<td>117</td>
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<tr>
<td>Change from baseline (n)</td>
<td>56</td>
<td>50</td>
<td>52</td>
<td>54</td>
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<tr>
<td>Initial plasma zinc concentration (µg/dL)</td>
<td>69.2 (66.5, 72.0)</td>
<td>69.8 (67.1, 72.7)</td>
<td>70.8 (68.0, 73.6)</td>
<td>70.5 (67.8, 73.4)</td>
<td>73.8 (71.0, 76.7)</td>
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<tr>
<td>Children with initial plasma zinc concentration &lt;65 µg/dL (%)</td>
<td>39.7</td>
<td>31.9</td>
<td>29.1</td>
<td>31.0</td>
<td>27.4</td>
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<tr>
<td>Change in plasma zinc concentration (µg/dL)</td>
<td>0.2a (−5.0, 5.8)</td>
<td>10.5a,b (4.1, 17.4)</td>
<td>16.4b (9.5, 23.9)</td>
<td>20.8b (14.2, 27.9)</td>
<td>16.6b (10.0, 23.8)</td>
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<td>Initial serum CRP (mg/L)</td>
<td>11.0</td>
<td>15.9</td>
<td>13.8</td>
<td>23.2</td>
<td>14.7</td>
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<td>Initial Hb concentration (g/dL)</td>
<td>12.8 ± 1.4</td>
<td>12.5 ± 1.5</td>
<td>12.7 ± 1.4</td>
<td>12.6 ± 1.3</td>
<td>12.8 ± 1.4</td>
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<tr>
<td>Change in Hb concentration (g/dL)</td>
<td>−0.68 (−1.05, −0.31)</td>
<td>−0.31 (−0.69, 0.07)</td>
<td>−0.43 (−0.84, −0.02)</td>
<td>−0.39 (−0.75, −0.04)</td>
<td>−0.48 (−0.86, −0.09)</td>
</tr>
<tr>
<td>Initial serum ferritin concentration (ng/mL)</td>
<td>28.1 (23.2, 34.1)</td>
<td>—</td>
<td>—</td>
<td>27.2 (22.3, 33.2)</td>
<td>27.6 (22.7, 33.6)</td>
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<tr>
<td>Change in serum ferritin concentration (ng/mL)</td>
<td>−8.2 (−11.2, −4.8)</td>
<td>—</td>
<td>—</td>
<td>−5.7 (−9.1, −1.6)</td>
<td>−6.4 (−9.7, −2.5)</td>
</tr>
</tbody>
</table>

1 CRP; C-reactive protein; Hb, hemoglobin. Values in a row with different superscript letters were significantly different, P < 0.01; the difference between the placebo group and the 3 mg Zn/d group was not significant, P = 0.08.
2 Actual samples analyzed and remaining in final analyses ranged from 44 to 56/group. Smaller sample sizes are due to inadequate sample volumes.
3 Geometric x̄ (95% CI); change in group means was based on the percentage change multiplied by the baseline overall geometric mean. Values were normalized by log transformation for analyses.
4 Group means were compared by using ANOVA; means in the table were not adjusted for covariates.
5 Group populations were compared by using a chi-square test.
6 ANCOVA; means in the table we adjusted for covariates.
7 Unadjusted x̄ ± SD.
8 Estimated median (95% CI).
TABLE 3
Initial anthropometric measurements and changes from baseline, by intervention group, in Ecuadorian children 12–36 mo old with low initial length-for-age during a 6-mo intervention

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>3 mg Zn/d</th>
<th>7 mg Zn/d</th>
<th>10 mg Zn/d</th>
<th>10 mg Zn + 0.5 mg Cu/d</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>127</td>
<td>123</td>
<td>126</td>
<td>126</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Baseline (n)</td>
<td>108</td>
<td>103</td>
<td>100</td>
<td>110</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>Change from baseline (n)</td>
<td>108</td>
<td>103</td>
<td>100</td>
<td>110</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>77.6 ± 4.7(^1)</td>
<td>77.3 ± 4.5</td>
<td>77.2 ± 4.6</td>
<td>77.2 ± 4.6</td>
<td>77.5 ± 4.7</td>
<td>0.93(^3)</td>
</tr>
<tr>
<td>Change (cm)</td>
<td>4.9 ± 1.1</td>
<td>4.9 ± 1.2</td>
<td>5.0 ± 1.0</td>
<td>4.8 ± 1.2</td>
<td>4.9 ± 1.1</td>
<td>0.34(^4)</td>
</tr>
<tr>
<td>LAZ</td>
<td>−2.3 ± 0.7</td>
<td>−2.3 ± 0.6</td>
<td>−2.3 ± 0.7</td>
<td>−2.2 ± 0.6</td>
<td>−2.3 ± 0.6</td>
<td>0.74(^4)</td>
</tr>
<tr>
<td>Change</td>
<td>0.11 ± 0.32</td>
<td>0.11 ± 0.30</td>
<td>0.15 ± 0.29</td>
<td>0.05 ± 0.33</td>
<td>0.10 ± 0.30</td>
<td>0.59(^4)</td>
</tr>
<tr>
<td>Weight</td>
<td>9.7 ± 1.3</td>
<td>9.8 ± 1.3</td>
<td>9.7 ± 1.2</td>
<td>9.7 ± 1.4</td>
<td>9.8 ± 1.2</td>
<td>0.82(^4)</td>
</tr>
<tr>
<td>Initial (kg)</td>
<td>1.0 ± 0.4</td>
<td>1.1 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>1.0 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>0.33(^4)</td>
</tr>
<tr>
<td>Change</td>
<td>0.04 ± 0.33</td>
<td>0.07 ± 0.45</td>
<td>0.06 ± 0.30</td>
<td>0.02 ± 0.41</td>
<td>0.06 ± 0.34</td>
<td>0.66(^4)</td>
</tr>
<tr>
<td>WLZ</td>
<td>−0.2 ± 0.8</td>
<td>−0.1 ± 0.9</td>
<td>−0.1 ± 0.8</td>
<td>−0.2 ± 1.0</td>
<td>−0.0 ± 0.8</td>
<td>0.36(^4)</td>
</tr>
<tr>
<td>Initial</td>
<td>0.04 ± 0.46</td>
<td>0.09 ± 0.65</td>
<td>0.05 ± 0.42</td>
<td>0.07 ± 0.57</td>
<td>0.07 ± 0.46</td>
<td>0.72(^4)</td>
</tr>
</tbody>
</table>

\(^1\) LAZ, length-for-age z score; WAZ, weight-for-age z score; WLZ, weight-for-length z score.
\(^2\) \(\bar{x}\) ± SD (all such values).
\(^3\) Group means were compared with ANOVA for baseline variables.
\(^4\) Group means were compared with ANCOVA for change variables, after control for significant covariates and baseline values.

very low consumption rates (consumption of at least half the dose on <60% of study days) came from all 5 study groups, but 3 of them were in the placebo group, and 2 left the study before reaching 30 d of data collection. There were no significant differences by treatment group in the percentage of children who were consuming the entire supplement \((P = 0.45; \text{Table 1})\) or at least half of the supplement \((P = 0.73)\).

Plasma zinc concentration

The mean change in plasma zinc concentrations from baseline increased progressively with higher doses of supplemental zinc \((P < 0.001)\) (Table 2). Regression analysis indicated that the increase in plasma zinc concentration did not deviate significantly from linearity within the range of doses provided in this study (Figure 2).

Morbidity

Among children consuming the placebo supplement, the estimated median incidence of diarrhea was 1.9 episodes/100 d of observation. The incidence of diarrhea was 21–42% lower in children consuming any dose of zinc than in those consuming placebo \((P < 0.01\) for group-wise comparison). Tukey-Kramer analyses indicated that the effect of treatment group on the incidence of diarrhea was significant for doses of 3 and 7 mg Zn/d \((P = 0.04\) and \(P < 0.01\), respectively) relative to placebo (Figure 3). Similar patterns were observed among boys and girls (data not shown).

Initial age, WLZ, and parental SES factors were significantly \((P < 0.001\), \(P = 0.038\), and \(P = 0.028\), respectively) associated with diarrhea incidence. In particular, diarrhea rates decreased as initial age, parental SES factors, and initial WLZ increased. Further analyses showed a significant interaction between treatment group and age \((P < 0.001)\) for incidence. To assess these effects, adjusted group means were calculated at ages 17.5 and 23.5 mo, which were the cutoffs for age tertiles (ie, 11.5–17.4, 17.5–23.4, and 23.5–30 mo old at baseline). The general model is shown in Table 5.

Subanalyses by age tertile showed a significantly \((P < 0.001\) greater effect of zinc than of placebo on the incidence of diarrhea in children in the youngest age tertile who received any dose of zinc (Tukey-Kramer test; Figure 4). No significant group-wise differences were found among children in the 2 older age tertiles (≥17.5 mo at baseline). However, the 11–39% reductions in diarrhea incidence in the older children who received 3 or 7 mg Zn are of a magnitude similar to the changes reported by Bhutta et al (2) in a pooled analysis of zinc intervention studies, although with larger CIs. Thus, the lack of statistical significance among the older children in the present study may be due to an inadequate sample size. These patterns did not differ significantly by study site (data not shown).

The overall mean percentage (and 95% CI) of days on which other morbidity symptoms were present were as follows: 4.4% (3.9, 4.9) for poor appetite, 15.2% (13.9, 16.7) for cough, 2.5% (2.2, 2.7) for reported fever, 2.7% (2.5, 2.9) for purulent nasal discharge, and 2.3% (1.9, 2.7) for acute upper respiratory infection. The overall proportion of days with vomiting, earache or discharge from the ear, respiratory rate > 40 rpm, and acute lower respiratory infection was <1%. There were no significant
group-wise differences in any of these symptoms or illness categories (data not shown) or in any other symptoms or illness categories (Table 6).

**Anthropometry**

During the 6-mo intervention, the enrolled children gained 4.9 ± 1.1 cm in height and 1.1 ± 0.4 kg in weight. LAZ increased by 0.1 ± 0.3, WAZ by 0.05 ± 0.37, and WLZ by 0.07 ± 0.52.

However, no significant group-wise differences were found between changes in length, weight, or anthropometric z scores, even after control for initial age, LAZ, WLZ, parental SES factor, and study site or when analyses were restricted to children with initial LAZ of < −2.0 (Table 3).

**Markers of copper and iron status and serum lipoprotein concentrations**

Plasma copper and serum ceruloplasmin concentrations declined in all children during the course of the study; there were no

**FIGURE 2.** Mean (and 95% CI) change in plasma zinc concentration in Ecuadorian children 12–36 mo old with low initial length-for-age during the 6-mo intervention, by intervention group. Values with different letters are significantly different between groups (ANCOVA). Subjects by group: placebo, n = 49; 3 mg Zn/d, n = 45; 7 mg Zn/d, n = 44; 10 mg Zn/d, n = 53.

**FIGURE 3.** Estimated median (and 95% CI) incidence of diarrhea in Ecuadorian children 12–36 mo old with low initial length-for-age during the 6-mo intervention, by intervention group. Values with different letters are significantly different between groups (ANCOVA). Subjects by group: placebo, n = 116; 3 mg Zn/d, n = 117; 7 mg Zn/d, n = 116; 10 mg Zn/d, n = 118; 10 mg Zn + 0.5 mg Cu, n = 121.
significant group-wise differences in these changes (Table 4). There was no consistent or significant change in erythrocyte SOD activity among the 3 groups ($P = 0.12$). Mean hemoglobin and ferritin concentrations decreased during the study intervention in all groups, but there were no significant group-wise differences in the change in either of these markers of iron status (Table 2).

The mean serum HDL-cholesterol concentration increased in all groups by 4.9 ± 17.5 mg/dl during the intervention period. There was no change in total cholesterol during the 6-mo intervention, and there were no significant group-wise differences in the mean change in serum HDL or total cholesterol during the 6-mo intervention ($P = 0.83$ and $P = 0.66$, respectively; Table 4).

**DISCUSSION**

We found that the mean plasma zinc concentrations of the children increased progressively in relation to the dose of supplemental zinc. In addition, there were greater reductions in the incidence of diarrhea in children who received supplemental zinc than in those who received the placebo, and these differences were significant for groups that received 3 or 7 mg supplemental Zn/d. There were no other significant group-wise differences in symptoms of morbidity or rates of growth between children who received any dose of supplemental zinc and those who received the placebo. Finally, there were no adverse effects of 10 mg supplemental Zn/d on markers of copper and iron status or on lipoprotein concentrations. The randomized clinical trial design, blinding of investigators and participants to treatment group, selection of participants on the basis of the likelihood of finding zinc deficiency, and the use of intention-to-treat analyses lend strength to the findings of the present study.

**Response of plasma zinc concentration to zinc dose**

The increases in plasma zinc concentration that we observed in response to supplemental zinc confirm that the intervention was successfully administered (28, 29). Tracer studies in adults and children have shown that the total amount of absorbed zinc increases linearly in relation to the test dose, with doses ranging from 1 to 6 mg Zn (30–32). However, the magnitude of the increase in zinc absorption is progressively less with higher doses, i.e., 9–30 mg Zn/d (30–32). We observed no significant deviation from linearity in the response of plasma zinc concentrations to zinc doses ranging from 3 to 10 mg Zn/d (Figure 2).

**Response of diarrhea to zinc dose**

The reductions we observed in the incidence of diarrhea after zinc supplementation are consistent with reductions reported in a pooled analysis of zinc intervention studies in which the provided single doses of supplemental zinc ranged from 5 to 20 mg/d to infants and children (2). By simultaneously assessing 3 different doses of zinc, we were able to determine that 3 mg supplemental Zn/d was sufficient to reduce the incidence of diarrhea in the current study population, and the greatest effect occurred with doses of 3 and 7 mg Zn/d. These findings support the recommendation of the IZINC (13) to provide 5 mg prophylactic supplemental Zn/d to children at risk of zinc deficiency. This dose-response design also exposed an interesting interaction between age at baseline and the supplemental dose of zinc for rates of diarrhea, which suggested that the greatest effect of supplemental zinc occurred among the children in the youngest age tertile, and that the children responded to the lower doses of supplemental zinc but not to the highest dose.
In general, the risk of zinc deficiency in children decreases with age, as growth velocity slows and more food sources of zinc are introduced into the diet (33). Thus, there is reason to believe that the younger children in this study were at the greatest risk of zinc deficiency. Moreover, in the placebo groups, the proportion of days with diarrhea was nearly two-thirds less in children aged 12–17.5 mo old (2.3%) than in those aged <17.5 mo old (7.1%), which substantially reduces the power to detect differences among the older children. Nevertheless, there were no significant differences in mean baseline plasma zinc concentrations by age tertile, so we cannot confirm whether there were indeed age-related differences in zinc status.

The absence of a reduction in rates of diarrhea in the older children consuming 10 mg supplemental Zn/d could be due to the lack of a beneficial effect or to the presence of an adverse effect of this dose of supplemental zinc in these children. Intestinal perfusion studies (34, 35) showed positive net water and sodium absorption at lower zinc concentrations (30–270 μmol Zn/L) and net losses at higher concentrations (270–1070 μmol Zn/L), which suggested a possible reversal at high doses of zinc’s beneficial effect of reducing diarrhea. Because the incidence of other morbidity symptoms was low in the study population, it is likely that the sample size was inadequate to detect other groupwise differences in morbidity.

**Anthropometry**

It was surprising that we found no change in growth rate in response to zinc supplementation in these children with presumed zinc deficiency, even after exploring for possible modifying effects of initial age, LAZ, and plasma zinc concentration. Although it is more likely to see a growth response in younger than in older children, we found no significant interaction by initial age. As described previously, the doses of zinc provided were sufficient to increase the plasma zinc concentration (Table 2), which indicated that bioavailability of the zinc provided was not a limiting factor. Previous studies found growth responses in stunted children consuming doses of 10 mg supplemental Zn/d (9), which suggested that the 10-mg dose of supplemental zinc should have been adequate to promote a growth response in the population of the present study. Therefore, it is possible that zinc was not limiting the growth response of the children in the current study or that another factor was preventing these children from responding to zinc with increased growth rates. Analyses of dietary data may provide more insight into other macronutrient or micronutrient deficiencies that could have contributed to the stunting found among the children in the present study.

**Possible adverse effects of zinc**

Our findings that markers of copper and iron status and lipoprotein concentrations did not differ significantly between children receiving 10 mg supplemental Zn/d, with or without copper, and those receiving placebo are consistent with the results of most studies among young children in which copper markers were analyzed (7, 36–42). Five of these 8 studies provided 10 mg supplemental Zn/d. We do not have an explanation for the downward trend in copper markers observed from baseline to 6 mo among all treatment groups in the current study. However, these results are consistent with the changes observed in the placebo group of other zinc intervention studies that enrolled children 1–3 y old (37–40, 42) and reported 5–13% decreases in erythrocyte SOD activity or serum copper concentrations after 4–15 mo of intervention.

**Conclusions**

The results of the present study confirm that a rise in plasma zinc concentration may be used as an indicator of successful provision of supplemental zinc in a population of young children, even at doses as low as 3 mg/d. The results also indicate that it may be possible to provide as little as 3–7 mg supplemental Zn/d to reduce the incidence of diarrhea in young children at risk of zinc deficiency. Indeed, there is some suggestion that the beneficial effect of zinc on diarrheal morbidity may not occur with larger doses of zinc, especially in older children. More dose-response studies are needed in other populations of young children to confirm these findings. We observed no adverse effects of 10 mg supplemental Zn/d on indicators of copper and iron status in this study population, which indicates that the tolerable UL of zinc recommended by the Institute of Medicine and the IZiNCG may be set unnecessarily low. Studies reporting the effect of zinc on copper and iron status markers and on lipoprotein concentrations in children should be reviewed to determine whether there is now sufficient

### Table 6

Duration of diarrhea and percentage of days with selected symptoms of illness, by intervention group, in Ecuadorian children 12–36 mo old with low initial length-for-age during a 6-mo intervention

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>3 mg Zn/d</th>
<th>7 mg Zn/d</th>
<th>10 mg Zn/d</th>
<th>10 mg Zn + 0.5 mg Cu/d</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>116</td>
<td>117</td>
<td>116</td>
<td>118</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>Duration of episodes of diarrhea&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.71 (1.57, 1.87)</td>
<td>1.81 (1.63, 2.02)</td>
<td>1.82 (1.65, 2.01)</td>
<td>1.72 (1.59, 1.86)</td>
<td>1.72 (1.56, 1.90)</td>
<td>0.82&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Days with low appetite (%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.6 (3.6, 5.8)</td>
<td>4.2 (3.2, 5.3)</td>
<td>4.1 (3.1, 5.2)</td>
<td>4.6 (3.6, 5.7)</td>
<td>4.9 (3.8, 6.0)</td>
<td>0.76&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Days with cough (%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>18.8 (16.1, 21.7)</td>
<td>14.8 (12.4, 17.5)</td>
<td>15.4 (12.9, 18.1)</td>
<td>14.7 (12.3, 17.3)</td>
<td>10.5 (14.5, 19.9)</td>
<td>0.14&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Days with reported fever (%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.9 (2.4, 3.4)</td>
<td>2.1 (1.7, 2.6)</td>
<td>2.3 (1.9, 2.8)</td>
<td>2.1 (1.7, 2.6)</td>
<td>2.5 (2.0, 3.0)</td>
<td>0.18&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Days with nasal discharge (%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.1 (1.5, 2.7)</td>
<td>2.0 (1.5, 2.6)</td>
<td>1.3 (0.9, 1.8)</td>
<td>1.5 (1.1, 2.0)</td>
<td>1.5 (1.1, 2.1)</td>
<td>0.16&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Geometric mean; 95% CI in parentheses.
<sup>2</sup> Values were normalized for ANCOVA by log transformation.
<sup>3</sup> Values were normalized for ANCOVA by square root transformation.
<sup>4</sup> Estimated median; 95% CI in parentheses.
information to reassess the recommended tolerable UL of zinc for young children.

We acknowledge the hard work of all the health workers, office and laboratory staffs, nutritionists, and doctors who were instrumental in completing this study in Ecuador, and we acknowledge Janet Peerson for assistance with the statistical analyses. In particular, we thank each of the children and the parents whose participation made this study possible.

The authors’ responsibilities were as follows—SEW: the study design, implementation of the project in the field, data analysis, interpretation of results, and preparation of the manuscript; KHB: the study design, overall supervision of the research team, interpretation of results, and preparation of the manuscript; and FS: contributions to the study design, implementation of the project in the field, and interpretation of the results. None of the authors had any personal or financial conflict of interest.

REFERENCES


