Zinc supplementation improves bone density in patients with thalassemia: a double-blind, randomized, placebo-controlled trial[1–3]

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ABSTRACT

Background: Patients with thalassemia major (Thal) frequently have low plasma zinc, which has been associated with low bone mass.

Objective: The objective was to determine the effect of zinc supplementation on bone mass in patients with Thal.

Design: Forty-two subjects (21 females aged 10–30 y) with Thal and low bone mass were randomly assigned to receive 25 mg Zn/d or placebo. Bone mineral content (BMC) and areal bone mineral density (aBMD) were assessed by using dual-energy X-ray absorptiometry, and fasting blood was collected for the measurement of plasma zinc at 0, 12, and 18 mo.

Results: Thirty-two subjects, 81% of whom were transfusion dependent, completed the study (mean ± SD: 17.1 ± 5.2 y). Plasma zinc was ≤70 μg/dL in 11 subjects at baseline and increased significantly with zinc supplementation ($P = 0.014$). Use of intention-to-treat analysis and linear models for longitudinal data, adjusted for baseline and pubertal stage, showed that the zinc group had significantly greater increases in whole-body BMC (adjusted mean ± SE: 63 ± 15 g; $P = 0.02$), and aBMD (0.023 ± 0.006 g/cm^2; $P = 0.04$) than did the placebo group after 18 mo. Furthermore, adjusted spine and hip aBMD z scores each decreased by 0.3 SDs (both $P = 0.04$) in the placebo compared with the zinc group over the 18-mo study.

Conclusions: In young patients with Thal, zinc supplementation resulted in greater gains in total-body bone mass than did placebo. Zinc was well tolerated and is worthy of investigation in larger trials in Thal patients across a range of ages and disease severity. This trial was registered at clinicaltrials.gov as NCT00459732. *Am J Clin Nutr* 2013;98:960–71.

INTRODUCTION

Thalassemia major (Thal)[4] is a group of autosomal recessive genetic disorders that lead to limited or absent production of the α or β globin chain of hemoglobin. The disease is characterized by ineffective erythropoiesis, poor oxygenation, and hemolysis. Many patients require frequent blood transfusions to sustain life, improve growth, and prevent severe skeletal deformities. Children and adolescents with Thal frequently have poor growth and delayed pubertal development, whereas many adults with Thal have low bone mass, hypogonadism, growth hormone deficiency, diabetes, and depressed immune function (1). Coincidently, deficiency in zinc is also characterized by growth retardation, hypogonadism, diabetes, red blood cell fragility, poor immune function, and poor bone mineralization (2–5).

Previous reports suggest that up to 60% of adult patients with Thal have reduced bone mass for age (6). Low bone mass is related to an increased risk of fracture risk and significant bone pain (6–8). Bone deficits appear linked not only to an increase in bone resorption (9), but to a decrease in bone formation (10). Many factors likely contribute to the etiology of low bone mass in Thal, including nutritional deficiencies of calcium, vitamin D, and zinc. Suboptimal zinc status has been observed in patients with Thal, as suggested by decreased plasma and leukocyte zinc concentrations, decreased alkaline phosphatase activity, and increased urinary zinc excretion (11–14). Zinc supplementation has been shown to improve growth in children with Thal, which indicates that zinc deficiency is growth limiting in some patients (15).

Low dietary zinc intake and/or low plasma zinc concentrations have been shown to be correlated with low bone mass in animal studies (16), postmenopausal osteoporosis (17–19), and adolescent patients with Thal residing in Iran (20). Combined supplementation regimens of zinc and calcium in postmenopausal osteoporotic women improved bone mineral density (BMD) more than did calcium supplementation alone (21).

It is hypothesized that patients with Thal have low bone mass, in part because of zinc deficiency. The primary aim of this study was to determine the effect of zinc (25 mg/d) compared with that of placebo on bone density in young patients (10–30 y) with Thal and a low bone mass (BMD z score < -1.0). The secondary

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[4] Abbreviations used: aBMD, areal bone mineral density; BMC, bone mineral content; BMD, bone mineral density; CHOP, Children's Hospital of Philadelphia; CHRCO, Children's Hospital & Research Center Oakland; DXA, dual-energy X-ray absorptiometry; IGF-I, insulin-like growth factor I; PA, posterior-anterior; RDA, Recommended Dietary Allowance; Thal, thalassemia major; UCSF, University of California at San Francisco; 25(OH)D, 25-hydroxyvitamin D.

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aims of the study were to explore the effects of zinc supplementation on markers of bone formation and resorption.

SUBJECTS AND METHODS

An 18-mo double-blind, placebo-controlled, multicenter trial of zinc supplementation in subjects with Thal and low bone mass was conducted. Subjects were recruited and stratified according to sex and pubertal stage before randomization. Half of the subjects received zinc (25 mg/d as zinc sulfate) and the others a placebo capsule. Evaluations of anthropometric measures, diet, clinical status, zinc metabolites, and markers of bone formation and resorption were made at baseline and 3, 6, 12, and 18 mo (±2 wk); evaluations by dual-energy X-ray absorptiometry (DXA) were made at baseline, 12 mo, and 18 mo.

Subjects

Subjects with Thal were recruited from 3 hematology clinics in the United States: the Children's Hospital & Research Center, Oakland (CHRCO); the Children's Hospital of Philadelphia (CHOP); and the University of California at San Francisco (UCSF). Subjects with Thal were considered eligible if they were between 6 and 30 y of age and had a DXA-derived areal BMD (aBMD) z score <-1.0 at the spine, hip, or whole body within the previous 2 y. Subjects were excluded if they had other chronic medical conditions known to affect bone health, had received a bone marrow transplant (successful or unsuccessful engraftment), were currently participating in a trial with a treatment known to affect bone health, were pregnant, or had taken a bisphosphonate in the previous year. Subjects who were currently taking zinc (alone or in a multivitamin) were eligible to participate after a 3-mo washout period. Three months was considered a sufficient washout period because we previously showed that zinc absorption changed promptly (within 24 h) with changes in dietary zinc (22). Also, when zinc supplementation is initiated or discontinued, plasma zinc concentrations respond to the changes in zinc intakes within 5 d (23). Thus, 3-mo washout period ensured that a subject was in a steady state without zinc supplementation before study entry. Subjects were provided multivitamins without zinc as part of the study design. Subjects currently prescribed growth hormone, estrogen, or testosterone replacement were eligible if they had been taking the hormone for ≥6 mo before entry. Potentially eligible subjects, on the basis of age and diagnosis, were identified and approached by one of the co-investigators of the study to answer study-related questions. After all concerns were addressed, a screening visit was scheduled, at which time serum copper and vitamin D were assessed and screening health questions were reviewed according to the protocol outlined below.

Subject recruitment began in April 2006 and was completed in May 2008. Informed written consent was obtained from all subjects or legal guardians and assent from subjects aged <18 y by the co-investigators at each site. The protocol was approved by the Committee for the Protection of Human Subjects of the Institutional Review Board at CHRCO, CHOP, and UCSF.

Supplement intervention and adherence assessment

Zinc was provided to the intervention group as zinc sulfate capsules containing 25 mg elemental zinc/d (Tyson Nutraceuticals Inc). A placebo capsule was developed to be indistinguishable from the zinc capsule and dispensed from the CHRCO clinical pharmacy. Both were analyzed by inductively coupled plasma atomic emission spectroscopy after study completion by the Elemental Analytic Facility at the Children's Hospital Oakland Research Institute [placebo: 0.0 mg; zinc (mean ± SD): 21.6 ± 0.6 mg; n = 4 capsules in duplicate]. Subjects were asked to take one capsule each morning of the 18-mo long trial. If subjects were prescribed oral chelation (deferasirox), they were asked to take their study supplement at a separate time from their oral chelator medication. Subjects were recruited, stratified according to sex and pubertal stage (Tanner 1 and 2 compared with Tanner 3, 4, and 5), and randomly assigned (1:1). The study statistician (GG) provided a block randomization table (4 strati × 2 intervention groups) to the clinical pharmacy before study initiation. All investigators and subjects were blinded to study group. Randomization code was provided to the principal investigator by the clinical pharmacy after the last subject completed the 18-mo time point. A 3-mo supply of capsules (100 capsules per bottle) was provided to each study participant at baseline and 3, 6, 9, 12, and 15 mo. A study calendar was also provided to each subject as well as pill containers, soft sided lunch boxes and refrigerator magnets with a study-specific logo (Think Zn) to remind subjects to take their supplements. Pill counts were made every 3 mo to assess adherence. One subject (female, adult) was initially randomly assigned to placebo; however, at the 3-mo time point, the research pharmacy erroneously provided her with zinc instead of placebo, at which time it was decided she should stay on this arm of the study rather than revert back to placebo. For the intention-to-treat analysis, and in the randomization scheme, **Figure 1**, this subject was considered part of the zinc group. Although the Principle Investigator was notified during the study of the pharmacy error, the subject and investigator remained blinded to the intervention group until completion of the project.

Rationale for supplement dosage

The dosage for the intervention, 25 mg elemental Zn, is 2–3 times the Recommended Dietary Allowance (RDA) for most age and sex groups (8–11 mg/d). Although it may seem high for a nutritional supplement, depressed plasma zinc has been observed frequently in this population, even in patients who are taking over-the-counter zinc supplements of 15 mg Zn/d (24, 25). Therefore, we reasoned that a routine, low-dose supplement might not be sufficient for some subjects to overcome the potential for marginal zinc deficiency observed. Moreover, in 1987, Arcasoy et al (15) used 22–90 mg/d in patients with Thal for 1 to 7 y to treat growth failure. In addition, on a clinical basis, 50 mg elemental Zn is routinely provided to patients with Thal who are prescribed the oral chelator deferiprone.

Methods

Study visits were conducted at 3 centers: CHRCO (n = 30), UCSF (n = 5), and CHOP (n = 9). Three of the 5 subjects from UCSF came to CHRCO for their baseline, 12-mo, and 18-mo visits, including all their DXA measures. Before enrollment, subjects had a screening blood test for copper and vitamin D status. If serum copper was <70 μg/dL, the subject was provided

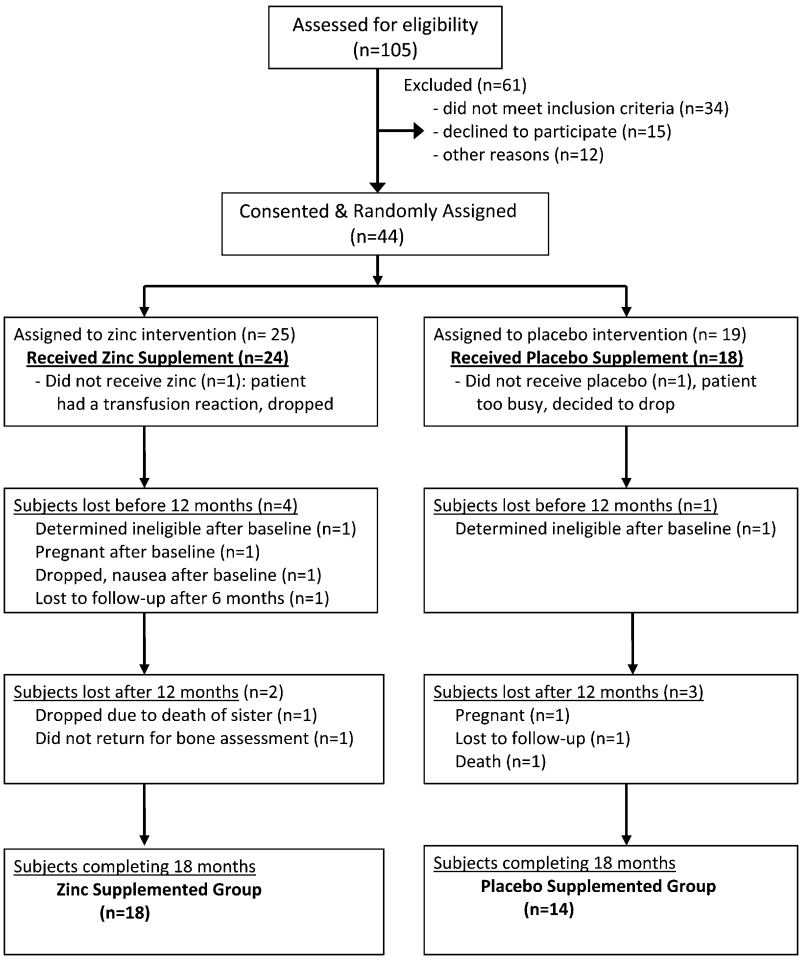

FIGURE 1. Recruitment and randomization scheme for subjects with thalassemia enrolled in the Think Zinc Trial.

2 mg Cu (as copper gluconate) throughout the study. Subjects with deficient concentrations of 25-hydroxyvitamin D [25(OH)D] (<11 ng/mL) were provided vitamin D supplements (400 IU/d as cholecalciferol), and their vitamin D concentration was retested. When their 25(OH)D concentration was ≥11 ng/mL, they were considered eligible for participation into the study. 25(OH) Vitamin D was monitored at each study visit; if the 25(OH)D concentration dropped to <20 ng/mL at any time point, they were provided supplemental cholecalciferol. The supplemental vitamin D dose was increased to 1000 IU in July 2007, when it was determined that 400 IU/d was not sufficient to maintain vitamin D status >20 ng/mL in this cohort of subjects. Once a subject's circulating concentrations of these nutrients were above the study threshold [70 μg/dL for copper, 20 ng/mL for 25(OH)D], they no longer were provided replacement supplementation.

Height, weight, and skinfold-thickness measurements were assessed by trained research anthropometrists in duplicate at each time point, and the mean was used in the analysis. In addition, subjects aged <20 y had a bone age examination. Sexual maturation was assessed in subjects <21 y of age by using a validated self-assessment pictoral questionnaire (26). Body composition was assessed from the DXA whole-body scan, fat (g), fat-free mass (g), and percentage total body fat used in the analysis.

After blood samples were collected, serum and plasma were separated from erythrocytes and stored at −70°C until analyzed. For transfused patients, blood samples were collected ≥2 wk after the previous transfusion, and subjects were instructed to refrain from taking their chelator medication for 24 h before blood sampling. A second morning void spot urine sample was collected, and 2 aliquots were saved, one of which was acidified before being frozen. Plasma and urinary zinc was assessed by inductively coupled plasma atomic emission spectroscopy as previously described (27). Urinary creatinine was analyzed by quantitative spectrophotometry (ARUP National Reference Laboratory). Serum ferritin was assessed by immunoassay and complete blood cell count with manual differential within each clinical laboratory. 25(OH)D was assessed by chemiluminescent immunoassay (ARUP). Serum osteocalcin, a marker of bone formation, was assessed by using the Metra Osteocalcin immunoassay (Quidel Corp). The reported interassay and intraassay variabilities are 4.8–10% and 4.8–9.8%, respectively, for osteocalcin. Serum N-telopeptide crosslinks, a marker of bone resorption, were assessed by using the OsteoMark ELISA/enzyme immunoassay (Wampole Laboratories Inc). The OsteoMark ELISA had an interassay range of 6.9% and an intraassay variability of 9.5%. One bone-formation marker (osteocalcin) and one bone-resorption marker (serum N-telopeptide crosslinks) was chosen to be analyzed from the serum of these subjects for several reasons.

Each of these markers has been published extensively in the literature, they had a reasonable amount of reference data available in adult and pediatric patients, and they had some of the lowest interassay variability ranges.

Data related to iron status, chelation, and transfusion history; hematologic variables; endocrine function; hormone therapy; orthopedic surgery; fracture history; and disease characteristics and severity were collected from each subject's medical record. Nutritional intake was assessed through a validated 110-item food-frequency questionnaire [Block 2005; Nutrition Quest (28, 29)]. Questionnaires were designed to estimate usual intake from a wide array of commonly consumed foods. The food lists were developed from NHANES (1999–2002) dietary-recall data; the nutrient database was developed from the US Department of Agriculture Food and Nutrient Database for Dietary Studies, version 1.0. For most subjects, the questionnaire took 30 min to complete. Portion size was quantified for each food according to a series of coded pictures. Completed questionnaires were sent to Nutrition Quest for analysis. Intake of individual nutrients pertinent to this study were quantified and calculated according to age- and sex-specific RDA (30, 31). Under- or overreporting was assessed by comparing an individual's kilocalorie intake with their estimated energy requirement for a sedentary lifestyle, calculated from their age, sex, height, and weight (32). Appetite was assessed by using a Likert scale starting at the 3-mo time point by using this question: "How hungry are you when it's time to eat?" Not hungry "0" to very hungry "4." Self-assessment immune function questions were also asked with these 2 questions: "When something is going around I usually catch it" and "I seem to get sick a little easier than other people I know"; subjects answered "mostly false = 1" to "mostly true = 4" for each question.

Nutrition counseling

All subjects were provided nutritional counseling within 2 wk of the baseline study visit by a trained dietitian within each institution's Clinical and Translational Science Institute program. Counseling emphasized nutrients known to affect bone density; subjects were encouraged to consume foods that were dense in such nutrients (eg, calcium, vitamin D, vitamin K, protein).

Bone assessments

Discovery A Quantitative Digital Radiography bone densitometers (Hologic Inc) were used to assess bone mineral content (BMC) and aBMD at the spine [L1-L4, fast array, posterior-anterior (PA) and lateral], left proximal femur (hip), and whole body in all subjects at baseline, 12 mo, and 18 mo according to the manufacturer's guidelines for subject positioning. The following variables were collected from each scan: BMC, aBMD, bone area, lateral spine BMC, and width-adjusted BMD from the lateral spine scan. Bone mineral apparent density was calculated according to the method of Carter et al (33) for the spine scans to estimate volumetric density. The z scores for adults and adolescents were calculated from manufacturer-specific (Hologic) adult and pediatric reference data, respectively.

The in vivo precision of DXA measurements was determined by duplicate measurement of 30 healthy subjects of an age similar to that of those enrolled in this study. The root mean square error of spine aBMD performed at CHRCO was 0.026 g/cm^2, of hip BMD was 0.029 g/cm^2, of whole-body aBMD was 0.023 g/cm^2, of spine BMC was 1.86 g, of hip BMC was 1.60 g, and of whole-body BMC was 62.3 g. The in vitro CV of the CHRCO, UCSF, and CHOP DXA instruments was $<1\%$ for standard spine phantoms. All DXA scans were analyzed by a single operator (EBF) with the use of Hologic software version 12.6.1.

Statistical analyses

Sample size

Our primary measure used to estimate sample size was the change in aBMD (ΔaBMD) calculated as the difference between the PA spine aBMD measured at baseline and the 18-mo time point. Data from longitudinal DXA studies conducted in children with sickle cell disease were used for estimating a sample size (34). The average change in BMD in untreated children with sickle cell disease between 9 and 20 y with a BMD z score of less than -1.0 was $3.4 \pm 4.7\%$ per year. Therefore, we chose a clinically sufficient effect size of 4% or the ability to detect a difference between treatment groups of 4%. With the use of a 2-tailed t test with $\alpha = 0.05$ and an effect size of 4% with an SD of 5%, we estimated that a sample size of 25 patients per arm (50 total) was needed to provide 80% power. We estimated a 10% dropout rate per arm ($n = 5$) for a total sample size of 60 subjects. However, recruitment was closed at 44 subjects, despite the fact that the a priori sample size was not reached because the subject pool at each of the 3 centers was exhausted and the funding timeline was limited. Post hoc, we calculated that a sample size of 44 would have 80% power to detect a difference in means of 4.0%, assuming that the common SD is 4.7%, by using a 2-group t test with a 2-sided significance level of 0.05.

Analyses

The z scores for weight and height were calculated by using Epi Info version 3.5.1. For subjects between 20 and 30 y of age, height z scores were calculated by using the oldest age in the reference database. The z scores for DXA aBMD were based on manufacturer reference curves (Hologic software version 12.6.1).

Statistical analyses were conducted by using Stata 9.2 (Stata Inc) and SAS version 9.3 (SAS Institute). Data were first plotted and normality tests were run to check for outliers, ranges, and distribution assumptions. Summary statistics were then computed, including means, SDs, and 95% CIs for all the variables on each time point in each group. Data throughout the manuscript are reported as means $\pm$ SDs unless stated otherwise. Supplement groups (zinc and placebo) were compared at baseline for continuous outcomes (eg, aBMD, BMC, weight-for age z score, and height-for-age z score) and were analyzed by using Student's t tests for normally distributed data and a Mann-Whitney U test for highly skewed data. Pearson's chi-square or Fisher's exact tests were used to assess differences in categorical variables (eg, sex, ethnicity) at baseline. Baseline demographic variables were also compared between those subjects who dropped out immediately after baseline ($n = 5$) and those who remained in the study ($n = 37$) by using Student's t tests and Fisher's exact tests.

Initial analyses explored differences in anthropometric measures, plasma zinc, urinary zinc, and bone-turnover markers within

serum and bone outcomes by DXA from the baseline, 12-mo, and 18-mo time points within each group (zinc or placebo) by using longitudinal models with the effects of time taken as a repeated measure. General linear mixed models incorporating the longitudinal structure of the data were then used to assess differences in continuous variables (eg, BMC, aBMD) between the groups (zinc compared with placebo) over time. This first set of models for each bone outcome at baseline, 12 mo, and 18 mo included effects due to time, group, and time × group interaction with control for an important covariate, pubertal development (Tanner stage as a continuous variable). Although there were no statistically significant differences in any bone outcomes between the placebo and zinc groups at baseline, PA aBMD spine and hip BMD *z* scores appeared to be lower (**Table 1**); therefore, we also adjusted for baseline measures in the models. If a significant interaction of group and time or time was indicated, we applied the Tukey-Kramer method of multiple comparisons to determine what changes occurred over time. Intention-to-treat analysis was used in developing all models; no one was excluded based on poor adherence. A significance level of 0.05 was used for all tests, whereas a trend was considered at $P < 0.1$.

TABLE 1
Demographic and disease characteristics of the subjects with thalassemia, by group at baseline[1]

	Placebo (n = 18)	Zinc (n = 24)
Age (y)	17.4 ± 4.7[2]	17.5 ± 5.7
Subjects aged >18 y [n (%)]	8 (44)	10 (42)
Sex, male [n (%)]	9 (50)	12 (50)
Diagnosis [n (%)]		
β-Thalassemia	14 (78)	15 (62)
E, β-Thalassemia	1 (5)	6 (25)
Hemoglobin H Constant Spring	1 (5)	3 (13)
β-Thalassemia intermedia	2 (12)	0 (0)
Chronically transfused subjects [n (%)]	16 (89)	18 (75)
Ethnicity [n (%)]		
Asian	11 (61)	18 (75)
White	6 (33)	6 (25)
Other	1 (6)	0 (0)
Average Tanner category [n (%)][3]		
Prepubertal	4 (22)	5 (21)
Peripubertal	9 (50)	14 (58)
Postpubertal	5 (28)	5 (21)
Serum ferritin (ng/mL)[4]	2033 ± 1715	1994 ± 1661
Subjects with diabetes [n (%)]	3 (17)	5 (21)
Dietary zinc (% of RDA)[5]	121 ± 82	123 ± 53
PA spine aBMD z score[6]	−2.32 ± 1.13	−1.88 ± 1.02
Hip aBMD z score	−1.47 ± 0.93	−1.18 ± 1.02
25(OH)D (ng/mL)	26.3 ± 11.2	23.0 ± 10.9
25(OH)D ≤20 ng/mL [n (%)]	7 (39)	12 (50)
Serum copper (μg/dL)	76.4 ± 18.9	81.2 ± 24.5
Serum copper ≤70 μg/dL [n (%)]	8 (44)	10 (42)
Plasma zinc (μg/dL)	80.6 ± 16.3	79.0 ± 14.0
Plasma zinc ≤70 μg/dL [n (%)]	6 (33)	5 (22)

[1] There were no differences between zinc and placebo groups in any of the baseline study characteristics noted. aBMD, areal bone mineral density; PA, posterior-anterior; RDA, Recommended Dietary Allowance; 25(OH)D, 25-hydroxyvitamin D.

[2] Mean ± SD (all such values).

[3] Tanner stage of gonad + hair for each individual patient, categorized as prepubertal (stage 1), peripubertal (stage 2 or 3), or postpubertal (stage 4 or 5).

[4] Reference range = 30–300 ng/mL.

[5] Presented as a percentage of the RDA for age (30).

[6] Determined by dual-energy X-ray absorptiometry.

RESULTS

A total of 105 subjects were screened for eligibility (Figure 1). Of these, 61 subjects were excluded because they did not meet the inclusion criteria (n = 34) [previous bone marrow transplant (n = 16), medical conditions known to affect bone health (n = 8), aBMD z scores > −1.0 (n = 5), current bisphosphonate therapy (n = 3), current pregnancy (n = 2)]; because they declined to participate (n = 15); or other reasons (n = 12) [lived too far for frequent research visits (n = 7), other research study conflict (n = 2), or death before contact (n = 3)]. Therefore, 44 subjects with Thal qualified, consented to participate in the study, and were randomized. Two dropped out immediately after randomization but before the baseline measurement, one after a transfusion reaction, and another who was too busy to continue. Therefore, 42 subjects with Thal (age 10–30.3 y) were enrolled, completed baseline, and are included in the baseline data for subject characteristics (Table 1). One of the subjects in the zinc group (17-y-old male) was determined after baseline assessment to have a BMD z score of only −0.9. He continued in the protocol through a protocol deviation. One subject in the placebo group (21-y-old male) voluntarily discontinued the study medication after the 9-mo time point; he returned for all study-related measures and therefore was kept in the study for the intention-to-treat analysis. One subject in the zinc group (13-y-old female) continued taking the supplement and completed some of the final study assessments, but did not return for her 18-mo DXA examination.

Most of the patients with Thal (81%) were transfused on a regular basis every 3–4 wk with 2–4 units of packed red blood cells to maintain a hemoglobin concentration >9 g/dL. Ten subjects with Thal received subcutaneous desferrioxamine at an average of 40 to 60 mg/kg per day for 5 to 7 nights/wk; an additional 22 subjects received desferasirox at a mean dose of 26.0 ± 7.7 mg/kg per day (range: 10–40 mg/kg per day), and 2 subjects received combination chelation therapy with desferrioxamine and desferasirox. There were no differences in the type of chelation therapy prescribed between treatment groups.

No differences in age, sex, ethnicity, puberty, or disease characteristics were found between the subjects randomly assigned to receive zinc or placebo (Table 1). Eleven subjects (26%) had low plasma zinc values at baseline, including both subjects on combination chelation therapy. No differences in age, sex, or ferritin concentration were observed between those with low or normal plasma zinc at baseline. Overall dietary energy intake, as a percentage of the estimated energy requirement for sedentary activity, averaged 97 ± 39% in the group as a whole at the baseline time point (100% is considered balanced intake to expenditure). Despite the large variability in the data, it appears that these subjects estimated their intake patterns consistently; therefore, the dietary data can be used with some confidence. Dietary intake of zinc averaged 11.4 ± 6.8 mg/d (123 ± 66% RDA) at baseline and did not differ between groups. In addition, neither calcium intake (850 ± 158 mg/d) nor vitamin D intake (143 ± 106 IU/d) differed by group at baseline. There were also

no differences observed in dietary zinc, calcium, or vitamin D intake with time of study.

Five subjects (4 zinc, 1 placebo) dropped out after baseline but before the 12-mo time point when bone was assessed by DXA (Figure 1). There were no differences in age between these 5 (19.3 ± 5.3 y) and the 37 subjects who continued in the study (17.2 ± 5.2 y), nor were there differences in sex distribution, transfusion therapy, Tanner stage, ferritin concentration, or 25(OH)D concentration (24.6 ± 11.0 compared with 22.6 ± 11.4 ng/mL, respectively). However, plasma zinc was higher ($P = 0.001$) in those who dropped out early (98.9 ± 20.2 μg/dL) than in those who did not (77.1 ± 12.1), and the PA spine aBMD z score was greater (−1.0 ± 0.7 compared with −2.2 ± 1.0; $P = 0.019$), as was the hip BMD z score (−0.3 ± 0.3 compared with −1.4 ± 0.9; $P = 0.017$).

Overall adherence averaged 82 ± 31% over the length of the study in the zinc group and was not different from that for the placebo group (78 ± 32%). There was a significant decrease in adherence over time in the placebo group (82% at 3 mo, 67% at 18 mo; $P = 0.04$) and a trend toward an increase in adherence over time in the zinc group (73% at 3 mo compared with 83% at 18 mo; $P = 0.09$). Although it was requested that the supplement not be taken with an oral chelator (desferasirox), 68% of the sample took their zinc supplement at the same time as other medications, most likely to facilitate recall. There was a trend toward a difference in appetite between groups at one time point only; 22% of subjects in the zinc group reported an improvement in appetite at 6 mo compared with only 6% in the placebo group($P = 0.051$). There were no differences in the self-assessment frequency of illness.

Plasma zinc increased by 14.7% and 15.2%, respectively, at 3 and 6 mo in the zinc group ($P = 0.014$; **Figure 2**A), although it did not change with time in the placebo group ($P = 0.18$). Urinary zinc excretion was rather variable, both within and between individuals. Although not statistically significant, there was a trend toward an increase in urinary zinc excretion in the zinc group ($P = 0.08$; Figure 2B). Urinary zinc excretion was significantly higher in those subjects with a diagnosis of diabetes ($n = 8$; 0.139 ± 0.035 μg/mg creatinine) than in the nondiabetic subjects at baseline ($n = 34$; 0.072 ± 0.006; $P = 0.01$). There was also no difference in urinary zinc excretion by transfusion status.

Insulin-like growth factor I (IGF-I) increased progressively with time of study in the adolescent subjects assigned zinc ($n = 14$; $P = 0.008$); this remained significant when the 3 subjects on growth hormone were removed from the analysis ($P = 0.019$). There was a trend toward an increase in IGF-I with time in the adolescents in the placebo group ($n = 10$; $P = 0.076$). No change in IGF-I was observed in the adults in either treatment group. No changes were observed in serum copper, vitamin D, osteocalcin (marker of bone formation), or N-telopeptide crosslinks (marker of bone resorption) in either the zinc or placebo group (**Table 2**).

Two subjects in the zinc group (11-y-old male and female) were prescribed growth hormone during the study for delayed linear growth and a low IGF-I concentration. Both subjects initiated this therapy on or soon after the 6-mo time point. Although growth hormone was an exclusionary criteria at the baseline time point, subjects who experienced a change in clinical criteria remained in the study. When ANOVA models (Table 2) were repeated without the addition of these 2 subjects taking growth hormone, results remained statistically significant. BMI

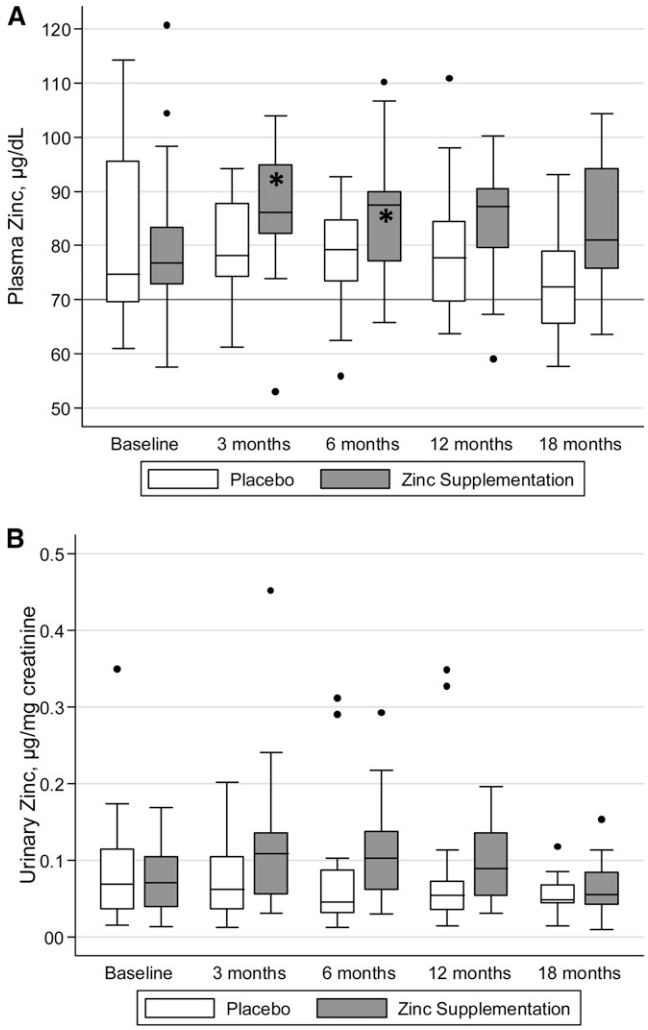

FIGURE 2. Fasting plasma zinc (A) and spot morning urinary zinc (B) by time and intervention group in subjects in the zinc-supplemented group ($n = 18$) and the placebo-supplemented group ($n = 14$) who completed the 18-mo intervention. Line = median; box = 25%–75%; bars = 5th to 95th. A significant increase was observed in plasma zinc at 3 mo and 6 mo compared with baseline in the zinc group, noted by an asterisk within the box plot, $P < 0.014$ (repeated-measures ANOVA). A trend toward an increase in urinary zinc excretion was observed from baseline in the zinc-supplemented group, $P = 0.08$ (repeated-measures ANOVA).

and lean mass index z scores were significantly lower for the placebo than for the zinc group at baseline, but did not change with time in either group.

Groups were then analyzed together by using general linear models to explore effects resulting from time, group, and time × group interactions, with adjustment for baseline value and pubertal development (**Table 3**). The zinc group had significantly greater increases in lateral spine and whole-body bone outcomes than did the placebo group, defined as either a significant group × time interaction, or, in the case of lateral aBMD, a significant group effect when no group × time interaction was observed. Although significance was not reached in the models for PA spine BMC, aBMD, or hip BMC and aBMD, trends were consistently observed. On average, lateral spine BMC was 1.4 ± 0.3 g (adjusted mean ± SE; $P = 0.016$) higher in the zinc than in the placebo group at the end of the study after adjustment for baseline BMC and pubertal development.

TABLE 2
Anthropometric measures, serum copper, vitamin D, and biochemical markers of bone turnover at baseline, 12 mo, and 18 mo in subjects with thalassemia who completed the 18-mo trial, categorized by placebo or zinc (25 mg/d) group[1]

	Placebo-supplemented group				Zinc-supplemented group			
Variable	Baseline (n = 14)	12 mo (n = 14)	18 mo (n = 14)	P value[2]	Baseline (n = 18)	12 mo (n = 18)	18 mo (n = 18)	P value[2]
Weight z score	−1.37 (−1.96, −0.78)	−1.32 (−2.00, −0.63)	−1.37 (−1.97, −0.76)	NS	−1.29 (−1.74, −0.84)	−1.15 (−1.63, −0.68)	−1.11 (−1.63, −0.60)	NS
Height z score	−1.26 (−1.84, −0.68)	−1.28 (−1.87, −0.68)	−1.33 (−1.96, −0.70)	NS	−1.98 (−2.39, −1.57)	−1.87 (−2.28, −1.45)	−1.84 (−2.29, −1.40)	NS
BMI z score	−0.71[3] (−1.06, −0.37)	−0.62 (−0.96, −0.27)	−0.66 (−0.95, −0.36)	NS	−0.16 (−0.51, 0.19)	−0.13 (−0.52, 0.25)	−0.14 (−0.56, 0.28)	NS
Lean mass index z score	−1.52[4] (−2.02, −1.03)	−1.45 (−1.92, −0.98)	−1.43 (−1.84, −1.01)	NS	−0.72 (−1.03, −0.41)	−0.68 (−1.07, −0.27)	−0.65 (−1.07, −0.23)	NS
Whole-body fat (%)	23.7 (20.4, 27.0)	24.4 (20.9, 27.8)	24.5 (21.3, 27.7)	NS	23.0 (19.6, 26.5)	23.3 (19.8, 26.8)	23.0 (19.1, 26.9)	NS
IGF-I (ng/mL)	166 (132, 205)	206 (139, 243)	235 (189, 280)	0.006	133 (95, 171)	185 (134, 235)	179 (143, 215)	0.028
Osteocalcin (ng/mL)	24.2 (12.6, 35.9)	29.5 (13.7, 45.2)	29.7 (10.7, 48.6)	NS	28.4 (15.9, 41.0)	34.7 (12.1, 57.2)	24.0 (20.0, 28.0)	NS
Serum NTx (nmol/L BCE)	50.2 (25.9, 74.5)	51.7 (28.5, 75.0)	48.1 (29.3, 66.9)	NS	38.5 (27.4, 49.7)	54.6 (17.4, 91.8)	48.8 (24.8, 72.8)	NS
Serum copper (μg/dL)	77.9 (66.5, 89.3)	76.1 (65.8, 86.3)	69.0 (61.7, 76.2)	NS	84.1 (68.8, 99.3)	82.6 (70.0, 95.2)	82.6 (67.4, 97.8)	NS
25(OH)D (g/mL)	24.1 (17.6, 30.6)	31.2 (23.7, 38.6)	27.6 (20.6, 34.6)	NS	23.0 (17.3, 28.8)	24.7 (19.0, 30.5)	26.6 (19.8, 33.3)	NS

[1] All values are means; 95% CIs in parentheses. BCE, bone collagen equivalents; IGF-I, insulin-like growth factor I; NTx, serum N-telopeptide crosslinks; 25(OH)D, 25-hydroxyvitamin D.
[2] Reflects a comparison of time effects from longitudinal models (repeated-measures ANOVA) of each group analyzed separately.
[3,4] Significantly different from zinc-supplemented group at baseline: [3]P = 0.025, [4]P = 0.005.

Significant increases were also observed for lateral spine aBMD (0.036 ± 0.01g/cm^2; P = 0.033), whole-body BMC (63 ± 15 g; P = 0.022), and whole-body aBMD (0.023 ± 0.006 g/cm^2; P = 0.037) compared with placebo, whereas the placebo group experienced an adjusted 0.32-SD decrease in spine aBMD z score compared with the zinc group over the 18-mo study (P = 0.041). Similar declines were observed in hip aBMD z scores in the placebo group, which dropped by 0.26 SDs (P = 0.036). Anthropometric and body-composition variables were also explored as dependent variables in the general linear models after adjustment for baseline and puberty. The only variable that proved significant was stature (Table 3). The zinc group had a slight improvement in adjusted height-for-age z scores compared with the placebo group, 0.14 SDs (P = 0.025).

Change in BMC and aBMD relative to the baseline value is shown graphically in **Figure 3** for the lateral spine and whole-body scans. Lateral spine BMC increased by a mean of 10.9% (95% CI: 4.3%, 17.6%) at the 18-mo time point relative to baseline in the zinc group, whereas there was only a 1.3% (95% CI: −8.0%, 10.7%) increase in the placebo group. Lateral spine aBMD increased by 3.8% in the zinc group and decreased by 2.1% (95% CI: −10.0%, 5.9%) in the placebo group; whole-body BMC increased by 5.7% (95% CI: 2.8%, 8.6%) in the zinc group and by 1.4% (95% CI: −3.6%, 6.4%) in the placebo group; and whole-body aBMD increased by 2.9% (95% CI: 1.3%, 4.4%) relative to baseline in the zinc group and decreased by 0.1% (95% CI: −3.5%, 3.4%) in the placebo group (Figure 3, A–D).

Adherence to the supplement (≥70% adherent compared with <70% adherent) and low vitamin D status (<20 compared with ≥20 ng/mL) were included in the general linear models as potential covariates. Adherence did not prove to be an important covariate. Initially, vitamin D status appeared to be important, that is, subjects provided zinc supplementation who had adequate vitamin D status had a more robust bone response than did those with vitamin D concentrations ≤20 ng/mL. However, when Tanner stage was included in each model, the importance of vitamin D was no longer significant.

Given the small sample size, any adverse events captured in this study are summarized below rather than in a formal "Adverse Events" table where percentage occurrence might be misleading. One adult female subject sustained a forearm fracture after the 6-mo time point, subsequent to a fall while cycling down a hill. This was not considered a fragility fracture given the magnitude of the load placed. One 16-y-old transfusion-dependent, female subject died after the 12-mo time point from heart failure related to severe cardiac iron overload. A total of 18 subjects (11 subjects in the placebo arm and 9 in the zinc arm) were prescribed the oral chelator deferasirox, and many commented that stomach cramping was associated with taking this medication. However, there were no significant differences in adverse events when presented as a percentage of all subject visits (3–18 mo) between the placebo and zinc groups, respectively, including diarrhea (9.7% compared with 9.2%), stomach upset (17.7% compared with 6.6%), and nausea (14.5% compared with 18.4%). Serum copper concentrations were monitored throughout the study, because zinc supplementation can suppress copper absorption; copper supplements were provided if serum copper dropped to <70 μg/dL. A total of 16 subjects required copper supplements

TABLE 3
Longitudinal mixed-model analysis of the effect of a 25-mg Zn supplement on bone and height outcomes in subjects with thalassemia categorized by supplement group after adjustments for baseline value and pubertal stage[1]

	Time of study			*P* value[2]		
Dependent variable and supplement group	Baseline (n = 42)	12 mo (n = 37)	18 mo (n = 32)	Group	Time	Group × time
PA spine BMC (g)	—	—	—	—	—	—
Placebo	33.57 ± 0.51	34.23 ± 0.52	34.70 ± 0.57	0.054	<0.001	NS
Zinc	33.67 ± 0.45	35.70 ± 0.47	36.34 ± 0.51[3]	—	—	—
PA spine aBMD (g/cm^2)	—	—	—	0.058	0.002	0.063
Placebo	0.702 ± 0.007	0.711 ± 0.007	0.710 ± 0.008	—	—	—
Zinc	0.702 ± 0.006	0.726 ± 0.007	0.739 ± 0.007[3]	—	—	—
PA spine aBMD (z score)	—	—	—	0.041	0.071	0.061
Placebo	−2.11 ± 0.08	−2.26 ± 0.08	−2.43 ± 0.09[3]	—	—	—
Zinc	−2.09 ± 0.07	−2.11 ± 0.07	−2.09 ± 0.08	—	—	—
PA spine BMAD (g/cm^3)[4]	—	—	—	NS	0.044	0.055
Placebo	0.103 ± 0.001	0.104 ± 0.001	0.103 ± 0.001	—	—	—
Zinc	0.103 ± 0.001	0.105 ± 0.001	0.106 ± 0.001[3]	—	—	—
Spine lateral BMC (g)	—	—	—	NS	NS	0.016
Placebo	13.24 ± 0.25	13.39 ± 0.29	13.31 ± 0.31	—	—	—
Zinc	13.29 ± 0.24	14.19 ± 0.25	14.71 ± 0.26[3]	—	—	—
Spine lateral aBMD (g/cm^2)	—	—	—	0.033	NS	NS
Placebo	0.619 ± 0.010	0.607 ± 0.011	0.612 ± 0.012	—	—	—
Zinc	0.621 ± 0.009	0.639 ± 0.09	0.648 ± 0.010	—	—	—
Hip BMC (g)	—	—	—	0.056	0.054	0.089
Placebo	23.05 ± 0.35	23.10 ± 0.36	23.15 ± 0.40	—	—	—
Zinc	23.04 ± 0.31	24.00 ± 0.33	24.40 ± 0.35[3]	—	—	—
Hip aBMD (g/cm^2)	—	—	—	0.084	NS	NS
Placebo	0.776 ± 0.007	0.773 ± 0.007	0.775 ± 0.008	—	—	—
Zinc	0.776 ± 0.006	0.789 ± 0.007	0.798 ± 0.007	—	—	—
Hip aBMD (z score)	—	—	—	0.036	0.021	0.052
Placebo	−1.34 ± 0.06	−1.52 ± 0.06	−1.60 ± 0.07[3]	—	—	—
Zinc	−1.33 ± 0.05	−1.37 ± 0.06	−1.34 ± 0.06	—	—	—
Whole-body BMC (g)	—	—	—	0.018	NS	0.022
Placebo	1449 ± 14	1454 ± 15	1463 ± 16	—	—	—
Zinc	1447 ± 12	1504 ± 13	1524 ± 15[3]	—	—	—
Whole-body aBMD (g/cm^2)	—	—	—	0.030	NS	0.037
Placebo	0.874 ± 0.006	0.876 ± 0.006	0.876 ± 0.006	—	—	—
Zinc	0.873 ± 0.005	0.892 ± 0.005	0.899 ± 0.006[3]	—	—	—
Whole-body aBMD (z score)[5]	—	—	—	NS	0.031	NS
Placebo	−1.96 ± 0.13	−2.16 ± 0.14	−2.26 ± 0.15	—	—	—
Zinc	−1.96 ± 0.11	−2.14 ± 0.12	−2.23 ± 0.14	—	—	—
Height-for-age (z score)	—	—	—	0.025	0.075	0.089
Placebo	−1.71 ± 0.03	−1.70 ± 0.03	−1.79 ± 0.04	—	—	—
Zinc	−1.71 ± 0.03	−1.60 ± 0.03	−1.65 ± 0.04[3]	—	—	—

[1] All values are adjusted means ± SEs; n = 111 observations for each outcome used to develop the models in the table. aBMD, areal bone mineral density; BMAD, bone mineral apparent density; BMC, bone mineral content; PA, posterior-anterior.

[2] Linear mixed models for longitudinal data were used to derive *P* values, after control for baseline values and pubertal stage. Pubertal stage was included in the model as a continuous variable (according to Tanner: stages 1–5), placebo was considered the reference group, and all 3 time points were used, with baseline considered as the reference. The effect of zinc supplementation on each bone outcome was considered significant if $P < 0.05$ for the group × time interaction or when there was no group × time interaction, for the group effect alone. *P* values for time and the group × time interaction for the models above are presented only if $P < 0.10$. Similar models were also developed for weight-for-age z score, BMI z score, lean mass index z score, whole-body fat, osteocalcin, N-telopeptide crosslinks, and insulin-like growth factor I; however, they are not presented here because they were not significant.

[3] Within-group significant differences over time of study, $P < 0.01$ (Tukey-Kramer's multiple comparisons).

[4] Determined by dual-energy X-ray absorptiometry.

[5] n = 98 because of the elimination of adult male subjects for whom there were no manufacturer reference data to calculate z scores.

at some time point during the study for low serum copper concentrations (n = 8 assigned to zinc, n = 8 assigned to placebo).

DISCUSSION

This was the first zinc supplementation study aimed at improving bone health in patients with Thal. The findings from this randomized, placebo-controlled trial showed that there was a functional zinc deficiency despite apparently adequate dietary zinc intakes (122% of RDA), and plasma zinc concentrations were within the normal range in most (74%) subjects. After adjustment for baseline and puberty, zinc supplementation increased BMC within the spine and in the whole body in a heterogeneous population of subjects with low bone mass. Assuming

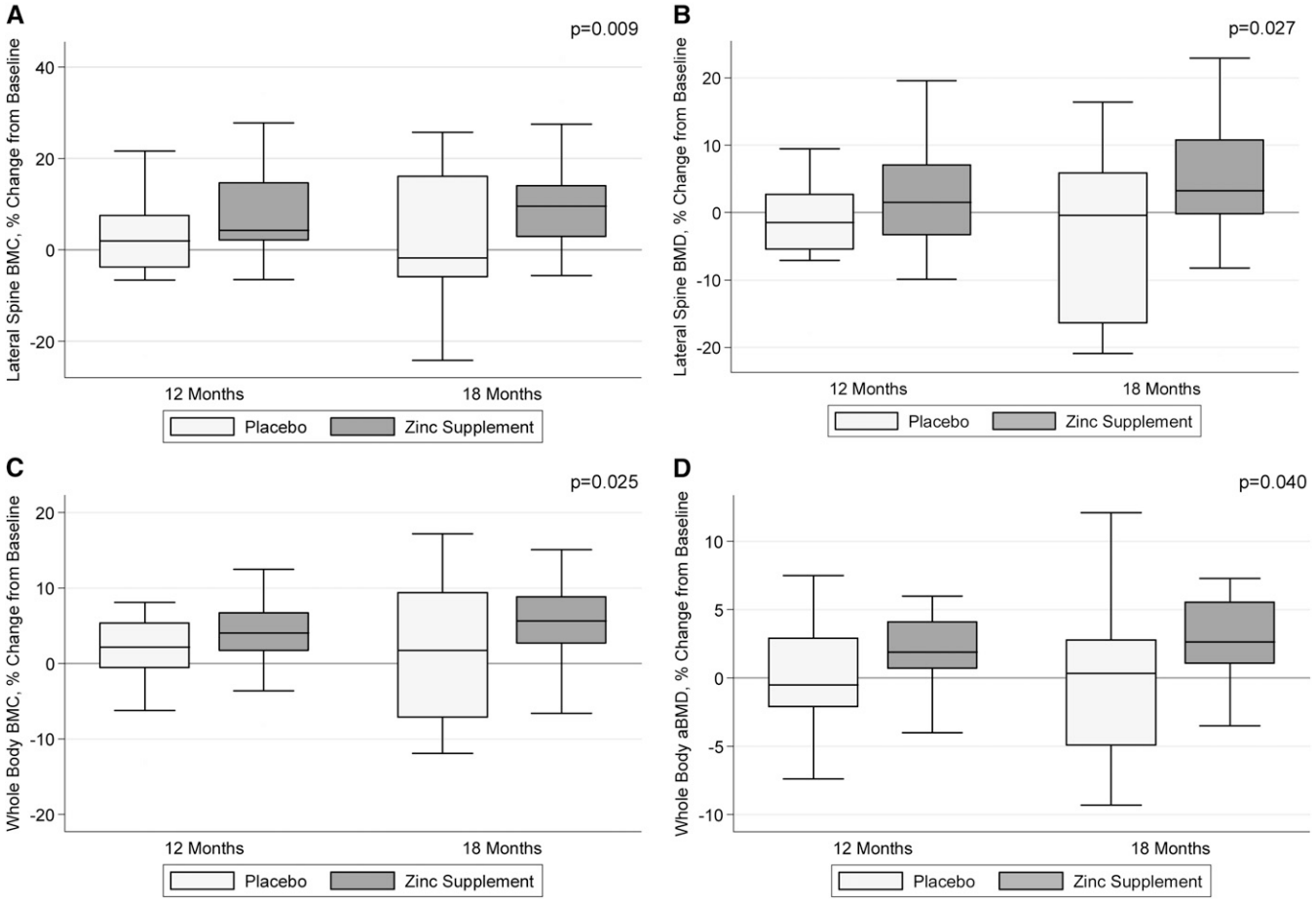

FIGURE 3. Percentage change in lateral spine BMC (A), lateral spine aBMD (B), whole-body BMC (C), and whole-body aBMD (D) assessed by dual-energy X-ray absorptiometry from baseline to 12 and 18 mo, by intervention group [zinc ($n = 20$), placebo ($n = 17$)], in subjects who completed ≥12 mo of the study. *P* value for the mixed-effects linear model after control for baseline: group × time interaction (NS). $P = 0.009$ for difference by group for lateral spine BMC, $P = 0.027$ for lateral spine aBMD, $P = 0.025$ for whole-body BMC, and $P = 0.04$ for whole-body aBMD. Line = median; box = 25%–75%; bars = 5th to 95th. aBMD, areal bone mineral density; BMC, bone mineral content; BMD, bone mineral density.

that copper status is monitored, there does not appear to be any serious adverse events related to taking 3 times the recommended dietary intake of zinc.

Zinc deficiency in thalassemia

In this study, roughly 25% of subjects had low plasma zinc concentration at the beginning of the study when evaluated under the most optimal conditions. A plasma zinc concentration ≤70 μg/dL is used as a clinical marker of suboptimal zinc status. However, in patients with hemoglobinopathies, even trace hemolysis during sampling can mask true status (35). In these subjects with Thal, a daily 25-mg Zn supplement produced a transient rise in plasma zinc and a mild increase in appetite above that observed in the placebo group. The effect of zinc on appetite suppression has been observed in other populations previously (36). However, by far the most robust response was on bone mass, which suggests functional zinc deficiency.

Etiology of zinc deficiency

Patients with Thal have multiple risk factors for marginal zinc status. These include ineffective erythrocytophoresis, which leads to increased zinc requirements, increased hemolysis, and the routine use of chelators, which can lead to increased urinary and/or fecal zinc (14, 37). The oral chelator deferiprone has been shown to induce subclinical zinc deficiency in 15% of patients (38); it remains unclear whether deferasirox is also associated with subclinical zinc deficiency, particularly in those with low total-body iron stores. Moreover, many patients with Thal and iron overload also have diabetes, which leads to increased zinc losses (5, 37). Eight of the subjects in the current study had diabetes, 5 of whom were in the zinc group. These subjects clearly had increased urinary zinc losses. Unfortunately, the numbers were too small for further exploration.

Alternative therapies for low bone mass in thalassemia

Low bone mass is one of the most frequent comorbidities in patients with Thal (6). The bone deficit observed in Thal appears to result from inadequate bone formation with or without increased bone resorption (39). Voskaridou et al (40) has shown that Dickkopf-1, an inhibitor of Wnt signaling, is increased in Thal and possibly causes poorly differentiated osteoblast cells and reduced bone formation. Histomorphometric studies in young patients with transfusion-dependent Thal have shown increased iron deposits within bone and defective mineralization (41).

Although bisphosphonates have been shown to increase bone density and reduce fracture risk (42, 43), these agents act primarily to reduce bone resorption. For this reason, bisphosphonates are most effective in Thal patients with "high turnover" bone disease (44). In many institutions, bisphosphonate therapy is reserved for adults with low bone mass and a history of fragility fractures. A few rare, but serious, adverse events are associated with long-term bisphosphonate use (45, 46); therefore, initiating these agents in teens or young adults and continuing them for life may be problematic. Therefore, establishing alternative therapies that have an anabolic effect on bone, such as zinc supplementation, offers many advantages, particularly for younger Thal patients.

In this study, supplementation with zinc increased whole-body bone mineral by 2–4% above that in the placebo group. These gains compare favorably with those observed previously with bisphosphonate therapy in Thal. Pamidronate has been shown to increase aBMD in the spine of adult patients with Thal by 4% (47). A recent meta-analysis of zoledronate reports increases in aBMD of 10% over 2 y or by 0.69 SD (95% CI: 0.47, 0.90) (48). By comparison, spine aBMD SD scores (z scores) in the current study increased on average by 0.37 in the zinc group above those in the placebo group.

Zinc and bone mass

Zinc supplementation may have influenced bone health in these subjects through growth hormone. Reduced growth hormone and IGF-I are commonly observed in patients with Thal (49). In this sample, 42% had low IGF-I concentrations at baseline. A decade ago, Domrongkitchaiporn et al (50) showed that low bone mass in Thal patients is likely modulated through a reduction in circulatory IGF-I. In rodent models, zinc deficiency not only effects bone growth but also mineralization, and these effects appear mediated by the expression of IGF-I (51). In this study, IGF-I increased significantly in the adolescents provided zinc, perhaps modulating the increase in bone density observed.

Other factors that may have influenced changes in BMC in patients with Thal included vitamin D status, endocrinopathies, or a change in hematopoietic activity. Neither transfusion nor chelation regimens changed dramatically in any of the subjects, and, other than growth hormone therapy, all other endocrine replacement remained consistent. No significant changes in any dietary intake variables assessed by food frequency were found.

Adherence and adverse events

Subjects in this study were quite adherent to this noninvasive therapy, as evidenced by pill counts (82%) and the transient increase in urinary zinc excretion. We observed no serious adverse events as long as copper status was actively monitored. Sixteen of the initial 42 subjects required copper supplements at some point during the study for low serum copper concentrations, equally distributed among the zinc and placebo groups. This is clearly a consideration before initiating any zinc supplementation regimen in patients with Thal.

Limitations

This study had some limitations. Most of the patients (80%) were transfusion dependent; thus, the results cannot be easily generalizable to the non–transfusion-dependent patient with Thal. The cohort was relatively small, and we had a large dropout rate (25%), which precluded detection of multiple clinical variables that could have contributed to skeletal status. However, it is possible that we may have increased our probability of detecting differences by chance alone given the number of bone variables assessed in this small sample. Of interest, the Thal subjects assessed at Oakland represented approximately half of the transfused patients who regularly attend the Thal clinic at this site. Roughly, half of the estimated 700 patients with Thal in North America are transfusion dependent (1). Therefore, we believe that the observations from these 42 subjects may be generalizable to other transfusion-dependent patients in the United States.

The subjects in this study were limited to patients previously identified to have low bone mass by DXA (BMD < -1.0). For this reason, we could not examine whether bone density would have changed in less severely affected subjects with this disorder. Finally, our main outcomes were assessed by DXA; although this measure is a strong surrogate for fracture risk, it does not provide information on bone microarchitecture, which can contribute to bone strength. Future studies using high-resolution peripheral quantitative computed tomography would be valuable. Despite these limitations, we believe that the robust study design added value to the literature related to skeletal therapies for Thal.

Conclusions

Low bone mass is common in Thal and may be caused in part by a subclinical zinc deficiency. We found that zinc supplementation in young patients with Thal and low bone mass increased both BMC and aBMD, particularly in the spine and whole body. Zinc was well tolerated. Assuming that copper status is monitored, there are no apparent adverse effects related to the consumption of 3 times the RDA of zinc. The potential to provide an anabolic stimulus to bone through a simple and safe nutritional intervention is promising and worthy of study in a larger cohort with a broader age range and disease severity.

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The authors' responsibilities were as follows—EBF: designed and conducted the research, analyzed the data, wrote the initial draft of the manuscript, and had primary responsibility for the data; EPV: designed the research, provided oversight of the data collection at CHRCO, and assisted with the writing of the manuscript; JCK: designed the research and assisted with the analysis and the writing of the manuscript; JLK: provided oversight of data collection at CHOP and assisted with the writing of the manuscript; JNH: provided oversight of the data collection at UCSF and assisted with the writing of the manuscript; and CG: provided statistical support for the study, analyzed the data, and assisted with the writing of the manuscript. None of the authors had any conflicts of interest related to this project.

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