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The Effect of Zinc Supplementation on Biomarkers of Zinc, Iron, and Copper Status in School Children in Guatemala

by

Vinh Quang Bui

Doctor of Philosophy

Division of Biological and Biomedical Sciences

Nutrition and Health Sciences

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Preventive zinc supplementation is beneficial for children with high risk of zinc deficiency, but its adverse effect on biomarkers of iron and copper status is still a great concern.

A randomized controlled trial of zinc supplementation was conducted in five public schools in a low-income urban area in Guatemala City from January to October 2006. Eligible children aged 6-11 years were randomly allocated to receive daily chewable tablets of 10 mg zinc oxide or placebo for six months. All the children concurrently received fortified milk (daily 200 ml of whole milk, containing 1.6 mg zinc, and 1.1 mg iron). Participants (n= 720) had high risk of zinc deficiency (21.6% low serum zinc and 39.4% low dietary zinc intakes). At baseline, serum ferritin and copper concentrations correlated with serum C-reactive protein concentration ($p < 0.001$), but serum zinc and zinc-related biomarkers did not ($p > 0.05$). Serum ferritin and copper concentrations were associated with high CRP levels, starting from the CRP >0.5 mg/L level ($p < 0.001$). Among zinc biomarkers (i.e., serum zinc, ALP, and albumin), only serum zinc concentration was associated with dietary zinc intakes both before and after 6-month interventions ($p \leq 0.06$). All significant associations and agreements were weak ($r \leq 0.21$, kappa ≤ 0.12). At six-month follow-up, compared to placebo, zinc supplementation induced higher serum zinc concentration and ALP activity ($p \leq 0.03$), but had no effect on iron or copper biomarkers ($p > 0.05$). In the

placebo group, fortified milk program improved serum zinc concentration and ALP activity ($p < 0.0001$), but decreased serum ferritin and copper concentrations ($p < 0.0001$).

Overall, serum CRP was associated with serum ferritin and copper, but not with serum zinc. Serum zinc concentration was consistently associated with dietary zinc intakes. Daily zinc supplementation increased zinc status but did not induce changes in iron and copper status. The fortified milk program improved zinc biomarkers but decreased iron and copper biomarkers. This is the first study that observed a positive association between serum CRP and copper concentrations. The study confirms the beneficial impact of zinc supplementation on zinc status and its safety on iron and copper status.

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TABLES OF CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

ACT	α-1 antichymotrypsin
AGP	α-1 acid glycoprotein
AHA	American Heart Association
ALP	Alkaline phosphatase
APP	Acute-phase protein
APR	Acute-phase reactant
ATP	Adenosyl triphosphate
CDC	Centers for Disease Control and Prevention
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
DMT	Divalent metal transporter
DSMC	Data Safety Monitoring Committee
EAR	Estimated Average Requirement
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization (United Nations)
FFQ	Food frequency questionnaire
hs-CRP	High-sensitive C-reactive protein
IAIA	International Atomic Energy Association
IL	Interleukin
INCAP	Institute of Nutrition of Central America and Panama
IOM	Institute of Medicine of the National Academies (U.S.)
IZiNCG	International Zinc Nutrition Collaborative Group
JUPITER	Justification for the Use of Statins in Primary Prevention
MMN	Multiple micronutrients
NAS	National Academy of Sciences (U.S.)
NIH	National Institute of Health (U.S.)
RCT	Randomized controlled trial
RDA	Recommended dietary allowance
RSPH	Rollins School of Public Health (Emory University)
SD	Standard deviation
SOD	Superoxide dimutase
TfR	Transferin receptor
TNF	Tumor necrosis factor
WHO	World Health Organization (United Nations)
ZIP	Zinc irt-like protein

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

There has been growing interest in zinc intervention programs to control zinc deficiency in developing countries. Zinc deficiency is common among children in developing countries. It is estimated that about 20% of the world's population may be at risk of zinc deficiency based on dietary patterns, and 4.4% of all childhood deaths are related to zinc deficiency (1, 2). A report on the global burden of disease in 2001 states that zinc deficiency is ranked as the 11th risk factor of mortality and the 12th risk factor of disease burden (15). Among 30 proposals for confronting ten great global changes in the Copenhagen consensus 2008, micronutrient supplements for children, including zinc and vitamin A, were given the highest priority based on cost and benefits (16). Current evidence indicates a beneficial impact of zinc interventions on zinc status and zinc related functional outcomes. Therapeutic zinc supplementation during episodes of diarrhea reduces the duration and severity of the illness (17). Preventive zinc supplementation reduces the incidence of diarrhea and acute lower respiratory tract infection among young children and decreases mortality in children over 12 months of age (14). Previous reviews observed the effect on growth in children, especially in stunted children (14, 18, 19), but a recent review did not show the effect on children less than 5 years of age (20). Zinc fortification increases zinc intake and total absorbed zinc and has been demonstrated to have a positive impact on zinc status (21). The World Health Organization (WHO) recommends developing zinc interventions to control zinc deficiency in the context of existing public health and nutrition programs (14).

Among the diverse zinc interventions, zinc supplementation is an important option. However, long-term use of preventive zinc supplementation alone in children induces several problems, such as difficulty with compliance with daily regimens, concurrent micronutrient deficiencies, and reduced absorption of other minerals (22). Specifically, prolonged zinc supplementation may be associated with impaired iron status and copper deficiency (lower serum copper and decreased copper-dependent enzymes) (23-26). A recent systematic review (Brown *et al.*) showed that preventive zinc supplementation with the RDA dosage had significant positive effect on indicators of zinc status, with no adverse effect on iron and copper status, but some concerns still remains (14). The effect of zinc supplementation on serum ALP in children is inconclusive. In patients with severe zinc deficiency, serum ALP activity decreased and returned to normal with zinc supplementation (27-29). In healthy people, the response to serum ALP is inconsistent. Recent literature suggests that serum ALP is not a reliable marker of zinc status (30, 31), but these existing studies were mainly in adults. Two studies reported effects of zinc supplementation on serum ALP in healthy children; one observed a significant increase in serum ALP activity, but the other did not observe any associations (32, 33). Few studies, including four by Brown *et al.,* have investigated the effect of preventive zinc supplementation on copper concentration (14). Further studies are needed to assess the effects of zinc supplementation on other nutrient biomarkers in children.

The effect of zinc supplementation on biomarkers of trace nutrients may be influenced by several confounding factors. Inflammation may induce transient changes in serum trace nutrient concentrations, such as decreased serum zinc concentration, and increased serum ferritin and copper concentrations (13, 34). In healthy people, inflammation

is measured by biomarkers as acute-phase proteins, such as serum C-reactive protein (CRP), serum α1-acid glycoprotein (AGP), and alpha-1-antichymotrypsin (ACT) (35). Although randomized controlled trials can distribute equally potential confounders in intervened and control groups, ignoring the effect of inflammation in the analysis may reduce the precision in estimating the measurement of association and bias the estimates toward the null. In addition, serum zinc concentration may be correlated with other zinc-related variables, such as dietary zinc intakes, serum ALP activity, and albumin concentration. Their reciprocal relationships are unknown, but if strong, they may need to be adjusted to improve the estimates. Finally, measurement errors in the assessment of variables, particularly dietary zinc intake from food frequency questionnaires may reduce the precision of estimates.

This study was conducted in school-aged children in an urban low-income area in Guatemala, a developing country in Central America with more than half of the population below the national poverty line (<2 US$ per day) (36), high child mortality (48/1000 live births in 2004) (37), and low public health expenditure (5.4% GDP in 2003) (37). It is among countries with the highest malnutrition in the world with 49% stunted children under 5 years and 30% of pregnant women having nutritional deficits (37). Nearly 90% of energy requirements are provided by maize, sugars, and beans, with the diets deficient in total fats, proteins of animal origin, and micronutrients (36). The Guatemalan population would therefore be at a high risk of zinc deficiency.

This dissertation aimed to investigate the effect of preventive zinc supplementation on biomarkers of zinc, iron, and copper status. It also examined the associations between inflammation and those biomarkers, and the associations among zinc-related variables.

Specifically, the study was conducted to test the following primary hypotheses:

1. Serum CRP concentration is associated with the concentrations of biomarkers of zinc, iron, and copper;
2. Biomarkers of zinc status, including dietary zinc intakes, serum zinc, alkaline phosphatase, and albumin, are mutually associated;
3. Preventive zinc supplementation is associated positively with biomarkers of zinc status, but not with biomarkers of iron and copper status.

This dissertation is comprised of seven chapters. Chapter 1 presents an introduction and objectives of the study. Chapter 2 reviews the literature on zinc nutrition and biomarkers for zinc, iron, and copper status; then evaluates the effect of inflammation on biomarkers, measurement errors on variables, and pairwise interactions among trace nutrient status; and finally, examines the effect of zinc supplementation on specific biomarkers. Chapter 3 describes the methods and reviews the characteristics of the subjects of the study. The following three chapters: 4, 5, and 6 presents three papers to be published. Chapter 4 investigates the associations between serum C-reactive protein and biomarkers of zinc, iron, and copper status. Chapter 5 studies the associations among four zinc-related variables, including dietary zinc intake, serum zinc, ALP, and albumin. Chapter 6 studies the effect of six-month zinc supplementation on biomarkers of zinc, iron, and copper. Finally, chapter 7 presents a summary of the findings in the 3 papers and conclusions.

Findings from the proposed study will contribute new knowledge about the relationships between inflammation and biomarkers of trace nutrient status. Understanding the mutual relationships among zinc-related variables in healthy individuals may help select

additional biomarkers in the assessment of marginal zinc status at the individual level. Furthermore, knowledge of the benefits and adverse effects of zinc supplementation on biomarkers is important to develop appropriate strategies to control zinc deficiency in children, especially in developing countries with high risk of zinc deficiency.

CHAPTER 2

LITERATURE REVIEW

2.1. Overview of Zinc Nutrition

Magnitude of zinc deficiency

History. Zinc was first recognized as an essential factor for the growth of *Aspergillus niger* in 1869 (38). Reversible zinc deficiency in the rat was produced in the early 1930s (39). Studies in Chinese patients with beriberi in the 1940s showed low zinc content in the hair and toenails and suggested the concomitance of thiamin and zinc deficiency (6, 40). However, scientists could not detect characteristic manifestations in people with zinc deficiency. In 1961, Prasad and colleagues described a syndrome of iron-deficiency anemia, hepatosplenomegaly, hypogonadism, and dwarfism among young males in Iran who ate little animal protein, subsisted on unleavened wheat bread, and practiced geophagia (41, 42). The symptoms reversed after zinc supplementation: their pubic hair grew, their genitalia size increased, and their growth increased (42, 43). Later, Moynahan showed that a rare inherited disorder, acrodermatitis enteropathica, was attributable to a defect in zinc absorption and reversible by zinc supplementation (44).

Epidemiology. Zinc is a trace element with atomic number 30, oxidation stage of +2, and standard atomic weight of 65g/mol. Although zinc is the 24th most abundant element in the earth's crust (45), zinc deficiency is an important factor contributing to increased morbidity, mortality, and impaired development of children in unprivileged settings (46).

Estimates suggest that 20% of the world's population may be at risk of inadequate dietary intake of zinc, and 4.4% of childhood deaths are related to zinc deficiency (1, 2). Zinc deficiency was ranked as the 11th risk factor of mortality and the 12th risk factor of disease burden in the world in 2001 (15). The populations at the highest risk are concentrated in South and South East Asia, Sub-Saharan Africa, Central America, and the Andean region. The most vulnerable groups are premature, small-for-age infants, and young children, particularly those 6- 23 months of age.

Mechanisms of zinc deficiency. Zinc deficiency may generally occur due to one or more mechanisms, including inadequate zinc intake, interference of other dietary factors with the absorption and bioavailability of dietary zinc, increased loss of zinc, impaired utilization of zinc, and increased requirement for zinc during physiological conditions, such as pregnancy, lactation, and periods of rapid growth (47).

Importance and functions of zinc

Zinc functions. Zinc is an essential trace element critical to a large number of structural proteins, enzymatic processes, and transcription factors (48). In its structural role, zinc interacts with certain protein domains; facilitating protein folding and producing structures, such as 'zinc fingers'. Thus, zinc is crucial for the production of biologically active molecules. In enzymatic processes, zinc is involved in the structure and stabilization of about 300 zinc metalloenzymes (49), such as oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. As a component of transcription factors, zinc is essential for the regulation of gene regulation. For example, zinc regulates the expression of metallothionein, which has functions in intracellular zinc compartmentalization and antioxidant function. The binding

of zinc to the metal response element transcription factor 1 (MTF1) activates the transcription of metallothionein and many other genes. Therefore, zinc is essential for many biological functions, including immunity, growth, neurological transmission, and reproduction (50).

Causes of zinc deficiency. Zinc deficiency may be secondary after various disorders or primary due to low dietary zinc intakes (47). Secondary zinc deficiency may occur in various conditions, such as acrodermatitis enteropathica, among patients fed total parenteral nutrition without zinc, or sickle cell anemia. Human nutritional zinc deficiency may coincide with the consumption of diets with low dietary zinc intakes, low zinc bioavailability due to high phytic acid content (in the case of Iranian adults), or age-associated decreases in intestinal absorption efficiency in the elderly. Severe protein energy malnutrition is also associated with zinc deficiency (47).

Manifestations of zinc deficiency. Clinical manifestations of zinc deficiency are nonspecific, vary widely, and depend on the severity of zinc deficiency (22). Frank zinc deficiency, due to secondary disorders or severe dietary zinc restriction, may have clinical sequelae, such as prominent skin rash or growth stunting, and can be confirmed by low zinc concentrations in blood and various tissues. In the original clinical observations in Iran, the male adolescents had hypogonadism, delayed sexual maturation, and severe growth retardation (41). Zinc deficiency in the elderly may be associated with poor wound healing. On the other hand, marginal zinc deficiency may be asymptomatic or associated with nonspecific clinical manifestations, such as growth retardation, increased frequency of infections, failure to thrive, and impaired taste (hypogeusia) (22).

Zinc intervention strategies

Benefits of zinc supplementation. Evidence of randomized controlled trials of zinc supplementation provides strong basis for the importance of zinc in reducing stunting, diarrhea, pneumonia, and childhood mortality (46). In a recent meta-analysis of zinc supplementation trials among children, zinc supplementation reduced the incidence of diarrhea by 20%, but the effect was limited to studies enrolling children with mean initial age greater than 12 months (reduced by 27%) (14). It also reduced the incidence of acute lower respiratory infections by 15%. It had a marginal 6% impact on overall child mortality, but an 18% reduction in deaths among children older than 12 months of age. Previous reviews suggest that zinc supplementation increase growth, especially in stunted children (14, 18, 19). A meta-analysis concluded that zinc supplementation did not influence growth, because recent studies showed that children had improved nutritional status compared to those in previous studies (20) Therefore, the available evidence supports the need for intervention programs to enhance zinc status to reduce child morbidity and mortality.

Potential program options for improving population zinc status include zinc supplementation, zinc fortification of staple foods at the national level or specific foods for specific subpopulations, and dietary modifications to increase zinc bioavailability based on nutrition education and agricultural interventions (46).

Zinc supplementation. Zinc supplementation to young children in at-risk populations on a routine basis and/or during diarrheal treatments is expected to have a high impact on zinc and health status, particularly among populations with high rates of low-birth-weight or small-for-gestational-age infants, stunting, diarrhea, or lower respiratory infections. The

WHO/UNICEF recommendation is a 10- to 14-day course of zinc supplementation (20 mg and 10 mg/day for children ≥6 months and <6 months of age, respectively) in addition to oral rehydration solution for the treatment of acute diarrhea (51, 52). For preventive supplementation, both clinical and operational research is needed to determine optional doses and dosing schedules (e.g., daily versus weekly supplementation). Despite the well-documented benefits of supplemental zinc in at-risk populations, there are currently no formal recommendations for preventive programs (46).

Zinc fortification. Zinc fortification of complementary foods is expected to have a moderate to high impact on zinc status and health in young children (46). Although universal zinc fortification of staple foods has not been shown to improve health outcomes, it is considered an important action for improving the adequacy of zinc intakes in overall populations with low zinc intakes. Well-designed trials are needed to determine efficacious-fortification methods to improve zinc status.

Dietary diversification/modification. Evidence of the impact of food-based strategies on zinc status (e.g., increased intake of animal-source foods, reduced dietary content of phytate, and zinc biofortification of staple foods) is still limited. We need more intervention studies to develop these strategies (46).

Digestion, absorption, transportation, storage, and requirement of zinc

Dietary sources of zinc. The zinc content of food is closely correlated with the protein content because zinc binds strongly to proteins in food. The richest dietary sources of zinc are animal products, such as shellfish, red meat, liver, and poultry. Plant protein sources, such as beans, lentils, chickpeas, and peas are also relatively rich in zinc. However, in whole

cereal grains, zinc is contained in the bran and the germ portions, which are normally lost through milling. Consequently, white rice and white bread are relatively poor zinc sources. Dairy products contain only moderate amounts of zinc, and drinking water is a minor source of zinc. Even in developed countries, zinc intakes are commonly suboptimal because of the excessive consumption of very poor zinc sources, such as soft drinks, refined bread, cakes and sweets, and potato chips. In many populations in developing countries, the consumption of meat and animal products is low; therefore, suboptimal zinc intakes are very common. Food taboos in Asia among pregnant and lactating women and young children may prevent them from eating protein-rich foods (22).

Zinc digestion. Zinc is released from its protein matrixes in food as free ions during digestion. Pancreatic secretions help digest foods and also deliver a large amount of zinc into the duodenum. Zinc in plant foods is generally less available for absorption than the zinc in animal foods because of the presence of many inhibiting factors, especially phytate (53, 54). Phytate is the magnesium, calcium, or potassium salt of phytic acid (myo-inositol hexaphosphate) and is present in high content in seeds, cereal grains, nuts, and legumes, and in low content in other plant foods, such as fruits, leaves, and other vegetables. In cereal grains, it is concentrated in the bran; in maize, the majority of phytate exists in the germ; in legumes, phytate is uniformly distributed and associated with protein. Since phytate is a strong chelator of minerals, including zinc, it forms insoluble complexes with zinc at alkaline or neutral pH, and cannot be digested and absorbed in the intestinal tract. The inhibition effect of phytate on zinc absorption appears to follow a dose-dependent response; the phytate:zinc molar ratio of the diet has been used to estimate the proportion of absorbable zinc. Diets with molar ratio > 15 have been associated with biochemical zinc deficiency in

human subjects (55-57). The IZiNCG divided diets into high and low phytate:zinc molar ratios by cutoff of 18 (58). In general, seeds, nuts, legumes, and unrefined cereal grains have the highest phytate:zinc molar ratios (ranged from 22-88); other plant foods have ratios ranging from 0- 42 (58). The phytate:zinc molar ratios in the diets of children from Malawi, Kenya, Mexico, and Guatemala who consume maize-based diets are very high (59).

Zinc bioavailability. Many factors change the phytate content and affect the bioavailability of zinc. Soaking and fermentation of plant foods can reduce the pH, hydrolyze phytate to other metabolites that have lower capacity to bind zinc and therefore, reduces the intake of phytic acid (60). The leavening of bread through the action of yeast also reduces phytate content (22). Increased protein in the diet enhances zinc absorption, especially if the protein is from animal sources (61). Organic acids (citric, lactic, acetic, butric, propionic, and formic acids) produced during fermentation can form soluble lignand with zinc in the gastrointestinal tract and thus facilitate zinc absorption (61). Soy-protein based infant formulas may have increased phytate content which reduces zinc absorption (62, 63). Diarrheal diseases interfere with the absorption of zinc and accelerate fecal losses of both dietary and endogenous zinc.

In addition to phytate, other factors such as fiber, oxalate, tannin, and lignin, which are abundantly present in plant foods also reduce the absorption of zinc (64). Polyphenols may reduce zinc bioavailability (65). Calcium may inhibit zinc absorption and augment the inhibition of zinc absorption by phytate (66, 67); the dietary phytate: zinc and phytate×calcium:zinc molar ratios are predictive of the risk of zinc deficiency (67, 68). High dietary calcium may impair zinc absorption in the presence of high phytate intakes (69). Other factors include high concentrations of ferrous iron in iron supplements (70), and

pharmacologic intakes of folic acid (71). Various forms of zinc supplements have a wide range of bioavailability, ranging from very low (e.g., zinc oxide), to relatively high (e.g., zinc acetates) (58).

Zinc absorption. Zinc absorption occurs at all levels of the small intestine, primarily in the jejunum (22). Zinc is absorbed both by passive diffusion and through a carrier-mediated process on the brush border of enterocytes (72, 73). Within the enterocytes, absorbed zinc is bound to many different molecules, including metallothionein and cystein-rich intestinal protein which are important in transmucosal transport (74). Zinc absorption strictly depends on both zinc status and dietary zinc intake. This tight regulation is achieved via numerous transporters. All zinc transporters have transmembrane domains and are encoded by 2 solute-linked carrier (SCL) gene families: ZnT and Zip (Zrt-, Irt- like proteins). Humans have 9 ZnT and 15 Zip transporters (22). ZnT transporters decrease intracellular zinc concentrations by promoting either zinc efflux from cells or zinc movement into intracellular vesicles; thus, increasing zinc absorption (22). Increased zinc intake results in the up-regulation of mRNA to increase the synthesis of ZnT1, which helps to transfer zinc from enterocytes to the circulation (41, 75). Zinc depletion results in an increase in ZnT4, which probably helps to transport zinc in milk (76). Inversely, Zip transporters increase intracellular zinc concentration by promoting zinc influx into cells or intracellular vesicular release of zinc and by decreasing zinc excretion to the intestinal lumen (22). Among them, Zip4 is up-regulated by zinc deficiency (77-81). During periods of high zinc intake, zinc absorption decreases and the secretion of endogenous zinc into the gastrointestinal lumen is enhanced. In contrast, during periods of low zinc intake, zinc absorption is enhanced and the secretion

of endogenous zinc is suppressed. During pregnancy and lactation, women increase their zinc absorption (82, 83).

Zinc transport. Zinc released from intestinal enterocytes into the mesenteric capillaries and the portal circulation is bound to albumin initially. Peripheral circulating zinc is firmly bound to α_3-macroglobulin (30- 40%), loosely bound to albumin (60- 70%), and chelated by amino acids (7%) (84, 85). The latter fraction is important in homeostatic mechanisms to keep serum zinc levels constant, within a range of 10- 15 μmol/L (48). Consumption of a low-zinc, low-energy diet induces the mobilization of amino acids from muscle to liver for gluconeogenesis and releases zinc into circulation to maintain serum zinc levels (22). Therefore, in zinc-depletion studies with low-zinc in high-energy diets, significant reductions in serum zinc only occur after 3- 6 weeks (29, 86).

Zinc storage. Zinc is found in all tissues and fluids in the body, but it is primarily an intracellular ion. It is estimated that 60% of total body zinc is in striated muscle, 20% in bone, 5% in blood and liver, and 3% in the skin and gastrointestinal tract (22). Kinetic studies with isotopes suggested that there are 2 major zinc pools in the body, one with a short half-life and another with a long half-life (87-89). The short-term pool in zinc, also called exchangeable pool of zinc (EZP), has a turnover of about 12.5 days and represents only about 10% of total body zinc (88, 90, 91). However, it comprises the most metabolically active form of zinc in plasma, extracellular fluid, liver, kidney, and intestine and can be mobilized for zinc-dependent function (91). In a study where lactating women consumed low-zinc diets, the EZP size was reduced to half of expected values, plasma zinc turnover rate doubled expected values, but total body turnover rates seemed normal (83). Total EZP size was reduced to respond to severe restrictions of dietary zinc intake (i.e., < 1mg/d for 4-

5 weeks induced 36% reduction) (87), but not to modest shorter-changes in zinc intake (i.e., 4-6 mg/d) (92). When this EZP is depleted, zinc deficiency ensues (90).

Zinc homeostasis

Primary homeostatic adjustments. Zinc homeostasis is the ability to maintain a constant internal state of cellular zinc nutrition regardless of varying external conditions. Typically, a healthy adult with average weight of 50 kg has zinc intakes ranging from 107 to 231 μmol/d equivalent to 14- 30 mg/kg, but the serum zinc concentration does not change even though zinc intakes are as little as 22 μmol/d (2.8 mg/kg) or as much as 306 μmol/d (40 mg/kg) (93). Thus humans appear to have the capacity to regulate whole body zinc content over a 10-fold change in dietary zinc intake. A review of experimental studies in animals and humans showed that the primary means of maintaining zinc homeostasis are the adjustments in endogenous intestinal excretion and zinc absorption (94). Both mechanisms are synergistic, but the shifts in endogenous excretion appear to occur quickly with changes in zinc intakes just above or below optimal intakes. The absorption of zinc responds more slowly, but it has the capacity to cope with large fluctuations in intakes. For example, when dietary zinc intake increased from 110 to 230 and 473 μmol/d in a study in adults, endogenous fecal zinc excretion (EFZ) increased promptly (i.e. in the first 4 days). Marked changes in zinc absorption did not occur until the 5th day and urinary zinc excretion was unchanged during the interventions (95). When dietary zinc intakes were reduced from 85 to 12 μmol/d in another study, EFZ dropped by 16 μmol/d, but the up-regulation of zinc absorption conserved only 6.5 μmol/d (96). When zinc intakes were low in a 6-month study in men (97), after 2 months on the low zinc diet (63 μmol/d), endogenous fecal loss fell from 65 to 48 μmol/d and fractional zinc absorption increased from 44% to 65% but the

total absorbed zinc declined from 85 to 41 μmol/d. The fractional zinc absorption and total absorbed zinc remained relatively constant, but the endogenous fecal losses continued to decline from 65 μmol/d during baseline to 48, 40, and 27 μmol/d at 2, 4, and 6 months, respectively. After 6 months, this reduction in EFZ allowed the men to achieve constant plasma zinc concentrations and to attain a positive zinc balance of 4.7 μmol/d.

Secondary homeostatic adjustments. In conditions of extremely low zinc intakes or prolonged marginal deficient intakes, secondary homeostatic adjustments may occur and enhance gastrointestinal changes. These secondary adjustments include changes in urinary zinc excretion, a strong retention of zinc released from selected tissues, and a shift in plasma zinc turnover rates (94). Urinary zinc excretion declined only when zinc intakes were reduced to < 51.6 μmol/d (93), but the change may occur very quickly (94). With severe zinc deficiency, such as <0.06 μmol/g diet in experimental studies in animals and humans, a negative zinc balance occurred with a reduction of about 30% in the whole body zinc content, but zinc concentrations in plasma, liver, bone, and testes dropped significantly while those in hair, skin, heart, and skeletal muscles remained constant (98). Bone is a significant source of endogenous zinc during low dietary zinc intakes to maintain plasma zinc concentration (98). Decline in bone during depletion may reflect a reduction in bone zinc uptake rather than an increased release. On the contrary, muscle zinc is maintained or even gained in depleted subjects. Weaning rats after receiving a severely deficient diet for 24 days in a study observed a net gain in total muscle zinc of 25 μmol and a net loss in bone zinc of 20 μmol (99). Therefore, muscle tissue gained weight and maintained its zinc concentration by incorporating any zinc release from bone and small amounts obtained from the diet. Hair zinc concentration did not change in experimental studies in animals (94), but it changed

with long-term, marginal intakes and has been used as a measure of zinc status in humans (100). In addition, an increase in the rate of fractional zinc turnover helps to maintain plasma zinc concentration during zinc deficiency.

Zinc turnover rate and kinetic studies. Plasma zinc contains nearly 3.5 mg of zinc, <0.1% of whole body zinc, in adults. Since all the absorbed zinc passes through plasma, approximately 50- 75 μmol/d, the flux of zinc out of the plasma must be rapid to maintain a constant concentration (94). The turnover rates are higher in the short-term pool (EZP) than in the long-term pool. Kinetic studies using a stable isotopic tracer of zinc, ^{70}Zn, by oral or venous routes permit us to monitor the amount of zinc in plasma, urine, and fecal samples; to describe zinc absorption, fecal and urinary endogenous excretions, and the sizes and turnover rates of tissues that exchange with plasma zinc; and formulate compartmental models of zinc metabolism. The EZP is then assumed as the sum of the zinc amount in plasma and tissue-fast pool (with high turnover rate), tissues-slow pool (slow turnover rate); the total body zinc mass is assumed as the sum of EZP and tissues-very-slow pool (too slow to be detected by a tracer experiment over 6 days) (87). A study showed that at baseline, plasma zinc turned over ~ 150 times/d, and ~ 7400 μmol of zinc---equivalent to one third of the whole body zinc---was moved in and out of the tissues daily. However, at the end of depletion when plasma zinc concentrations had declined by two-thirds from 12 to 4 μmol/L, the fractional turnover rate increased about one third from 150 to 200 times/d (94). In a study where 5 men administered with intravenous zinc isotope tracers at baseline (12.3 mg Zn/d) and under a diet restricting zinc severely (0.23 mg Zn/d), the plasma zinc mass fell on average by 65% (from 3.36 mg at baseline to 1.17 mg at depletion) after 5 weeks. The compartmental model and simulation estimated that zinc absorption increased from 26% to

100%; the rate constants for zinc excretion in urine and gastrointestinal tract decreased 96% and 74%, respectively; the total EZP decreased by 36% (from 166 to 106 mg) (87).

Interrelationship between key variables of zinc homeostasis. Kinetic studies show that quantity of ingested zinc is inversely associated with the fractional absorption of that zinc (101). Total absorbed zinc correlates positively with endogenous fecal zinc, which strictly reflects the intestinal endogenous zinc (101-103). The quantity of endogenous zinc excreted in the feces depends on both recent zinc absorption and zinc status of the host over a wide range of typical dietary intakes (97, 102). Among various zinc-containing tissues, the EZP with high zinc turnover rates is very important to maintain normal plasma zinc within wide changes of dietary zinc intakes (94, 103); the size of EZP is normally positively correlated with the quantity of endogenous zinc in the feces (101). However, the changes in EZP were less sensitive than the changes in plasma zinc concentration to a loss of whole-body zinc during an acute depletion study; therefore, it may not be a good indicator of zinc status following an acute change in dietary zinc intake (104). When homeostatic mechanisms fail to ensure the requirements, plasma zinc concentration drop, changes in tissue zinc concentrations occur, and clinical symptoms of zinc depletion develop (94).

Distinctive features of zinc homeostasis. Unlike many other nutrients there is no separate functional reserve or body store of available zinc. Therefore, when dietary intakes are inadequate, the growth rate in children, or rate of zinc excretion among adults, is reduced in an effort to conserve tissue zinc levels and maintain homeostasis (30). When homeostasis is sufficient, no further biochemical and functional measurements change. However, when plasma zinc concentration drops, metabolic changes develop rapidly. Zinc balance becomes negative, with a net loss of zinc from a small rapidly exchangeable zinc pool, resulting in

general tissue dysfunction. Therefore, zinc is classified as a type-2 nutrient like other nutrients as essential amino acids, N, P, Mg, and K. In type-2 nutrient deficiency, the body limits growth and/or reduces excretion in an effort to conserve the limiting nutrient. As a result, even in severe zinc deficiency, tissue zinc concentrations may not be low because the body conserves zinc. The signs and symptoms associated with type-2 nutrient deficiencies are normally non-specific; and for zinc, they may include stunting, wasting and loss of function in tissues with rapid turnovers (e.g., immune function and skin) (59).

Dietary zinc requirements

Zinc dietary requirements. Reference data for dietary zinc requirements are theoretically derived based on knowledge of zinc absorption and excretion determined from small-scale, controlled, clinical studies in adults. The estimates were developed by expert committees of the World Health Organization/Food and Agriculture Organization/International Atomic Energy Agency (WHO/FAO/IAEA) in 1996 and reviewed in 2004 (105, 106). They used a factorial method to estimate the average physiological zinc requirement, which is the amount of zinc that must be absorbed to compensate daily losses of body zinc from intestinal and non-intestinal sites, and zinc requirement for tissue accumulation in growing children, pregnant women, and lactating women (**Figure 2.1.1**) (12). This was then divided by the estimated average absorption of zinc from usual diets to estimate the total dietary zinc requirement, also called the estimated average requirement (EAR) from the diet (12). The EAR represents the dietary intake level at which 50% of individuals would meet their physiological requirement (**Table 2.1.1**). From the EAR, we can estimate the recommended daily allowance (RDA) or the recommended nutrient intakes (RNI) (**Table 2.1.2**), which is the recommended dietary intake level that is sufficient to meet the nutrient requirement of

nearly all (97.5%) healthy individual in a particular life stage and gender group (IOM 2003) (107).

Diet types. The percentage zinc absorption, also called fractional zinc absorption, varies depending on the phytate:zinc molar ratios, which are largely dependent on diet types. Three types of diets---cereal-based diet, refined diet with low cereals and high animal foods, and mixed diet---are associated with 3 categories of low, high, and moderate bioavailability (106). Diets with a high availability of zinc (50%) are characterized by a low cereal fiber content, low phytate content, and adequate protein content of meats and fish. Diets with a moderate availability of zinc (30%) include mixed diets containing animal or fish protein: lacto-ovo, ovo-vegetarian, or vegan diets that are not primarily based on unrefined cereals; and diets with a moderately high phytate:zinc ratio. Diets with a low availability of zinc (15%) include diets high in unrefined, unfermented, and ingeminated cereal grain, diets with a high phytate:zinc ratio, diets with high phytate-soya protein, diets high in energy intake from high-phytate, and diets high in inorganic calcium salts (107). The phytate:zinc molar ratio is high if > 18 following IZiNCG (58).

Tables and Figures 2.1.

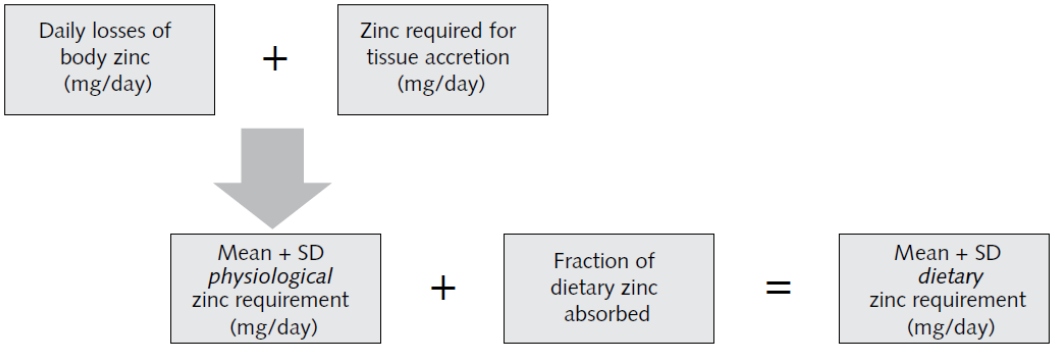

Figure 2.1.1. Information used to estimate average dietary requirement. Reprinted from Hotz C. Dietary indicators for assessing the adequacy of population zinc intakes. Food Nutr Bull. 2007 Sep;28:S430-53 (12).

Table 2.1.1. Revised estimated average requirement (EAR) for zinc by life stage and diet type, as suggested by IZiNCG

Age	Sex	Reference body weight (kg)	Revisions suggested by IZiNCG for EAR for zinc (mg/d)	
			Mixed or refined vegetar-ian diets	Unrefined, cereal-based diets
6–11 mo	M + F	9	3	4
1–3 yr	M + F	12	2	2
4–8 yr	M + F	21	3	4
9–13 yr	M + F	38	5	7
14–18 yr	M	64	8	11
14–18 yr	F	56	7	9
Pregnancy	F	—	9	12
Lactation	F	—	8	9
≥ 19 yr	M	65	10	15
≥ 19 yr	F	55	6	7
Pregnancy	F	—	8	10
Lactation	F	—	7	8

Reprinted from Brown KH, Rivera JA, Bhutta Z, Gibson RS, King JC, Lonnerdal B, Ruel MT, Sandtrom B, Wasantwisut E, Hotz C. International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. Food Nutr Bull. 2004 Mar;25:S99-203 (58).

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Table 2.1.2. Recommended nutrient intakes (RNIs) for dietary zinc (mg/day) to meet the normative storage requirements for different population groups from diets differing in zinc bioavailability

Population group	*Assumed body weight (kg)*	*High bioavailability*	*Moderate bioavailability*	*Low bioavailability*
Infants and children				
0–6 months	6	1.1[b]	2.8[c]	6.6[c]
7–12 months	9	0.8[b], 2.5[c]	4.1	8.4
1–3 years	12	2.4	4.1	8.3
4–6 years	17	12.9	4.8	9.6
7–9 years	25	3.3	5.6	11.2
Adolescents				
Females, 10–18 years	47	4.3	7.2	14.4
Males, 10–18 years	49	5.1	8.6	17.1
Adults				
Females, 19–65 years	55	3.0	4.9	9.8
Males, 19–65 years	65	4.2	7.0	14.0
Females, 65+ years	55	3.0	4.9	9.8
Males, 65+ years	65	4.2	7.0	14.0
Pregnant women				
First trimester	–	3.4	5.5	11.0
Second trimester	–	4.2	7.0	14.0
Third trimester	–	6.0	10.0	20.0
Lactating women				
0–3 months	–	5.8	9.5	
3–6 months	–	5.3	8.8	
6–12 months	–	4.3	7.2	

[a]Based on World Health Organization/Food and Agricultural Organization estimates of zinc availability in three general types of diet [65].
[b]Infants fed exclusively human milk.
[c]Formula-fed infants.

Reprinted from IOM. Dietary Reference Intakes: Applications in dietary planning: Institute of Medicine of the National Academies; 2003 (107).

2.2. Measures of Zinc, Iron, and Copper Status

Nutritional assessment measures for a specific nutrient status may be based on the stages in the development of a nutritional deficiency state. The changes may start from a change of dietary intakes, then proceed to changes of the nutrient concentrations and its dependent functional activity in tissues (e.g., enzymes, mRNA, protein synthesis), and advance to changes in physiologic functions and anatomic lesions. Corresponding to the stages of nutritional depletion are a series of dietary, laboratory, anthropometric, functional, and clinical methods (**Table 2.2.1**) (108). In fact, the choice of appropriate measures for a specific nutrient status in research studies may mainly depend on study purposes. It is important to distinguish between the applications of nutritional assessment methods to evaluate the nutrient status of either individuals or populations. Assessment of individuals is used to guide case specific treatment and counseling; therefore, the results of the assessment must be valid for the particular individuals in question. By contrast, assessment of populations is used to plan and evaluate population-based interventions; therefore, the results of the assessment do not need to provide certainty with regard to any particular individual's true nutrient status.

Measures of zinc status at the individual level

Diagnosis of zinc deficiency at the individual level is still hampered by the lack of a single, specific, and sensitive biochemical index of zinc status that reflects the entire spectrum of zinc status from deficiency to adequate, excess, and toxicity (109). There is no universally accepted assessment measure for the zinc status of individual, but the most often used approach to assess zinc status is the measurement of serum zinc levels (31, 110).

Developing a reliable and sensitive measure of marginal zinc status is a challenge; such measures must change after a depletion intervention with restricted zinc intakes in diets and be restored with zinc supplementation in healthy people.

Dietary zinc intakes. Assessment of dietary zinc intakes is impeded by lack of adequate data on zinc content of local foods (111). Furthermore, zinc bioavailability is an important determinant of zinc adequacy but the methods for estimation of zinc bioavailability are not easily available. Even if accurate and available, dietary zinc intakes at the individual level may not be useful, because of the ability of the body to adapt to very low or very high zinc intakes for some time in healthy people (110). A recent systematic review examined the effects of zinc supplementation or zinc depletion on the concentrations of potential biomarkers for zinc status (31). The studies included randomized controlled trials (RCTs), controlled clinical trials, and before-after studies (B/A). Zinc compounds for supplementation included zinc sulfate, gluconate, methionine, and acetate; all were readily absorbable (112). The durations of interventions were at least 2 weeks. To claim that a biomarker was effective in reflecting changes in zinc status, 3 conditions needed to be met: 1) statistical significant pooled estimates (95% CI did not include 0 or $P < 0.05$), 2) $\geq$ 3 trials, and 3) $\geq$ 50 participants in intervention or controlled arm or both. To claim that a biomarker was ineffective, 4 conditions had to meet: 1) lack of statistical significant pooled estimates, 2) $\geq$ 3 trials, and 3) $\geq$ 50 participants in intervention or controlled arm or both, and 4) roughly similar results (heterogeneity levels were acceptable with $I^2 < 50\%$). After analyzing data on 32 potential biomarkers from 46 publications, the authors found that plasma, urinary, and hair zinc concentrations were reliable biomarkers of zinc status. Inversely, zinc

concentrations in the erythrocyte, platelet, polymorphonuclear, mononuclear cells, and serum phosphatase alkaline activity did not appear to be effective biomarkers for zinc status.

Plasma or serum zinc concentration. Plasma or serum zinc concentration responded to dietary manipulation in adults, women, men, pregnant and lactating women, the elderly, and those at low and moderate baseline zinc status (31). Combining data from 10 depletion studies and 35 supplementation studies (1454 participants), plasma zinc concentration significantly responded to dietary zinc intake with weighted mean difference (WMD) of 2.9 μmol/L (95% CI: 2.2, 3.5; I^2 = 94%; P <0.00001; I^2 = 93.6%) (31). In supplementation studies, the WMD increased in a dose-dependent manner. In depletion studies, plasma zinc concentration fell in response to a marginal depleted diet. Overall, the result of the primary analysis yielded conclusive evidence that plasma zinc reflects and is a reliable marker of zinc status (31).

Homeostatic mechanisms maintain serum zinc concentrations in healthy persons within a narrow range (about 12 to 15 μmol/L; 78 to 98 μg/dL) even in the presence of varying zinc intakes (30). The lower cutoffs of serum zinc concentration are currently based on statistical definitions derived from the 2.5th percentiles referent population data, which is the National Health and Nutrition Examination Survey (NHANES) II survey conducted in the United States from 1976 to 1980 (113). Specific lower cutoffs may differ depending on age, gender, time of measurement, fasting status (**Table 2.2.2**) (114). For example, the lower cutoffs are 65, 66, and 70 μg/dL for children < 10 years, women, and man, respectively, using non-fasting blood samples. The NHANES II did not measure zinc status among children less than 3 years of age; however, information from many other studies in children showed no significant differences in the mean concentrations among different age

subgroups. Therefore, it seems reasonable to use the same lower cutoffs established for children less than 10 years based on the NHANES II data (113). Although few pregnant women and lactating women were measured in the NHANES II, they may use the same lower cutoffs derived from non-pregnant, non-lactating women (113).

Urinary zinc excretion. There were 5 supplementation studies and 4 depletion studies (403 participants). Supplementation studies significantly increase urinary zinc excretion (WMD: 0.31 mmol/mol creatinine; 95% CI: 0.20, 0.43; I^2 = 0%). Depletion studies significantly decreased urinary zinc excretion (WMD: 3.89 μmol/d; 95% CI: 1.02, 6.76; I^2 = 92.9%). Moreover, urinary zinc was a useful marker of zinc status only in those with moderate zinc status at baseline (31).

Hair zinc concentration. Data were analyzed from 3 supplementation studies which included 93 participants with either low or moderate baseline status and intakes in the ranges of 15-25, 26-50, and 51- 100 mg/d. Primary analysis revealed that hair zinc concentration was significantly elevated after supplementation (WMD: 13.24 ppm; 95% CI: 11.91, 14.56; I^2 = 0%) (31).

Plasma alkaline phosphatase activity. Zinc has its primary metabolic role as a component of more than 70 metalloenzymes (115). Of them, alkaline phosphatase is the most frequently used in clinical settings as a signal for a variety of diseases involving particularly the liver and the bone. The enzyme catalyzes the transfer of phosphate from an ester to a second alcohol (116), and may be involved in lipid transport in the intestine and the calcification process in bone (117). A fully active alkaline phosphatase molecule has two dimmers; each dimmer contains two Zn and a Mg ions (116). Specifically, total alkaline phosphatase in serum

includes several isoenzymes encoded by different genes, such as placenta, intestine, liver, bone, and kidney (118). Serum of healthy humans contains mainly bone- and liver-type alkaline phophatases, which are products of the same gene and differ only by post-translational modifications (**Figure 2.2.1**). These two types can be distinguished by traditional methods, including heat inactivation, gel electrophoresis and wheat germ lectin precipitation; however, these methods are unsuitable for routine use. Recent immunoassays have improved sensitivity and are commercially available (118).

The activities of both phosphatases are markedly age dependent (**Figure 2.2.2**) (9). In children, bone alkaline phosphatase is the main form and depends on physiological osteoblastic activity of bone growth. There are two peaks during childhood: one during infancy, and another, during puberty (119). The second peak occurs earlier in girls than in boys, and induces a significant gender difference in bone alkaline phosphatase and total alkaline phosphatases in children (119, 120). After puberty, the activity of bone ALP markedly decreases in adults (10-fold lesser than in puberty girls) (121), although there is some increases in the elderly (9). In another way, liver phosphatase activity increases steadily throughout life (9). Children show higher ALP activity than healthy adults as a result of the leakage of bone ALP from osteoblasts during bone growth. Using the International Federal of Chemical Industry (IFCC) procedure at 37°C, the central 95th percentile reference intervals are shown in **Table 2.2.3** (117, 122, 123).

An increase of up to two or three times normal ALP has been observed in women in the third trimester of pregnancy, with the additional enzyme of placental origin (117). Transient, benign increases in serum ALP may be observed in infants and children, with changes often more than 10 times the upper reference limit involving both the liver and the

bone forms. These changes seem to reflect a reduction in the removal of ALP from blood caused by transient modification of enzyme glycosylation (124). Recently, studies in adults showed that serum ALP is correlated with serum CRP concentrations (125, 126), and is related to the risk of cardiovascular disease, metabolic syndrome, and diabetes (127, 128).

Low serum alkaline phosphatase activity has been reported in human zinc deficiency due to acrodermatitis enteropathica (129), and severely restricted zinc intakes, either experimentally-induced or from total parenteral nutrition unsupplemented with zinc (42, 130, 131). The low activity returned to normal upon supplementation with zinc. However, when only moderately zinc-restricted diets have been fed, there has been no significant change in the activity of serum zinc alkaline phosphatase during experimental zinc depletion for women (132, 133), and men (92, 134). In a review by Lowe *et al.* in 2009 (31), data were obtained from 3 supplementation (RCTs) and 3 depletion (before/after) studies (n = 410 participants) (135-140). Overall, the primary analysis revealed no significant effect of zinc intakes on plasma alkaline phosphatase activity (WMD: 4.14 IU/L; 95% CI: -2.38, 10.66; I^2 = 56.5%). It suggests that this is not a useful zinc biomarker. However, all 6 studies were performed in adults, and none in children.

Other ineffective or uncertain measures of zinc status. The review showed sufficient evidence that zinc concentrations of erythrocyte, platelet, and mono- or polymorpho-nuclear cells were ineffective markers for zinc status (95). Other potential markers are fecal zinc, endogenous zinc excretion, exchangeable zinc pool, nail zinc; metallothionein concentrations in the erythrocyte, monocyte and T lymphocyte; enzymes aminolevulinic acid dehydrase, plasma extracellular superoxide dismutase, lymphocyte exto-5'-nucleotidase, plasma angiotensin-converting enzyme, plasma 5'-nucleotidase, carbonic anhydrase, neutrophile α-

D-mannosidase, neutrophile alkaline phosphatase, erythrocyte membrane alkaline phosphatase. Insufficient studies show whether they were effective markers of zinc status.

Limitations. Results of the systematic review of *Lowe et al.* may be cautiously interpreted because of many limitations. There were few studies of infant, adolescents, and non-white population groups (31). The pooled estimates from primary analyses did not consider confounding effects of other factors that were unrelated to zinc status or dietary zinc intakes but that influenced biomarker concentrations. For example, plasma zinc concentrations can fall in response to infection, inflammation, stress, or trauma; and increase due to tissue catabolism, sample hemolysis, or contamination from collection. In addition, measurement errors may decrease the ability to detect significant changes in biochemical indicators.

Measures of zinc status at the population level

At the population level, serum or plasma zinc is used for identifying subgroups at risk of zinc deficiency (58). The WHO/UNICEF/IAEA/IZiNCG formed a working group meeting in 2004 and 2007 to review methods for assessing population zinc status. They recommended using 3 indicators to identify specific subgroups with elevated risk: the prevalence of serum zinc concentration less than specific cutoffs (>20%), the prevalence of zinc intakes below the appropriate estimated average requirement (EAR, >25%), and the prevalence of low height-for-age (≥20%) (141). The efficiency of such indicators and other zinc biomarkers in reflecting zinc status at the population level was recently demonstrated in a recent review of Gibson *et al.* (30).

Prevalence of inadequate dietary zinc intakes. The dietary zinc intakes of a population are usually measured by either a weighed food record or a 24-hour recall on at least 2 non-consecutive days on each individual or on at least a subsample of individuals (30- 40 individuals per stratum). Data are then analyzed to calculate zinc and phytate intakes, dietary phytate:zinc molar ratio; adjusted for within day-to-day variation in individual intake; and dichotomized by the EAR cut-point to estimate the prevalence of population zinc intake inadequacy (12, 58). The EAR cut-points were defined based on the physiological requirements of zinc intakes and varied depending on age, gender, life cycle, and diet types. In fact, the prevalence of dietary zinc inadequate is chosen as an indicator for population zinc status because it reflects the proportion of the population, who are not meeting physiological requirements for absorbed zinc and the prevalence of low serum zinc concentrations in several subpopulations (30). In a survey of Mexican preschool children, the prevalence of low dietary zinc intakes (EAR < 3.3 mg/d) and of low serum zinc level (< 65 μg/dL) was similar in the subgroups of low, moderate, and high socio-economic status (142).

However, among studies in children, there is less consistency between the prevalence of inadequate zinc intake and low serum zinc concentrations. This may be caused by the methods used to estimate the EARs for zinc in children; endogenous zinc losses and zinc absorption in children are extrapolated from those of adults. In addition, other possible factors, such as the presence of infection in children, may explain the inconsistencies in children (30).

Prevalence of low serum zinc level. Serum zinc concentration was recommended as a measure of population zinc status for 2 reasons: 1) serum zinc concentration responds to

changes in zinc intakes and zinc supplementation; and 2) serum zinc concentration at baseline affects functional responses to zinc interventions. First, a review of Hess and colleagues (113), using data from 14 studies including controlled zinc depletion-repletion, zinc supplementation, or observational studies, showed that in severe dietary zinc restriction (i.e., <2-3 mg/d), serum zinc concentration sharply decreased within 2 weeks, increased again during repletion diet when zinc intakes were greater than 2-3 mg/d, and achieved a plateau when zinc intakes reach 25- 30 mg/d. In moderate dietary zinc restriction (3- 5 mg/d), the response was inconsistent, with a slight decrease or no change in serum zinc concentrations. In zinc supplementation studies, serum zinc concentration consistently increased and returned to baseline shortly after discontinuation of the supplements. However, in observational studies, the relationship between serum zinc concentrations and dietary zinc intakes was less convincing; only few studies reported weak relationships between serum zinc concentration and dietary zinc intakes (143), phytate:zinc molar ratios (142, 144), or indicators of dietary quality (e.g. inclusion or exclusion of red meat) (145). This may be caused by inaccurate measurements of dietary zinc intakes, technical errors and biological factors (e.g., inflammation) affecting serum zinc concentrations, which were not considered or controlled in the analysis. In addition, in studies with lower mean serum zinc concentrations at baseline, the growth responses to zinc supplementation (e.g., weight gain and weight-for-height Z-score) and the changes in serum zinc concentration were larger (113). Overall, the mean serum zinc concentration for the population may reflect the average dietary zinc intakes and predict the growth response of a population to zinc interventions.

Prevalence of stunting. Among several functional consequences of zinc deficiency (e.g., stunting, susceptibility to infectious diseases, or impaired cognitive development), stunting

has been suggested as a functional indicator for zinc status of a population. In a review by Fisher-Walker and Black of data from 28 zinc supplementation RCTs of children from birth to 17 years, 21 studies reported a positive effect of zinc on at least one measured indicator of growth (19). The results are consistent with two reviews of RCTs of zinc supplementation among pre-pubertal children by Brown *et al.* (14, 18). A recent review of 43 studies of zinc supplementation by Ramakrishnan *et al.* observed a small positive effect on change in weight-for-height z-ratio but no significant effect on height or weight gain (20). The insignificant effect of zinc supplementation on growth may be related to children's nutrition status which has shown improvement in more recent studies, and the selection of studies limited in children less than 5 years in the latter review (20). The greatest effect of zinc supplementation on linear growth can be expected among children with low HAZ scores at recruitment. Among many anthropometric measures of growth, height- or length-for-age was selected as the functional variable for estimating zinc deficiency in population because it is maybe to be the primary response to increased zinc intake, while weight gain is likely to arise as a result of increased linear growth. In addition, it is used in routine health and nutrition examinations and can be classified into normal or stunting based on available reference data. The cutoff for stunting was based on the WHO classification as height-for-age Z-score < -2 (146).

Other potential indicators for zinc status in population. Other functional indicators may be related to zinc deficiency, such as the incidence and prevalence of both diarrhea and pneumonia, and psychomotor development. However, the prevalence of infectious diseases is modified by the risks of exposure to infectious agents in specific seasons and areas; therefore, it is not useful for quantifying the prevalence of zinc deficiency in the population

(19). The effect of zinc deficiency on child development is not consistent and difficult to measure; therefore, child development is not considered as a useful indicator of population zinc status. Other biomarkers have been investigated. Low hair zinc concentration in children reflects chronic suboptimal zinc status (e.g. impaired taste acuity, low growth percentiles, and high dietary phytate:zinc molar ratio) in the absence of protein-energy malnutrition; however, more studies are needed to indentify the cutoffs specific for age and gender. Activity of zinc-dependent enzymes may decrease in severe dietary zinc restriction, and respond positively in zinc-depletion-repletion studies, but the responses are not consistent in mild zinc deficiency (30). Zinc concentrations in various cell types, including erythrocytes, platelets, leucocytes, and neutrophils have mixed responses in depletion-repletion and supplementation studies, and even no response when there has been evidence of functional zinc deficiency based on impaired taste acuity and immune function (147). Metallothionein, a zinc-binding protein measured in plasma and in erythrocytes, decreases in response to both severe and moderate restrictions in zinc intakes; and rises again during the repletion phase (90, 148). Total exchangeable zinc pool (EZP), measured by kinetic studies with isotope markers, appears to respond to severe restriction of dietary zinc (i.e., < 1 mg/d), but not to modest short-term changes in zinc intakes (i.e., 4-6 mg/d) (92). Therefore, the size of EZP does not appear to be a useful indicator for identifying short-term marginal zinc intakes. Plasma zinc turnover rates increased in acute, severe zinc depletion (i.e., 0.23 mg/d) and was correlated with net loss or gain of body zinc (87). However, the correlation was weaker than that between serum zinc concentration and changes in body zinc (87), and its changes were not observed in modest zinc depletion (92). In addition, stable isotope

studies are invasive, costly, complex and time-consuming. Therefore, kinetic markers currently are not useful indicators for zinc status in community settings.

Limitations. There are many limitations in methodology to define appropriate indicators for zinc status at the population level. First, measurement of dietary intakes may encounter measurement errors and misclassification of deficient intake levels. The stunting prevalence may not reflect recent zinc status of children because stunting occurs in children under 2 years of age. Rates of growth in length after 3 years of age are generally similar between poor and well nourished populations as demonstrated in the INCAP studies (149). Next, to explore the relationship between 2 indicators (e.g., between dietary intakes and serum zinc concentration) at the population level, the unit of analysis for correlational studies should be the population. Therefore, we need to obtain estimates of average measurements or the prevalence of deficient levels from many studies (113). However, there are very few studies using appropriate methods to compare dietary intakes and serum zinc concentrations in different populations or subgroups. In addition, data on the prevalence of low dietary intakes and stunting in children published by the WHO did not always concur with many countries (58). Therefore, conclusions about zinc status of a population should be cautiously interpreted.

Measures of iron status

Iron deficiency may develop into many stages as depleted iron stores, iron deficiency, and anemia. *Depleted iron stores* represent the mildest form of iron deficiency when there are no functional impairments of anemia. *Iron deficiency* is defined as one or more abnormal iron biochemical tests. *Anemia* is defined as hemoglobin concentration below -2 standard

deviations (SD) of the age- and sex- specific normal reference. The most commonly used cutoff for anemia is hemoglobin below 110 g/L for children under 5 years old and pregnant women, below 120 g/L for non-pregnant women, and below 130 g/L for men (150). *Iron deficiency anemia* is defined as meeting both criteria for iron deficiency and anemia.

Many hematologic and biochemical measures permit the assessment of iron status. In 2004, the WHO/CDC reviewed and selected 5 indicators of iron status as the most appropriate in practice, including hemoglobin concentration, mean cell volume, serum feritin, serum transferrin receptor, and zinc protoporphyrin (35).

Serum ferritin. Plasma or serum ferritin is considered a useful indicator of depleted iron stores and the best indicator of the impact of an iron intervention (35). Low serum ferritin per se is regarded as an indicator of low or depleted iron stores: when the concentration is ≥ 15 μg/L, iron stores are not depleted; higher concentrations reflect the size of the iron store; when the concentration is low (<12-15 μg/L) then iron stores are depleted (35). It can be determined in venous or capillary bloods or dried blood spots using enzyme-linked immunosorbent assays (ELISAs) or immunoradiometric assays (109, 151). However, serum ferritin is strongly affected by inflammation. Infections and other causes of inflammations can falsely elevate the levels and are a concern in areas where infections and parasitic infestations are common.

Transferrin receptor concentration. Transferrin receptor in serum is derived mostly from transferrin receptors on surface membrane of developing red blood cells, and reflects the intensity of erythropoiesis and the demand of iron. When iron stores are exhausted, the

concentration of serum transferrin receptor increases. Clinical studies indicate that serum transferrin receptor is less affected by inflammation than serum ferritin (152).

Hemoglobin concentration. Hemoglobin concentration is a measure of anemia, a condition that has important outcomes for health and child development. Not all anemias are caused by iron deficiency. The prevalence of anemia is an important public health indicator and when it is used with other measures of iron status, it can provide information about the severity of iron deficiency (35).

To assess the iron status of populations, the measurement of serum ferritin and transferrin receptor is the best approach. Combining both measures helps to identify the severity of iron deficiency in populations in which infectious diseases are common (35). The joint WHO/CDC consultation recommended, if possible, to measure hemoglobin concentration, serum ferritin, transferrin receptor, and at least one acute phase proteins, with CRP, ACT, and AGP as the first choices (35).

Measures of copper status

Copper (Cu) is an essential nutrient for man. The recommended dietary allowance (RDA) of copper is 0.9 mg/d for the general population, and the upper limit is 10 mg. The US National Academy of Sciences states that copper deficiency is common in US, and experts conclude from the 'EU Voluntary Risk Assessment for copper and copper compounds' that Cu deficiency may be more common than generally thought in Europe (153). However, the prevalence of copper deficiency is unknown due to the lack of sensitive and specific biomarkers for copper. Severe copper deficiency is well understood; its diagnosis is by serum copper and ceruloplasmin levels, which are reduced to a remarkable

degree in deficient individuals, e.g., to 30% of the normal levels and return to normal within a few days after copper therapy (154). Although chronic copper deficiency may induce common pathological characteristics as anemia, neutropenia, osteoporosis, arthritis, or cerebellar disorders (Menkes disease) (155), it is virtually impossible to identify marginal deficiency (156). Neither plasma copper nor plasma cuproenzymes reflects copper status (157-159). In healthy adult volunteers that had an estimated daily intake of 0.9 mg Cu/day (approximately 15 μg/kg/d), exposure to additional 50- 60 μg of copper/kg/d for 3 months or up to 150 μg/kg/d for 2 months did not show significant changes of either copper concentrations (in serum, erythrocytes, and mononuclear cells), serum ceruloplasmin, or superoxide dismutase (SOD) activity in erythrocytes (158). In 4-19 years old school children, the plasma copper concentration did not correlate with dietary copper intakes measured by 7-day weighed food records (157). In addition, copper absorption is strongly dependent on dietary copper. An absorption study in young adults receiving dietary intakes varying from 0.79 mg/d (low), to 1.68 mg/d (normal), and to 7.53 mg/d (high) had their copper absorption average 55.6 ± 0.9%, 36.3 ± 1.3%, and 12.4 ± 0.9%, respectively (160). Expression of CCS, a copper chaperone, may be a promising biomarker for copper status. Chaperones are a series of intracellular proteins which transport intracellular copper to its target proteins. In rat models, CCS protein levels were inversely proportional to copper intakes (161). Copper deficiency induced by feeding rats with increased zinc in the diet was detected by erythrocyte CCS (162). However, its validity in humans remains yet to be tested (156).

Currently, the prevalence of copper deficiency is based on dietary copper intake and exposure assessments, but its measurements may be flawed with measurement errors and

precision (163). Recent research studies measure plasma or serum concentrations. The prevalence of low copper levels (i.e., <90 mcg/dL) may be very high in developing countries, up to 24% in urban healthy Iranian adults, 58.2% in Nigerian women, and 25% in urban India school children (163-165). The prevalence of low serum copper levels may be lower or higher than the prevalence of low serum zinc (164, 165). In patients after bariatric surgery with biblio-pancreatic diversion, the prevalence of low copper levels was very high, such as 50.6% with at least once, and 30.3% with repeated measurements (166).

Tables and Figures 2.2.

Table 2.2.1. Generalized scheme for the development of a nutritional deficiency

Depletion stage	Method(s) used
1. Dietary inadequacy	Dietary
2. Decreased level in reserve tissue store	Biochemical
3. Decreased level in body fluids	Biochemical
4. Decreased functional level in tissues	Anthropometric/ Biochemical
5. Decreased activity of nutrient-dependent enzymes or mRNA for some proteins	Biochemical/ Molecular techniques
6. Functional change	Behavioral/ Physiology
7. Clinical symptoms	Clinical
8. Anatomical sign	Clinical

From Martorell, R. (1984). Measuring the impact of nutrition intervention on physical growth. Methods for the Evaluation of the Impact of Food and Nutrition Programs. D. E. Sahn, Lockwood, R., Scrimshaw, N.S. Tokyo, Japan, United Nation University**:** 65-93. Reprinted from Gibson, R. S. (2005). Principles of nutritional assessment. New York, NY, Oxford University Press (108, 167).

Table 2.2.2. Suggested lower cutoffs (2.5th percentile) for the assessment of serum zinc concentration in population studies, derived from NHANES II

	Serum zinc (μg/dL, [μmol/L])[1]		
		≥ 10 yr	
Time of measurement	< 10 yr	Females	Males
Morning fasting[2]	NA	70 [10.7]	74 [11.3]
Morning non-fasting	65 [9.9]	66 [10.1]	70 [10.7]
Afternoon	57 [8.7]	59 [9.0]	61 [9.3]

NA: not available.

[1] Conversion factor: μmol/L = μg/dL : 6.54.

[2] Based on data from subjects aged ≥ 20 years.

Reprinted from Hotz C, Peerson JM, Brown KH. Suggested lower cutoffs of serum zinc concentrations for assessing zinc status: reanalysis of the second National Health and Nutrition Examination Survey data (1976-1980). Am J Clin Nutr. 2003 Oct;78:756-64 (114).

Table 2.2.3. Reference interval of serum alkaline phosphatase

Sex	**Age**	**Reference Interval**
Males-females	4-15 yr	54-369 U/L
Males	20-50 yr	53-128 U/L
	≥50	56-119 U/L
Females	20-50 yr	42-98 U/L
	≥60 yr	53-141

Reprinted from Panteghini M, Bais, R., and van Soiling W.W. Enzymes. In: Burtis CA, editor. TIETZ Textbook of Clinical Chemistry and MOlecular Diagnostics. 4th ed. Missouri, US: Elsevier Saunders; 2006. p. 607(117).

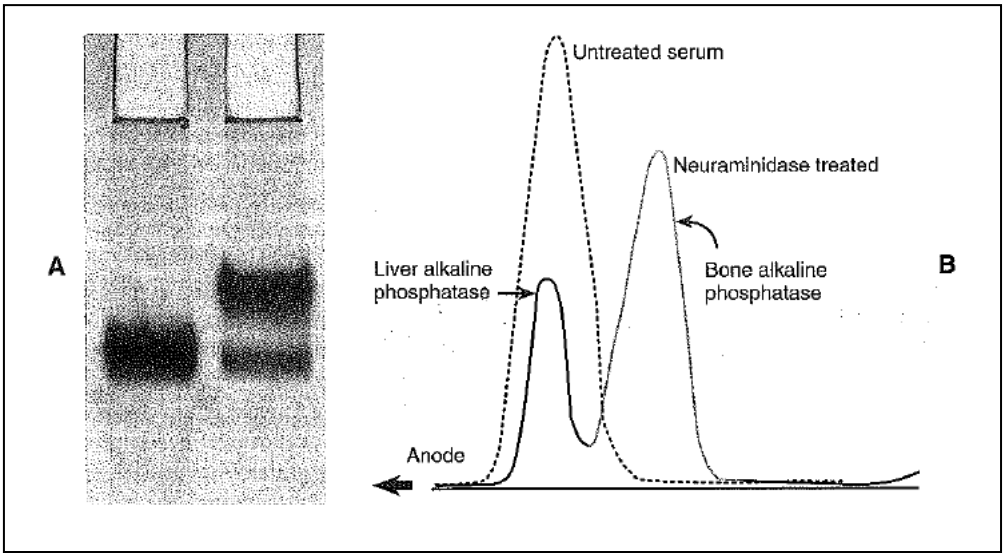

Figure 2.2.1. A, Polyacrylamide-gel electrophoresis of bone and liver alkaline phosphatases in human serum. B, Densitometric scans of electrophoretic patterns shown in A. Reprinted from Moss DW, Edwards, R.K. Improved electrophoretic resolution of bone and liver alkaline phosphatases resulting from partial digestion with neuraminidase. Clin Chim Acta. 1984;143:177-82 (5).

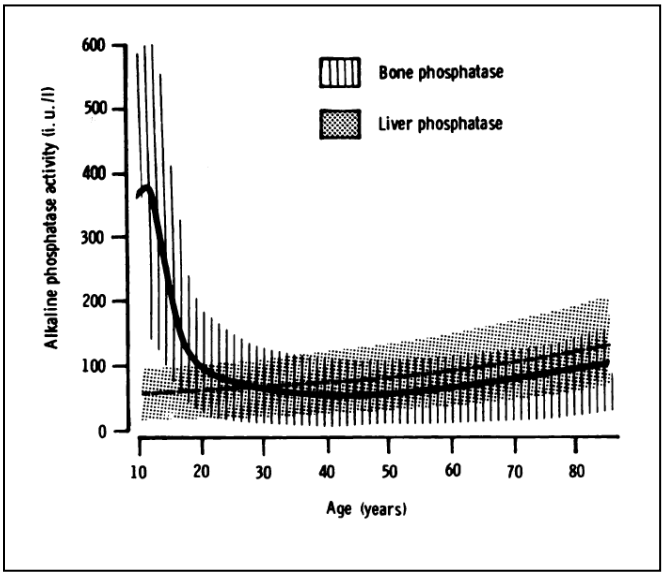

Figure 2.2.2. Changes in the relative activities of bone and liver alkaline phosphatases in human serum with age, as determined by the quantitative heat-inactivation technique of Moss and Whitby. Reprinted from Moss DW, Whitby LG. A simplified heat-inactivation method for investigating alkaline phosphatase isoenzymes in serum. Clin Chim Acta. 1975 May 15;61:63-71 (9).

2.3. Measurement Errors of Nutrition-Related Variables

Food frequency questionnaire

There are several methods to assess individual intakes of foods, nutrients, and total energy; they include single 24-hour recalls, food frequency questionnaires (FFQs), diet histories, food or diet records, and biomarkers. Among them, the FFQ has emerged as a preferred approach in large prospective studies because it is easy for participants to complete, can be processed by computers, and is inexpensive (168). The FFQ is based on 2 principles: 1) that average long-term diet is conceptually more important than short-term diet; and 2) that relative ranking of individual intakes is more important than absolute intakes in predicting chronic disease risk (169). The questionnaire consists of a structure listing of individual foods and beverages. Comprehensive FFQs in most epidemiologic studies generally include 60 to 180 food items (109). Collection of portion size information varies according to types of FFQ. In non-quantitative FFQs, portion size information is not collected. Such questionnaires cannot provide estimates of nutrient intakes and are typically used for screening purposes. In semi-quantitative FFQs (e.g., Willett's FFQ, the Block FFQ, and the NCI Diet History Questionnaire DHQ) portion sizes are specified as standardized portions or choices. Available evidence suggests that including portion size data, compared to not including, only marginally improves the validity of FFQs (169, 170). Subar *et al.* suggested that portion size information could improve estimates of absolute macronutrient intake (171), but not necessarily the validity of energy-adjusted nutrients. However, FFQs have significant limitations. First, they may not provide accurate estimates of absolute nutrient intakes because they lack the detail and specificity of diet records or recalls. Second, items and nutrient database should be updated in a timely manner to reflect constant

changes in food supplies and compositions. Third, the completion of FFQs requires memory and cognitive estimation skills. Fourth, FFQs need to be specific to individual cultures and populations. Low education may limit the usefulness of self-administered surveys (168). Therefore, FFQs are subject to both systemic and random errors.

Validity and reproducibility

Validity refers to "the degree to which the questionnaire actually measures the aspect of diet that it was designed to measure", or the difference between the true and the observed mean (167). Reproducibility or precision refers to "consistency of questionnaire measurements on more than one administration to the same person at different times" (169), or the difference between the observed estimates and the observed mean. A valid instrument is accurate in measuring unbiased true intake, while a precise reproducible instrument shows good agreement in repeated administrations. Ideally, an instrument should be accurate as well as precise. However, systemic errors and random errors can lead to inaccuracy and imprecision, respectively (172).

The precision of nutrient measurements can be reduced by large within-individual variation, which is caused by day-to-day true variation in intake, long-term cycles and trends, or errors in measurement. Random errors do not bias the mean of the specific nutrient measurement, but it increases the variance of nutrient estimates and its measures of associations with other outcome variables.

Correction of dietary estimates by FFQ

The effect of measurement errors on nutrient measurements can be systemic or random. Measurement errors can be corrected by many methods: validation study, repeated measurements, total energy adjustment, and regression calibration method (168, 169, 173).

In validation studies, the interest measurements are compared to one or more reference methods in a subset of participants or in another population. Diet records are the standard that is most commonly used to evaluate other dietary methods, especially FFQs. Multiple 24-hour recalls and biomarkers are also popular choices as reference methods. Observed correlations between the FFQ and the reference methods can be used to correct the estimated nutrient intakes by using the method of triads (174). The method is based on the assumption that the errors between the methods are independent. If the true dietary intake is measured by the FFQ, the biomarker, and the reference method (e.g., diet record) as Q, M, and R; and pairwise correlation coefficients among 3 methods are r_{QR}, r_{MR}, and r_{QM}, the validity coefficient (VC) between the true dietary intake (T) and the FFQ can be estimated as following (175):

$$VC_{QT} = \sqrt{(r_{QR} \times r_{QM})/r_{RM}}$$

Similarly, the validity coefficient between the biomarker or the reference method and the true dietary intake are calculated as:

$$VC_{QT} = \sqrt{(r_{QR} \times r_{QM})/r_{RM}}$$

$$VC_{RT} = \sqrt{(r_{QR} \times r_{RM})/r_{QM}}$$

For example, in a Hispanic population, after adjustment for energy intakes, the average correlations between nutrient intakes assessed by the FFQs and multiple diet records or repeated 24-hour recall ranged from 0.4 to 0.7 (169), and those between FFQs and biomarkers are in range of 0.3 to 0.5 (168). The median VCs for tocopherols/carotenoids estimated by repeat 24-hour recalls, the average of 2 FFQs, and plasma were 0.71, 0.60, and 0.52, respectively (175). In general, the biomarkers did not perform better than the FFQs, and thus, the authors concluded that biomarkers should be used to complement the FFQ rather than substitute for it.

In repeated measurements, we measure the averages of nutrient intakes measured by repeated FFQs or other dietary methods over time, and estimate the association between average dietary intakes and outcome variables. For biochemical measurements, the best measure of precision is the coefficient of variation (CV), as determined by the ratio of the standard deviation to the mean of the replicates. Ideally, the CV should be calculated for a sample at the bottom, middle, and top of the reference concentration range of the test, as determined on apparently healthy individuals (109). Some investigators have stipulated that the analytical CV for an assay used in epidemiology studies should not exceed 5% (176). Repeated measures from FFQs in prospective studies are more likely to represent long-term dietary intake, reduce random errors, and potentially improve the precision of dietary measures. A study found that use of the cumulative average of dietary intakes of fatty acids was more predictive of coronary heart disease (CHD) risk than use of only baseline diet (177).

In regression calibration studies, variations in dietary intakes measured by the FFQ are partitioned by the regression ANOVA method into within subject variation (day-to-day

variation) in the same subjects and between-subject variation. The approach can be used to correct correlation coefficients or odds ratios of within-subject variation. The deattenuated correlation coefficient and beta coefficient (slope of linear or logistic regression) for the nutrient of interest (e.g., FFQs) are calculated as the following (178).

$$r_{True} = r_{Observed} \cdot \sqrt{1 + \frac{\lambda_X}{n_X}}$$

$$b_{True} = b_{Observed} \cdot (1 + \lambda_X / n_X)$$

Adjustment for total energy intake. Underreporting of total energy intake by dietary instruments is widely recognized. A study comparing reported energy intakes by different dietary assessment methods with the reference energy expenditure measured by doubly labeled water showed that all the dietary methods were underreported (172). The mean energy intake: energy expenditure ratios were 0.87, 0.84, and 0.84 for FFQ, weighted records, and 24-hour recall (single or multiple). The FFQ may have greater degree of underreporting with FFQs than with 24-hour recalls (179). In most studies, underreporting is more common in the obese than in normal weight subjects. Therefore, we should adjust for total energy intake in measuring dietary nutrient intakes. The adjustment has several conceptual and practical advantages. First, control of total energy intakes allows us to compare macronutrient intakes in individuals under the assumption of the same energy intake. Second, adjustment of total energy intakes may reduce measurement errors for nutrient intakes, which are correlated with measurement errors for total energy intakes. Third, energy adjustment removes extraneous variation between subjects of different body size and physical activity levels. Finally, it is useful in estimating associations between specific nutrient

intakes and a disease risk because total energy may associate with both the nutrient intakes and the disease, and thus, confound the associations.

Total energy intake may be adjusted by nutrient density or nutrient residuals. Nutrient density is the percentage of calories contributed by a macronutrient, or the absolute intake of non-energy contributing nutrient per 1000 cal. It is the most commonly used method, but it does not control adequately for confounding by total energy intake. Nutrient residuals are proposed by Willett and Stamper (10). In this method, the energy-adjusted nutrient intake of an individual is calculated by adding the nutrient residual of this individual to the expected nutrient intake for a person with mean caloric intake. The nutrient residual of the individual is obtained from a regression model with absolute nutrient intake as the dependent variable and total energy intake as the independent variable (**Figure 2.3.2**) (10).

In analyses of nutrient-disease relationships, several statistical regression models are available for energy adjustments, such as the standard multivariate model, the nutrient residual model, the energy-partition model, and the multivariate nutrient density model (**Table 2.3.1**) (169).

Tables and Figures 2.3.

Table 2.3.1. Statistical models for adjusting for total energy intake in epidemiologic analyses

Model	*Relation Expressed*
Model 1A (standard multivariate)	Disease risk = β1 nutrient + α (total energy)
Model 1B (residual nutrient)	Disease risk = β1 nutrient residual* + β2 total energy
Model 1C (energy partition)	Disease risk = (α + β) nutrient + + α (energy from non nutrient sources)
Model 2 (multivariate nutrient density)	Disease risk = β3 nutrient/total energy + + β4 total energy

* "Nutrient residual" is the residual from the regression of a specific nutrient on energy.

Reprinted from Willet WC. Nutritional epidemiology, 2nd ed. New York: Oxford University Press; 1998 (10).

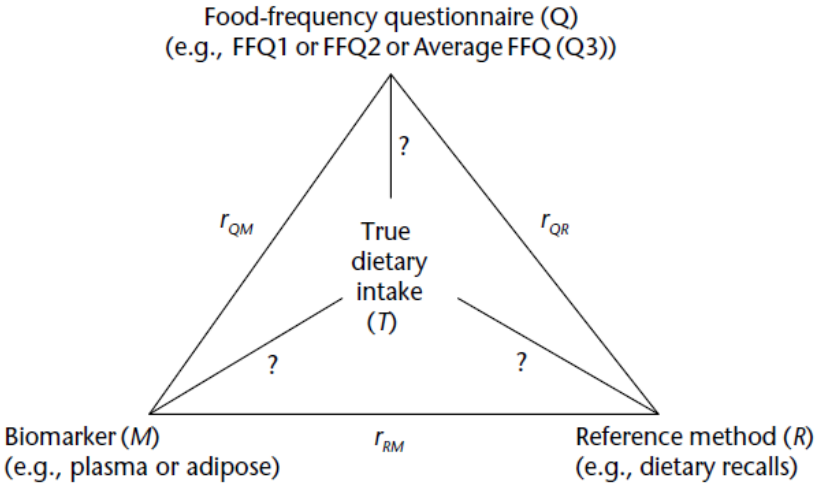

Figure 2.3.1. Diagrammatic representation of the method of triads used to estimate the correction between true long-term nutrient intake and the intake estimated using dietary assessment methods. Reprinted from Eggleton WG. The zinc content of epidermal structures in beriberi. Biochem J. 1939 Apr;33:403-6 (6).

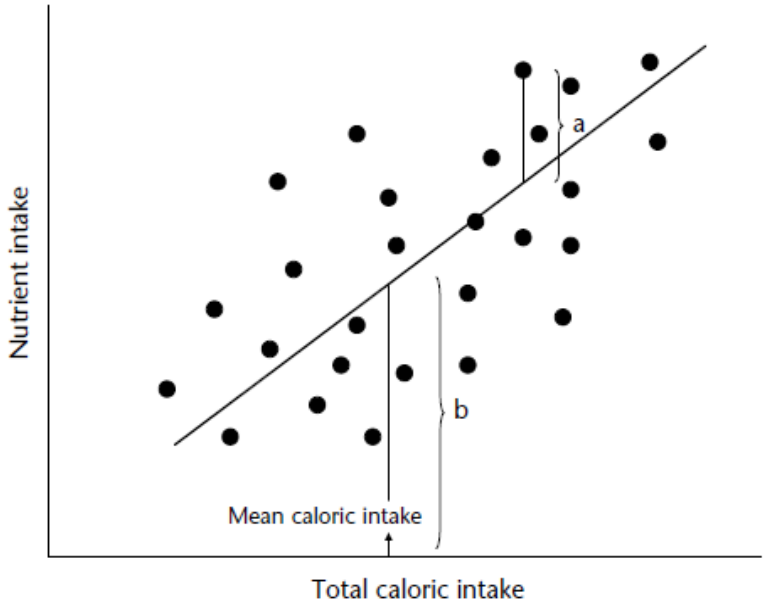

Figure 2.3.2. Adjustment for total calorie intake. Calorie-adjusted intake = a + b, where a = residual for subject from regression model with nutrient intake as the dependent variable and total caloric intake as the dependent variable, and b = the expected nutrient intake for a person with mean caloric intake. Reprinted from Willett W, Stampfer MJ. Total energy intake: implications for epidemiologic analyses. Am J Epidemiol. 1986 Jul;124:17-27 (10).

2.4. The Effect of Inflammation on Nutrition-Related Biomarkers

Changes in serum zinc, iron, copper concentrations during inflammation

Assessing micronutrient status in human samples is difficult because of many factors, such as the time of day of collection, previous consumed meals, recent exercise or stress, age, gender, and pregnancy. For example, serum zinc may fluctuate by as much as 20% during a 24-hour period (180), increase in overnight and daytime fasting (181), and decrease after meals (181, 182). It is lower in childhood, peaks during adolescent and young adulthood, and declines slightly with age thereafter. From adolescent onwards, men have higher serum zinc concentration than women (113). However, infections or inflammation processes transiently induce marked changes (183).

The effect of inflammation on micronutrient status has been recognized for many decades. The effect of inflammation on vitamin A, thiamin, riboflavin, ascorbic acid, vitamins D and K, iron, zinc, and copper was reviewed in a classic publication by Scrimshaw et al. in 1968 (184). The authors observed the association between severe clinical infections and low plasma levels of micronutrients in repeated measurements. Then, the effect of inflammation was confirmed in experimental studies among animal and human volunteers under controlled conditions (90, 185), and in community studies among adults or children (186-188). The changes in micronutrient concentrations occurred not only during the clinically apparent illness and during the periods of peak pyrexia, but also during the incubation and convalescent periods. Therefore, subclinical infections also played a key role in influencing micronutrient status (34).

Specifically, zinc, iron, and copper status may be affected by inflammation. In a study in 153 Peruvian children aged 11- 19 months (187), children with any evidence of infection (defined by reported signs of infection, elevated C-reactive protein or leukocytosis) had significantly higher serum copper and ferritin concentrations than those without infections ($p < 0.05$). Another randomized controlled trial in 418 Indonesian infants showed that infants with elevated acute phase proteins (defined by either elevated serum C-reactive protein (CRP), alpha-1 acid glycoprotein (AGP), or both at 6-month follow-up) had significantly lower plasma zinc and higher ferritin concentrations (186). Another study in Kenyan healthy adults with positive HIV but not reaching stage IV or clinical AIDS showed that participants with inflammation (defined by either elevated CRP or AGP) had lower plasma zinc than those without inflammation (183).

Mechanisms of changes in nutrient concentrations

Infection and inflammation processes induce changes in body metabolism as well as nutrient concentrations during acute phase response. Acute phase response is a non-specific process that includes the production of acute phase proteins (APPs) prior to the full activation of the immune response (35). The main purpose of the acute phase response is to prevent damage to tissue, and remove harmful molecules and pathogens. During such a response, the concentrations of some proteins, called positive APPs, increase in the plasma; and others, called negative APPs, decrease. Alterations in plasma levels of acute-phase proteins can be caused by many mechanisms, such as hemodilution, sequestration in interstitial space, and increased or decreased rates of synthesis or breakdown (34). Mechanisms for changes in the concentrations of APPs are largely due to changes in their production by hepatocytes, which in turn are regulated by cytokines, such as interleukin-1

(IL-1), interleukin-6 (IL-6) and tumor necrosis factor α (189). Positive APPs participate in host-adaptive and host-defense mechanisms by binding to foreign substances and by modulating phagocytic cell functions. Positive APPs include C-reactive protein (CRP), α-1 antichymotrypsin, alpha-1 glyco protein (AGP) also known as orosomucoid, serum amyloid A (SAA), fibrinogen, haptoglobin, ceruloplasmin and ferritin. The magnitude of the change in concentration of the APPs during an acute phase response varies considerably: ceruloplasmin can increase by about 50% whereas CRP can increase by as much as 1000-fold (190, 191). Negative APPs include transferrin, albumin, transthyretin and retinol binding protein. Those proteins do not have an immune function, but rather act as transport proteins; the plasma concentration of the specific nutrients they carry may be reduced during infection and inflammation (191).

Serum zinc. Plasma or serum zinc is reduced during the acute-phase response (34). Plasma metallothionein, an alternative but not robust indicator of zinc deficiency, is also reduced (34). The mechanism may involve considerable urinary zinc loss in systemic infection, particularly those with a pronounced metabolic stress leading to breakdown of muscle (192, 193), or calprotectin release from damaged neutrophils during inflammation (34). Circulating zinc reduction may be related to increased metallothionein mRNA and metallothionein in liver under the influences of inflammatory cytokines synthesized in response to stress (194-196). Consequently, the reduction of circulating zinc reduces zinc availability for microbial metabolism during infection and may provide an advantage to human's body (197, 198). However, the change in zinc concentration is small compared to striking change in serum ferritin concentration. In a study in Peruvian children aged 11-19 months, those with infection (diagnosed according to clinical signs or elevated CRP >10

mg/L), compared with those without infection, had small change in serum zinc concentrations (7.0 ± 2.3 vs. 7.5 ± 2.0 μmol/L, P = 0.16) but striking change in serum ferritin concentrations (10.0 ± 12.9 vs. 3.9 ± 4.4 μg/L, P <0.001) (187).

Serum iron and ferritin. A low plasma or serum *iron* concentration and a high serum ferritin concentration during inflammation are associated with a redistribution of iron into the liver and mononuclear phagocyte system, both mediated by cytokines (189). The administration of the cytokines TNF-α, IL-1 and IL-6 reduces serum iron concentration (hypoferraemia) and increases ferritin production in experimental animal, in vitro, and humans (189). The cytokines induce ferritin translation through an "acute-phase box" in the 5' region of its transcript (199, 200). Inflammatory cytokines, in particular IL-6 and IL-1β, also increase hepcidin production in hepatocytes and other cells of the immune system (e.g. macrophages and neutrophils) through a toll-like receptor-(TLR) dependent pathway (201-204). Hepcidin binds to ferroportin and induces its internalization and degradation (205). Reduced ferroportin levels limit dietary iron absorption and promote iron retention by the reticular endothelial system (206, 207). Combined up-regulation of ferritin and cell mediated down-regulation of ferroportin regulate iron absorption and induce iron retention and sequestration (208).

Serum copper. Copper is measured within the copper-binding protein complex of ceruloplasmin (209). This is a positive APP that is elevated during the acute and chronic inflammatory response (210). After major trauma or surgery, serum copper concentrations increases steadily and by day 7 are 30% higher (211). After less radical surgery, serum copper increases by 12% after 1 week (212). Such changes are a direct result of increased hepatic synthesis of ceruloplasmin mediated by cytokines IL-1 and IL-6 (213). The increases in

ceruloplasmin after injury or infection are similar to the changes in copper concentration while the copper:ceruloplasmin ratio does not change. Higher ceruloplasmin concentrations during illness are postulated to be beneficial since ceruloplasmin scavenges free radicals and helps to maintain iron in the reduced state; that is, it functions as an antioxidant (214).

Other nutrients. Besides serum zinc, ferritin and copper concentrations, inflammation may influence plasma concentrations of other nutrients. Plasma retinol, a biomarker for vitamin A status, is reduced during the acute phase of a wide range of infection (215-217), in HIV and AIDS (218), and after myocardial infarction (219). Its change may be explained by the reduction in synthesis of liver retinol binding protein (RBP), a transport protein of retinol in plasma. Plasma tocopherol, a biomarker of vitamin E, is known to decline during inflammation (220, 221), but there is no information on the association between tocopherol and APPs or chronic phase protein (222). Plasma or leukocyte ascorbic acids, biomarkers of vitamin C status, are obviously changed in inflammation, but there is no clear association between a level of AGP or CRP at which the changes occur (223). Serum folate is reduced during acute inflammation (34). HIV and ACT were independently associated with lower serum folate levels (224). Selenium levels in serum, plasma, and whole blood decrease during the acute phase response (34). Low levels of selenium occur in several infections (225, 226). It has been postulated that several viral infections stimulate the production of selenoproteins, leading to low serum selenium levels (227); or the process of quenching free radicals induces the consumption of selenium as an antioxidant (228).

Biomarkers for subclinical infection

Community-based surveys or studies usually recruit only apparently healthy participants; however, the people may have elevated inflammatory markers because of many common factors. For example, elevated serum ACT concentrations were associated with histories of diarrhea or fever in the previous 2 weeks in preschool Bangladeshi children (229), and with stunting in Nepali village children or rainy season in apparently healthy Gambian and Indian infants (230, 231). In fact, the mean plasma ACT concentrations may be elevated in more than 50% of apparently healthy Gambian infants every month over 1 year (232). Subclinical inflammation may account for the poor responses in plasma retinol concentrations to vitamin A supplementation in children in Papua New Guinea (233), and in plasma zinc concentrations to zinc-containing micronutrient supplementation in healthy HIV-positive adults in Kenya (183).

There are 3 acute-phase proteins that have been used to measure subclinical inflammation in several nutritional studies and that are accepted by the joint WHO/CDC and Prevention Technical Consultation: C-reactive protein (CRP), α-1 acid glycoprotein (AGP), and α-1 antichymotrypsin (ACT) (35, 188). CRP concentrations increase rapidly within a few hours of infection and achieve maximum concentration in the plasma within 24-48 h, often before the appearance of clinical symptoms (188, 191). ACT rises rapidly; however, it remains elevated longer than CRP, after clinical symptoms disappear (234). In contrast, AGP is slower to rise and it may take 4 to 5 days before maximum concentrations are reached (191, 235). The concentration of AGP may be a better indicator than CRP or ACT of the presence of chronic, subclinical infection, and may better reflect the changes in the concentration of ferritin during infections (35). When clinical symptoms of disease

disappear, the CRP concentration falls rapidly, whereas AGP remains elevated during convalescence (236, 237). Based on APPs, infection may develop through 4 stages: I, healthy or reference (normal CRP, ACT and AGP); II, incubation (elevated CRP and ACT); III, early convalescence (elevated CRP, ACT, and AGP); and IV, late convalescence (normal CRP and ACT, but elevated AGP) (**Figure 2.4.1**) (13).

Correction of inflammation effect on nutrient measurements

In a population with frequent exposure to endemic disease, such as children in developing countries, infection may distort estimates of nutrient status. Currently, the distortion is more prominent in the assessment of iron deficiency. Serum ferritin is the best indicator of iron status because it reflects both the impact of an iron intervention and depleted iron stores (35). However, it is also an acute phase protein that increases during inflammation. To avoid such distortions, some investigators have sought to use iron status indicators that are less perturbed by infections or inflammation, such as transferrin receptors (TfR) (238). However, TfR has wide day-to-day variation (between 12-24%) (239), varies in run-to-run assay performance, and lacks both an international standard for comparison and a specific range of normal values (35). In the absence of a nutrient-status indicator that is independent of inflammation, the WHO and CDC recommend the measurement of many iron indicators (e.g., hemoglobin concentration, serum ferritin, and TfR) and at least one acute phase protein, with CRP, ACT, and AGP as the first choices (35). There are many approaches to correct the inflammation effect. First, the cutoffs for the iron deficiency may be adjusted to account for this influence. For instance, in children < 5 year of age, the normal ferritin cutoffs of 12- 15 μg/L may be shifted to 30-50 μg/L if there is evidence of chronic disease or inflammation (35). This approach is problematic because it does not

provide a distinct international standard for inflammation and a single cutoff for ferritin levels (240). Second, some authors exclude individuals with inflammation from analysis. This approach is usually used with relatively high cutoffs of CRP (about 10 mg/L). However, the approach has two disadvantages: potentially valuable data are lost and some influence of inflammation may still remain because subjects with CRP values between 5 and 10 mg/L are still included (241). The problem is exaggerated for children in developing countries with high prevalence of chronic inflammation, such as malaria and hook worm infestation (186, 242). Furthermore, researchers rarely measure AGP; therefore, any influence of chronic inflammation on the data is ignored. Third, some authors adjust for the influence of inflammation measured by the APPs on iron status by regression modeling. This approach is useful in assessing the associations between iron status and other outcomes (e.g. anemia) (240). However, it is not useful in estimating the average concentration or prevalence of iron deficiency of a population. In addition, using the change in AGP is not useful in predicting the change in retinol or ferritin (243, 244), because individual APPs do not reflect exactly the behavior of nutritional biomarkers during inflammation, especially in apparently healthy people (241). In apparently healthy people, the correlations between APPs and nutritional biomarkers are poor and changes in APP explain very little of the variance in the nutritional biomarkers (244). The fourth approach proposed by Thurnham et al. uses a non-parametric method with correction factors to remove the influence of inflammation (241). Biomarker concentrations in a group are corrected by multiplying with a correction factor, which is calculated as the ratio of the median value of the biomarker for the reference group by the respective median value for this group. For example, based on measurements of CRP and AGP, the data are categorized into 4 groups: normal or reference group, with normal CRP

and AGP levels; incubation, with elevated CRP but normal AGP; early convalescence, with both elevated CRP and AGP; and late convalescence, with normal CRP but elevated AGP. The approach may be used for ferritin and other nutrients, such as serum retinol, carotenoids, hemoglobin, and zinc (183, 188, 241, 245). The reference group with normal biomarker levels may be defined usually by combinations of 2 APPs (e.g., CRP and AGP in 4-group analysis), and rarely, by one biomarker alone (e.g., CRP or AGP, in 2-group analysis (246). The 4-group and 2-group analyses for serum ferritin concentration and levels were evaluated in a recent review of 32 studies by Thurnham et al (246). Compared to the reference group (normal CRP and AGP), the averages of serum ferritin concentrations were overestimated by 30% without any correction, 13% with CRP correction, 14% with AGP correction, and 5% with both CRP and AGP corrections. Similarly, the prevalence of iron deficiency defined by low ferritin values was underestimated by 14% without any correction, 5% with CRP correction, 9% with AGP correction, and 0% with both CRP and AGP corrections (246). The reference group was defined as subjects with CRP $\leq$ 5 mg/L and AGP < 1 mg/L in this review. However, CRP cutoffs for adjustment of inflammation may be different depending on APP measurement methods.

CRP and its cutoffs

History of CRP. C-reactive protein was discovered by Tillett and Francis in 1930 as a material in the sera of patients with acute pneumonia that precipitated a non-type-specific polysaccharide fraction extracted from pneumococci (C polysaccharide, CPS) (247). The C-reactive material was not found in sera of recovered patients and normal healthy individuals. In 1941, Avery *et al.* characterized the C-reactive material as a protein which required calcium ions for its reaction with CPS, and introduced the term "acute phase" to refer to serum from

patients acutely ill with infectious disease and containing the C-reactive protein (248-250). In 1944, Lofstrom described a non-specific capsular-swelling reaction of some strains of pneumococci when mixed with acute-phase sera and subsequently showed that the substance responsible was CRP (251). Since its discovery, CRP has become a useful marker of acute inflammation (252). Later, CRP was also detected in non-infective inflammatory processes, including cellular and/or tissue necrosis (e.g., myocardial infarction), and malignant neoplasia (252, 253). Numerous epidemiologic studies demonstrated that increased serum CRP concentrations are positively associated with risk of future cardiovascular diseases (i.e., coronary artery disease, cerebrovascular disease, peripheral arterial disease) in apparently healthy men and women (254-258). CRP is also predictive of future events in patients with acute coronary syndromes, stable angina, and coronary artery stents (259-265). In 2003, the CDC/American Heart Association Consensus Panel recommended clinical use of hs-CRP assay for the risk of CVD (266). Based on the distributions of hs-CRP samples from >15 populations involving >40 000 persons, CRP levels of <1.0 mg/L, 1.0 to 3.0 mg/L, and >3.0 mg/L corresponded to low, average, and high risks of CVD in the adult population (266).

Properties of CRP. CRP is synthesized by hepatocytes and is normally presented as a trace constituent of plasma. It has molecular weight of 105500 and consists of 5 identical non-glucosylate polypeptide subunits (252). In addition to the reaction with pneumococcal C-polysaccharide, CRP undergoes calcium-dependent binding with choline phosphatides, polysaccharides, and peptidopolysaccharides which are presented in diverse bacteria, fungi, and parasites (252); and calcium-independent binding to polycations (267). Therefore, CRP precipitates soluble ligands and agglutinates particulate ligands (268, 269). Once complexed,

it becomes a potent activator of the classical complement pathway, and may bind to T lymphocytes or suppress platelet aggregation (270, 271). The role of CRP is unknown, although it can cause inflammation in some circumstances. Pepys suggested that the main role of CRP was to recognize in the plasma the potentially toxic autogenous materials released from damaged tissues, to bind to them, to detoxify them, and to facilitate their clearance (252).

CRP measurement methods. Most nutritional studies in healthy populations use traditional CRP methods. The conventional method for CRP measurements is an immunoturbidimetric assay (imCRP) (272). This method is available for routine testing laboratories and is suitable for measurement of CRP concentrations during infection. However, it is relatively insensitive for determination of concentrations within the normal range (< 10 mg/L) (272). Routine automated CRP methods with either immunonephelometric or immunoturbidimetric assays typically have limits of quantification of 3-8 mg/L (273). Traditional methods have low sensitivity and can detect only CRP concentrations >3 mg/L (189). These methods cannot reproducibly measure lower protein concentrations because of interference due to the presence of other light scattering species in the sample (274). Coupling of latex particles to antibodies can enhance the light scattering properties of specific antigen-antibody complex and permits measurements in low CRP concentrations (275). Over the years, high-sensitive CRP assays (hs-CRP) have been developed and can measure CRP at concentrations as low as 0.1 mg/L (272, 276, 277). Initial hs-CRP methods used ELISA methodology, and a single in-house ELISA assay was used for several population studies (254, 278, 279). However, this methodology is primarily for research and is not ideal for routine use in highly automated clinical laboratories. Other

hs-CRP methods, including latex-enhanced immunonephelometric, immunoturbidimetric, and immunoluminometric hs-CRP assays, are automated and available. Among diverse assays, the latex-enhanced immunonephelometric method is chosen as the comparison method because it was evaluated and validated in clinical settings (276, 280), and only its assay from Dade Behring was approved by the Food and Drug Administration (FDA) for use in the United States to assess the risk of cardiovascular and peripheral vascular disease (273). A study comparing the Dade Behring and 8 other hs-CRP assays in 388 apparently healthy individuals showed that they had high concordance; no subjects varied by more than one quartile of the distribution by any method (273).

CRP cutoffs for inflammation. Currently, there is no consensus to define inflammation based on clinical signs or biomarkers. There are two possible strategies for defining normal ranges of a biomarker concentration. One approach is to use a statistical definition. It is based on the observed distribution of the biomarker among presumably healthy individuals to define upper and lower cutoffs, using either the 2.5th and 97.5th percentiles or the 1st and 99th percentiles of the observed distribution in the reference population. A second, more physiological approach, is to determine a level of biomarker concentration below or above which some understandable functional outcome occurs (113). Currently, the CRP cutoffs for normal ranges are based on the first approach; statistical definitions are derived from reference population data. The median value in 468 healthy adults was 0.8 mg/ml, 90% were less than 3 mg/L, and 99% less than 10 mg/L in a study in 1981 (252). Therefore, the CRP cutoff of 10 mg/L for high CRP concentration has been used in many research studies to correct the prevalence of iron deficiency of inflammation effect (186, 187). Recently, in the National Health and Nutrition Survey (NHANES) III in United States during 1999-2000,

adult men and women had the median CRP concentrations of 1.6 and 2.7 mg/L, the 90th percentiles of 7.0 and 11.9 mg/L, and the 95th percentiles of 12.2 and 17.9 mg/L, respectively (281, 282). In children and adolescents in the same study, CRP concentrations increased with age and did not differ until 16-19 years of age; females had higher concentrations than males (283). Overall, children and young adults from 3 to 19 years of age had CRP median 0.4 mg/L, 90th percentile 3.6 mg/L, and 95th percentile 7.5 mg/L. If excluding participants with high CRP values (>10 mg/L), CRP concentrations had median 0.4 mg/L, 90th percentile 2.9 mg/L, and 95th percentile 4.4 mg/L(283). In another study of Soldin *et al.* in 2004 which measured hs-CRP by a standard method (Dade Behring RxL Dimension Analyzer, Newark, DE) in 800 in- and out-patients aged of 0-18 years, the 97.5th percentile ranged from 7.6-15.8 mg/L in males and 7.9-15.8 mg/L in females (284). Then, many nutritional studies used other CRP cutoffs smaller than 10 mg/L, such as 8 mg/L, 5 mg/L, and 3 mg/L, to adjust for inflammation effect (189, 240, 241, 244, 245, 285, 286). However, the uses of CRP cutoffs were based on the suggestions of manufacturers of CRP assays (244), or followed the cutoffs of previous studies (186, 240, 246, 286), which in turn, had not a clearly documented justification (187, 188, 287). In fact, commonly used CRP cutoffs are arbitrary.

In the second approach, many studies have demonstrated high CRP levels for the risk of cardiovascular diseases in apparently healthy adults (266). The Centers for Disease Control and Prevention and the American Heart Association (CDC/AHA) suggested that levels with hs-CRP <1 mg/L, 1 to <3, and ≥3 mg/L be used to represent low, moderate and high vascular risk (266). Compared to the reference level of CRP < 1 mg/L, high CRP concentrations (3-10 mg/L) predict increased risk of myocardial infarction and stroke in

prospective studies (266, 288). Other CRP levels ≥1 mg/L are associated with higher risk of common inflammatory conditions in men (e.g., chronic urinary tract diseases, peptic ulcers, chronic cough, bronchitis, asthma, rheumatoid arthritis, and any cancer) (289). Recent data from the "Justification for the Use of Statins in Primary Prevention: an International Trial Evaluation Rosuvastatin (JUPITER)" showed that the primary prevention with statin (vs. placebo) among individuals with CRP >2mg/L and low LDL cholesterol (<130 mg/L) significantly reduces the risk of major cardiovascular events (e.g., myocardial infarction, stroke, and death from CVD) (290); while the primary prevention with statins did not benefit other adult populations without CRP screening (291). Therefore, subclinical inflammation may occur and induce harmful health outcomes at moderate CRP levels (3-5 mg/L or > 1 mg/L). In children, however, there are few studies assessing the risk of chronic outcomes between high and low CRP levels. Although CRP was correlated with adiposity and several cardiovascular risk factors in children (292), hs-CRP is rarely used in research studies in children. A recent cross-sectional study showed that in American children aged 1 to 17 years, a CRP level of >1 mg/L is associated with high levels of obesity (≥99^{th} percentile) (293). A study in healthy Germany infants showed that the acute-phase response may be triggered at very low inflammatory levels; individuals with CRP > 0.6 mg/L (75^{th} percentile), compared to those with CRP ≤0.6 mg/L, had significant lower levels of serum iron, retinol, beta-carotene, and transthyretin (294). Overall, subclinical inflammation can induce acute-phase response at moderate CRP levels; however, a reference group with CRP <1 mg/L defined by hs-CRP measurements is advocated for use only in adults for screening the risk of CVD or metabolic diseases. There is no consensus about possible CRP cutoffs of a reference CRP group for nutrient assessments in adults and children.

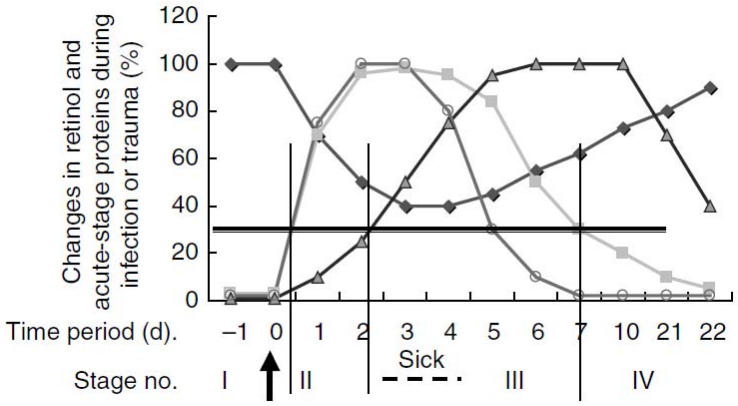

Figure 2.4.1. Idealized behavior of acute-phase proteins during the course of infection. Standardized changes in C-reactive protein (CRP; o), α1-antichymotrypsin (ACT; ▪), α1-acid glycoprotein (AGP; ▲) in response to trauma or infection. (---) threshold of elevated APPs. Reprinted from Thurnham DI, Mburu AS, Mwaniki DL, De Wagt A. Micronutrients in childhood and the influence of subclinical inflammation. Proc Nutr Soc. 2005 Nov;64:502-9 (13).

2.5. Pairwise Interactions among Zinc, Iron, and Copper

Zinc, iron, and copper have similar absorption and transport mechanisms, and are thought to compete for absorptive pathways (295).

Zinc regulation. Zinc absorption occurs at all levels of the small intestine. Zinc released from diets as divalent ions Zn^{2+} is absorbed both by passive diffusion and through a carrier-mediated process on the brush border of enterocytes (72, 73). All zinc transporters have transmembrane domains and are encoded by 2 solute-linked carrier (SCL) gene families: ZnT and Zip (Zrt-, Irt- like proteins). Humans have 9 ZnT and 15 Zip transporters (22). Members of the ZnT family mediate zinc efflux or sequestration into organelles/vesicles, whereas those of the ZIP family transport extracellular ir organelles/vesicular zinc into cytoplasm (**Table 2.5.1**) (296, 297). In zinc-depleted conditions, ZnT1 and ZnT2 are down-regulated and zinc absorption increases; in zinc-adequate conditions, Zip4 is up-regulated and zinc absorption decreases (296).

Iron regulation. Iron in diets consists of either nonheme iron (approximately 85%, in plant products) and heme iron (~ 15%, in animal products) (150). The two forms have separate mechanisms of absorption. Most of the iron in diets is in the ferric (Fe^{3+}) form. During digestion, gastric secretions dissolve the iron from nonheme and permit it to form soluble complexes with other substances that help reduce it to the ferrous (Fe^{2+}) form. Mostly all iron absorption occurs in the duodenum. Heme is *transported* into the enterocyte by a separate heme transporter (HT) (**Figure 2.5.2**) (4). The relative absorption of heme iron is two to three times greater than nonheme iron and is less affected by the overall composition of the diet (150). Ferric is converted to ferrous (Fe^{2+}) by ferric reductase, and

ferrous is transported into enterocytes by divalent metal transporter 1 (DMT1), the major intestinal iron importer (298, 299). Inside enterocytes, iron is transferred across the intestinal mucosa into the circulation by ferroportin at the basolateral membrane (135, 300, 301). In plasma, iron is bound to serum transferrin, delivered to peripheral tissues, received by transferring receptor-1 (TfR1) in cellular membranes, and picked up by receptor-mediated endocytosis. For macrophages, iron can be delivered not only by transferrin-transferrin receptor 1 but also by erythrophagocytosis. Iron absorption is regulated by hepcidin that is synthesized primarily in the liver. Hepcidin inhibits iron transport in enterocyte by binding to ferroportin and inducing its internalization and degradation (205). Because ferroportin mediates the entry of iron into circulating pools from both enterocytes at the basolateral membrane and macrophages, reduced ferroportin levels limit dietary iron absorption, promote iron retention by the reticulum endothelial system (206), and induce hypoferremia and anemia.

Copper regulation. Copper in the diet is absorbed mainly in the stomach and duodenum (11). Dietary copper is absorbed by enterocytes (**Figure 2.5.3**). The sequence of transport events by which copper is moved from the intestinal lumen into the portal circulation is not completely known. Recent evidence indicate that dietary copper absorption is absolutely dependent on at least 2 transporter proteins, the high-affinity copper transporter 1 (CTR1) transporting copper at the brush border of intestinal membrane, and intracellular ATP7A translocating cytosolic copper into the biosynthetic pathway (e.g., Golgi Apparatus) (11). In addition, copper chaperone ATOX1 is essential for dietary copper uptake by transiently binding copper inside the cell to deliver the metal ion to its proper intercellular destinations (e.g., to ATP7A) (302). More controversial or circumstantial

evidence has implicated possible roles of the divalent metal transporter 1 (DMT1), the low-affinity copper transporter 2 (CTR2), and metallo-reductases in copper absorption (11). DMT1 is a protein with 12 transmembrane regions that transport divalent metals (Fe^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, Pb^{2+}, and Zn^{2+}) across membranes by exchanging protons H^{+} in an energy-dependent manner (299). It is expressed in the brush border of duodenal enterocytes. It may simultaneously participate in the absorption of many metals, such as iron, zinc, and copper. However, its role may be small in copper absorption. Studies in Belgrade rats with inactivating mutation in DMT1 and in human patients with hereditary mutations in DMT1 observed iron deficient anemia but no copper deficiency (298, 303). CTR2 has structure and topology similar to CTR1, but lacks the Mets domains that are essential for high-affinity copper transport; therefore, it is weaker and only an alternative route of copper uptake (304, 305). A cupric reductase system with metallo-reductases may be necessary to reduce the Cu^{2+} to the reduced Cu^{3+} form, which is transported by specific CTR1 into enterocytes. However, its existence is only established in vitro models (306). On the contrary, intestine-specific CTR1 knockout mice displayed copper-deficiency (307). Therefore, CTR1 mediates the major copper uptake route in enterocytes (11).

Zinc and iron interaction. Although zinc and iron appear similar in having the same outer electronic configuration (+2), the mechanisms of zinc and iron absorption and their interactions are not completely similar. Zinc appears to be a less effective inhibitor of iron absorption than iron is of zinc (50). Under usual conditions, human zinc absorption is determined largely by zinc complex formation with food in the intestinal tract, and the influence of iron on zinc absorption may not be significant. However, if iron supplements are ingested, the increased iron could impair zinc absorption. For example, in human studies,

inorganic iron added to solutions of zinc salts in Fe/Zn ratios of 2.25 significantly lowered zinc absorption (308-311). In pregnant women, iron supplementation of 30-100 mg daily was associated with significant lower plasma zinc levels (312, 313). Inversely, zinc supplementation at a level of 50 mg daily over a 10-week period may impair iron status in adults (23). The combination of zinc and iron (20 mg:20 mg) in iron-deficient Iranian schoolboys had less impact on growth than supplementation of iron alone (24). High zinc intakes may interfere with iron status at many levels, by impairing the intestinal absorption of iron, interfering with iron uptake by the liver to decrease the storage of iron as ferritin, and competing with iron for transport proteins as transferrin (50). In addition, zinc deficiency or decreases in zinc concentration have been associated with enhanced iron absorption and increased levels of iron (314-316). However, providing zinc levels of the RDA does not decrease iron absorption and iron status in humans (58).

Zinc and copper interaction. The interaction between zinc and copper may be mutually antagonistic. In humans, chronic elevated intakes of zinc, 100 mg or more daily has been shown to induce copper deficiency (25, 26). Raising dietary copper to narrow the zinc:copper ratio may reverse the changes in copper status (14). In mechanisms, zinc and copper inhibit each other's intestinal absorption under certain conditions. Higher zinc intakes increase the synthesis of metallothionein synthesis in the intestinal cells. Inside the cells, absorbed copper is bound to metallothionein, which has higher affinity for copper than zinc, does not enter the body, and is returned to the intestinal lumen with the turnover of the intestinal cells (317). Inversely, excess copper exposure has no effect on metallothionein and zinc absorption (318).

Zinc excess. When zinc exposures are high, concentrations of zinc in blood plasma or serum, urine, and hair may increase. In humans, consumption of drinks with high zinc concentrations up to 2500 mg/L or an estimated 325-650 mg may cause acute toxicity with nausea, abdominal cramping, vomiting, tenesmus, and diarrhea with or without bleeding (319, 320). Excess zinc during embryogenesis can be teratogenic or lethal (319). However, zinc is neither a mutagen nor carcinogen in humans (319, 321). Chronic zinc toxicity occurring when oral zinc intake is disproportionately high relative to copper may induce copper deficiency. In humans, multiple effects include decreases in copper-dependent enzymes such as ceruloplasmin, superoxide dismutase, and cytochrome c oxidase, and changes in immunological parameters, cholesterol, and its lipoprotein distribution (322). In adults, nutritional copper deficiency is manifested by leucopenia and anemia (26). In infants and children, copper deficiency results in hypochromic microcytic anemia and neutropenia (323). Therefore, zinc and copper should be proportionate in dietary and supplemental intakes (324).

Figures 2.5.

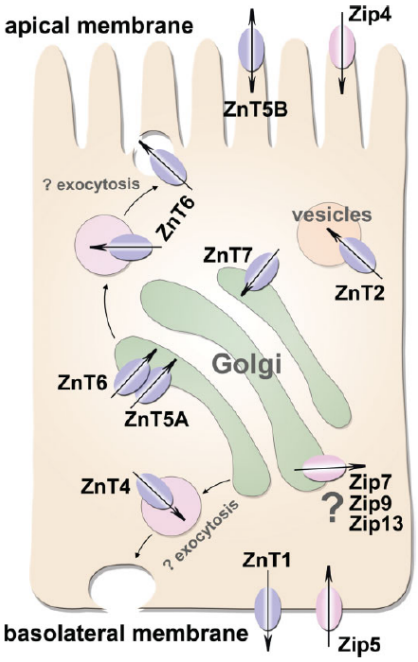

Figure 2.5.1. Zinc transportation in a polarized enterocyte. Reprinted from Wang, X. and B. Zhou (2010). "Dietary zinc absorption: A play of Zips and ZnTs in the gut." IUBMB Life **62**(3): 176-182 (8).

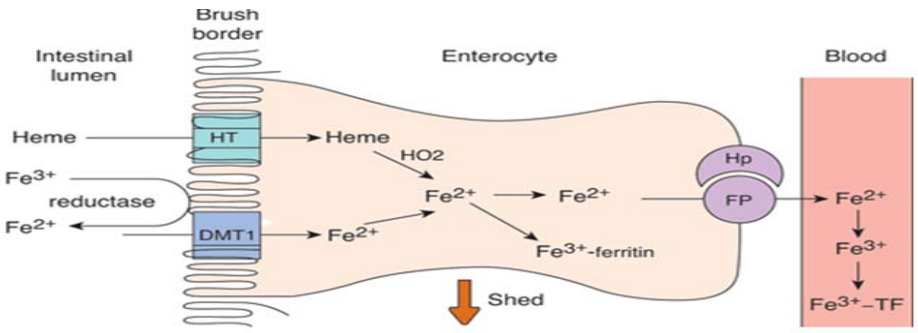

Figure 2.5.2. Iron absorption at the enterocyte. Reprinted from Barrett K, Barman, SM, Boitano, S, Brooks, H. Digestion, absorption, & nutritional principles. In: Barrett K, Barman, SM, Boitano, S, Brooks, H., editor. Ganong's review of medical physiology. 23th ed: McGraw-Hill Companies, Inc.; 2010 (4).

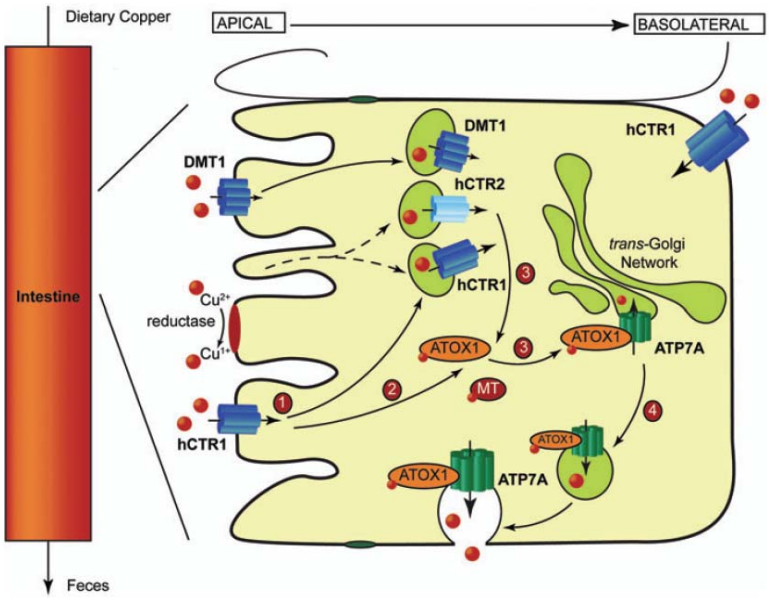

Figure 2.5.3. Copper absorption at the enterocyte. Reprinted from van den Berghe PV, Klomp LW. New developments in the regulation of intestinal copper absorption. Nutr Rev. 2009 Nov;67:658-72 (11).

2.6. The Effect of Zinc Supplementation on Biomarkers

Limitations of preventive zinc supplementation. Zinc supplementation is obviously an important option to use as a short-term solution in the treatment of infectious diseases, especially diarrhea (47). However, for more prolonged use of preventive purposes, there are several problems that need to be resolved. First, if supplements are given often (i.e., daily), it is generally difficult to maintain high levels of compliance with these regimens. Second, because zinc deficiency often occurs simultaneously with other micronutrient deficiencies (e.g., iron deficiency), giving zinc alone may have no impact or even exacerbate other deficiencies (325). However, combined iron and zinc supplements are not the ideal solution because these 2 elements show interactions in absorption, and combinations are less effective than providing either alone in improving their respective status indicators (326, 327). Therefore, the effect of zinc supplementation on biochemical measurements is important and needs further research.

Reviews. The effect of preventive zinc supplementation on biochemical outcomes among children has been investigated in numerous studies. In 2009, Brown *et al.* conducted a systematic review of relevant supplementation trials in infants and pre-pubertal children (14). From 1620 articles identified in PubMed, they found 55 individual randomized trials, which included 75 group-wise comparisons (zinc-containing vs. no-zinc-containing groups) and enrolled a total of 202,692 children (**Figure 2.6.1**). Of the 55 included studies, 7 were form Africa, 23 from Asia, 11 from North America, 1 from Australia, and 1 from Europe. The supplementation durations ranged from 2 weeks to 15 months. The periodic zinc supplementation doses ranged from 1 to 70 mg per dose (median 10 mg). The doses were provided daily (n = 50), several times per week (n = 30), or once per week (n = 6), resulting

in a daily dose equivalents ranging from 0.9 to 21.4 mg of zinc/day. Most studies provided zinc as zinc sulfate (n = 36), but other compounds were also provided, such as zinc acetate (n = 5), zinc gluconate (n = 5), zinc amino acid chelates (n = 3), and zinc oxide (n = 1). The mean initial age at enrollment varied greatly among studies. Some studies enrolled infants within a few days after birth, whereas one study enrolled children with a mean initial age of 11.1 years.

Effect on serum or plasma zinc concentration. Information on the change in serum or plasma zinc concentration was available from 22 intervention trials consisting of 30 group-wise comparisons (**Fig 2.6.2**). Serum zinc concentration increased significantly with an overall moderate effect size of 0.60 (95% CI, 0.44 to 0.77; $p < 0.0001$, random-effect model). There was significant heterogeneity of results ($p < 0.0001$). The daily zinc dose equivalents ranged from 2.9 to 21.4 mg of zinc/day, and the study lasted from 2 weeks to 14 months. The results are similar to those of previous meta-analysis (113).

Effect on serum ALP activity. The review of Brown *et al.* did not address the effect of preventive zinc supplementation on serum ALP activity. A review of Lowe *et al.* in 2009 collected six studies investigating the response of plasma ALP activity to changes in zinc intakes, including 3 randomized controlled trials with supplementation and 3 depletion (before/after) studies (31). No study recruited children. Overall, the primary analysis revealed no significant effect of zinc intakes on plasma ALP activity (weighted MD: 4.14 IU/L; 95% CI: -2.38, 10.66; $I^2 = 56.6$). Another review by Gibson *et al.* observed that the ALP activity was reduced in severe zinc deficiency states (87, 328), but not in more moderate zinc deficiency (92); only one study was in infants with persistent diarrhea (328). The authors suggested that the results have been inconsistent in community settings, probably because

this enzyme has poor specificity (30). Only two studies investigating the response of ALP activity to zinc supplementation in healthy children were found; one study observed significant changes (32), while another did not (33).

Effects on hemoglobin and iron status. A total of 11 studies, which included 19 group-wise comparisons, provided information on the change in hemoglobin concentration following zinc supplementation. The daily dose equivalents for those 19 sets of observations ranged from 2.9 to 21.4 mg of zinc/day. Iron supplements were also provided in 8 of these group-wise comparisons. Considering all of the available information, there was no overall effect of zinc supplementation on change in hemoglobin concentration (**Fig 2.6.3**). The estimated mean effect size was 0.02 (95% CI, -0.13 to 0.17; p = 0.80, random-effects model). There was significant heterogeneity among studies (p <0.0001), but no particular characteristics of studies were associated with the magnitude of hemoglobin response. Neither the daily zinc dose nor the presence of iron in the supplement was correlated with the effect size in hemoglobin concentration due to zinc.

Similarly, there was no overall effect of zinc supplementation on the change in serum or plasma ferritin concentration among the 17 available group-wise comparisons derived from 10 studies, 7 of which also provided iron (**Figure 2.6.4**). The estimated effect size was 0.05 (95% CI, -0.15 to 0.25; p = 0.60, random-effects model). There was significant heterogeneity among comparisons (p <0.0001). The magnitude of the change in serum ferritin concentration in relation to zinc supplementation was negatively correlated with the presence of iron in the supplement, the mean initial hemoglobin concentration, and the mean initial ferritin concentration (all p-values =0.02).

Effect on copper status. Four studies involving 8 group-wise comparisons provided results on the change of serum copper following zinc supplementation (**Figure 2.6.5**). There was no overall effect of zinc supplementation on the change in serum copper concentration. The estimated effect size was -0.04 (95% CI, -0.21 to 0.13; p = 0.59, random-effect model). The daily zinc dose was not associated with the change in serum copper concentration. However, it should be recognized that serum copper is a relatively insensitive biomarker of copper status (159). It is possible that more subtle changes in copper metabolism may have occurred; however, such changes, if they did occur, would be unlikely to have any functional significance.

Effect on other biological outcomes. A number of studies have examined the effects of zinc supplementation on iron absorption either by using isotopic tracers during short-term studies or by assessing biochemical and functional responses following longer-term supplementation. The tracer studies indicate that each mineral may interfere to some extent with absorption of the other, but only when they are provided simultaneously in aqueous solutions and in disproportionate molar doses (308). However, there is no evidence of interference when they are delivered in near isomolar amounts or with foods (329). Some longer-term studies also suggest that when given together, each mineral may reduce the magnitude of the response observed with single-nutrient supplementation (326, 327, 330). Less information is available for the interaction between zinc and copper, but some studies have found negative effect of large-dose zinc supplementation on indicators of copper status in adults (23, 133).

Figures 2.6.

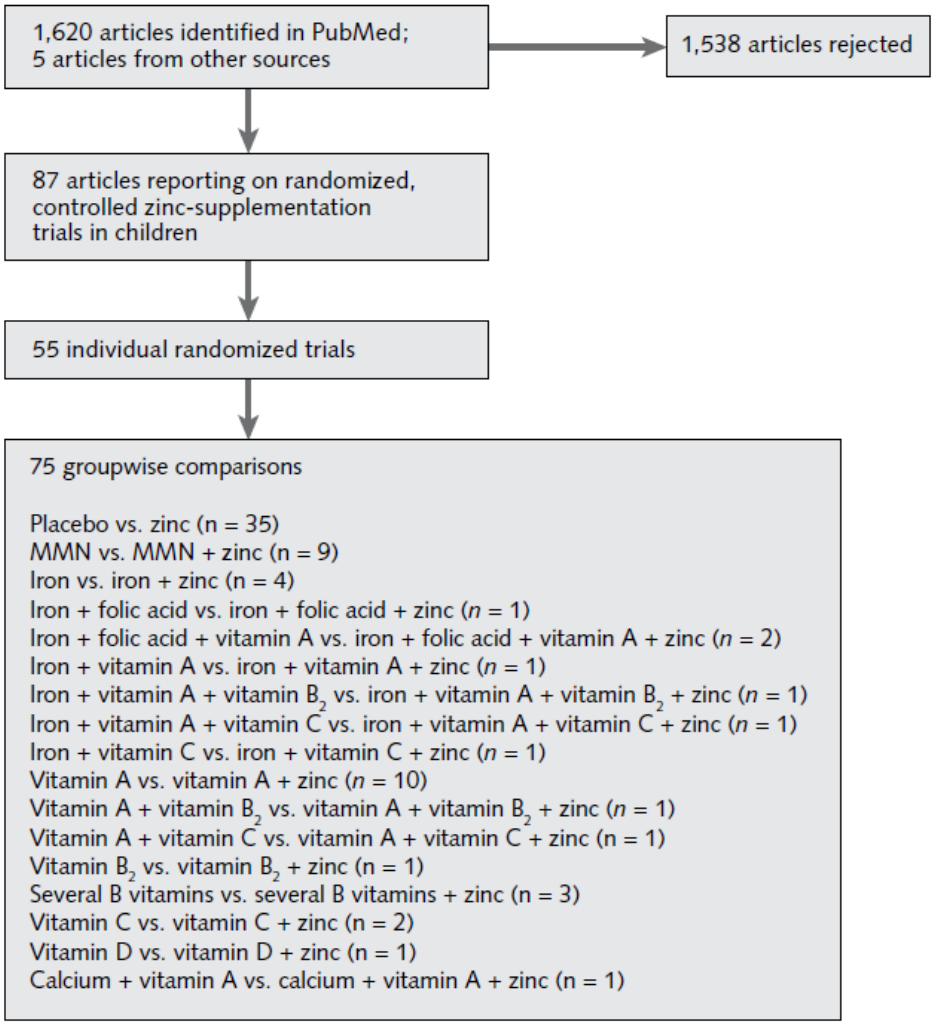

Figure 2.6.1. Flow chart of number of articles and individual studies included in the meta-analysis on preventive zinc supplementation in children. For group-wise comparisons, the two treatment groups differed only by the presence or absence of zinc in the supplement provided. MMN (multiple micronutrients) indicates at least 4 micronutrients. Reprinted from Brown KH, Peerson JM, Baker SK, Hess SY. Preventive zinc supplementation among infants, preschoolers, and older prepubertal children. Food Nutr Bull. 2009 Mar;30:S12-40 (14).

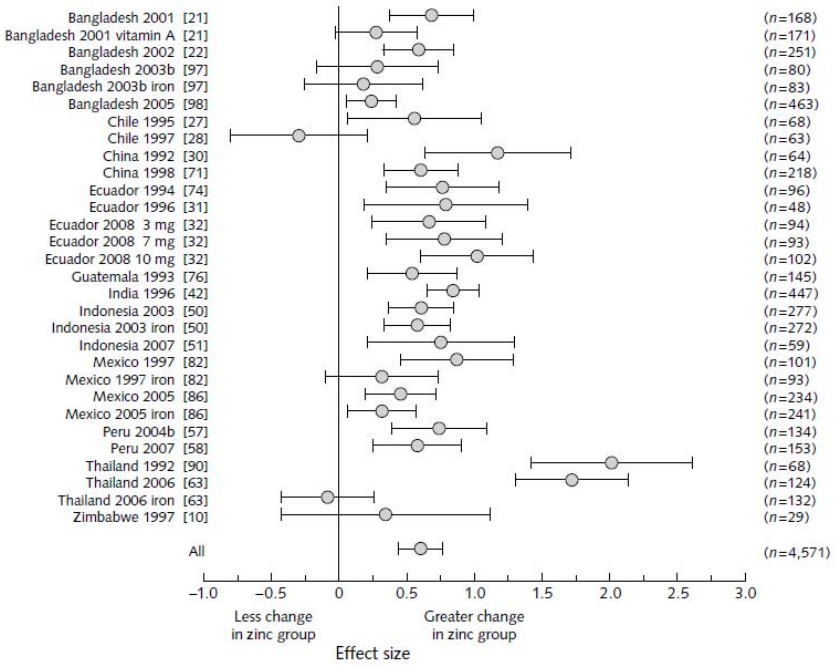

Figure 2.6.2. Effect of zinc supplementation on change in serum or plasma zinc concentration in children from 22 controlled supplementation trials with 30 group-wise comparisons in which the supplements differed only by the presence or absence of zinc. Reprinted from Brown KH, Peerson JM, Baker SK, Hess SY. Preventive zinc supplementation among infants, preschoolers, and older prepubertal children. Food Nutr Bull. 2009 Mar;30:S12-40 (14).

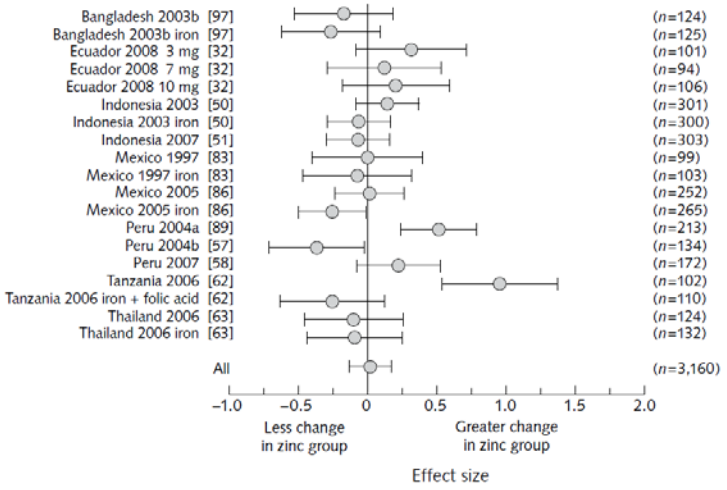

Figure 2.6.3. Effect of zinc supplementation on change in hemoglobin concentration among children from 11 controlled zinc supplementation trials with 19 group-wise comparisons in which the supplements differed only by the presence or absence of zinc. Reprinted from Brown KH, Peerson JM, Baker SK, Hess SY. Preventive zinc supplementation among infants, preschoolers, and older prepubertal children. Food Nutr Bull. 2009 Mar;30:S12-40.

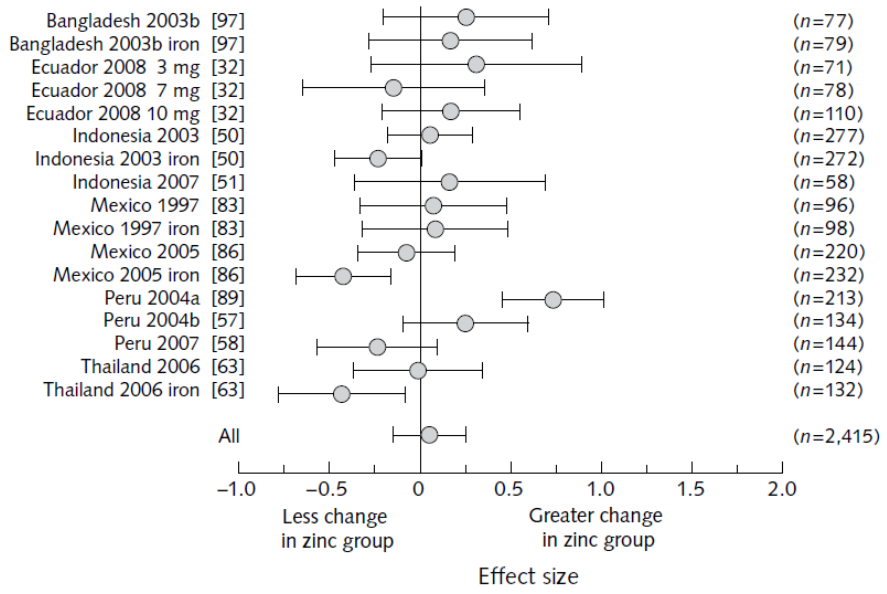

Figure 2.6.4. Effect of zinc supplementation on change in serum or plasma ferritin concentration in children from 10 controlled zinc supplementation trials including 17 group-wise comparisons in which the supplements differed only by the presence or absence of zinc. Reprinted from Brown KH, Peerson JM, Baker SK, Hess SY. Preventive zinc supplementation among infants, preschoolers, and older prepubertal children. Food Nutr Bull. 2009 Mar;30:S12-40 (14).

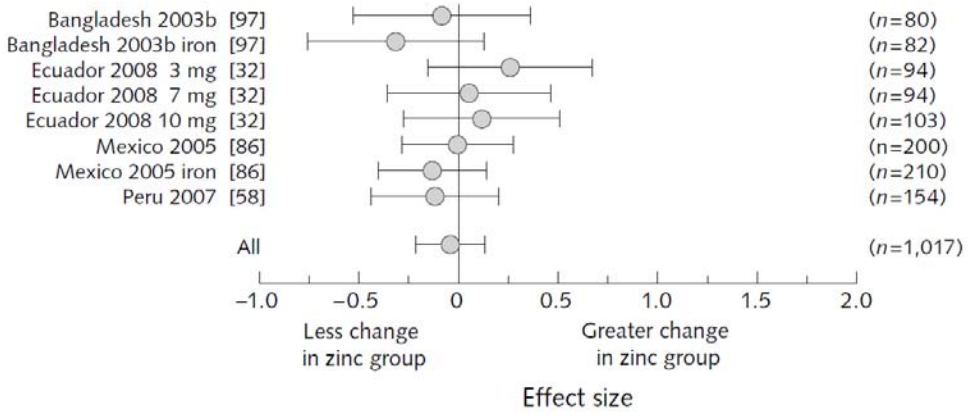

Figure 2.6.5. Effect of zinc supplementation on change in serum copper concentration in children from four controlled zinc supplementation trials with eight group-wise comparisons in which the supplements differed only by the presence or absence of zinc. Reprinted from Brown KH, Peerson JM, Baker SK, Hess SY. Preventive zinc supplementation among infants, preschoolers, and older prepubertal children. Food Nutr Bull. 2009 Mar;30:S12-40 (14).

2.7. Health and Nutritional Status of Children in Guatemala

Country and people in Guatemala

Guatemala is a democratic republic in Central America, bordering the North Pacific Ocean (between El Salvador and Mexico) and the Gulf of Honduras (between Honduras and Belize). Its total land area is 108 889 km2; however, most of the terrain is mountains and only 13.22% of land is cultivable (331). The country is divided by 2 mountain chains into 3 regions: the highlands in the center, the Pacific coast in the south, and the Peten region in the north. All major cities are located in the highlands and the Pacific coast region; Peten is sparsely populated (3). In history, the Mayan civilization flourished in Guatemala during the first millennium A.D (3, 37). After 3 decades as a Spanish colony, Guatemala won its independence in 1821. From 1944, it experienced many military and civilian governments, including a 36-year guerrilla war. In 1996, the Guatemalan civil war ended, and the country is currently in the process of restoring its social system after several decades of armed conflicts (3, 37).

The Guatemalan population was 13.5 million in 2010 (331). It is the most populous nation in Central America, with a largely poor, rural, young, and indigenous population. About 42% of the population is Ladino, also called Mestizo (mixed Amerindian and Spanish); 18% is White, primary of Spanish but also of Italian, German, British, and Scandinavian descents; and 38% is of indigenous origin, mostly Mayan people (3). Guatemala's crude birth rate is 27.4 births per 1000 population, with a total fertility rate of 3.4 children born per woman, the highest in the region. Life expectancy at birth was 68.2 years in 2006. It is a multiethnic, multicultural nation with 23 linguistic groups. The net

schooling rate at the primary level was 92.3% between 2000- 2004, with a literacy rate of 82.2% in the 15- 24 age group (332). Due to lack of sustainable growth and inadequate domestic redistribution, the main problems are the high concentration of land ownership, low taxation (around 9% of GDP), disparity in wealth, and difficulties in implementing effective social policies (37). Guatemala is among the most unequal countries in the world. Guatemala's GDP per capita was estimated at 5100 dollars in 2009, but more than half of the population is below the national poverty line, and 15% lives in extreme poverty (331). Between 1984- 2003, the income ratio was 5 times between the highest and lowest 20% of the population (37), and the richest 20% of the population received 60% of all income (333). More than half of the population, approximately 6 million inhabitants, had incomes lower than US$ 2 per day (36). Indigenous groups, living largely in rural areas, are more vulnerable with the proportion living in poverty and extreme poverty rising to 76% and 28%, respectively. An extremely low tax base means that the government has very little to work with to alleviate the poverty.

Health status in Guatemala

Guatemala's public health expenditure is among the lowest in the Americas (around 1% of GDP). Total health expenditure rose from 4.7% of GDP in 1999 to 5.4% in 2003, while government expenditure fell from 48.3% to 39.7% in the same period and private expenditure increased from 51.7% to 60.3% (37). Therefore, 20% of its population lack regular access to health services (332). Infant mortality was 39 per 1000 live births and under-5 mortality was 48 per 1000 live birth in 2004, the third and the fourth highest in America (37). Maternal mortality rate was high at 153 in 2000, and Guatemala has the third-lowest contraceptive use in America. Some 75% of the population had access to improved

source of drinking water in 2002. The HIV/AIDS epidemic is growing with low antiretroviral coverage (prevalence of 0.8%, 59 000 HIV-infected patients, 3900 deaths in 2007) (331). The risk of infections is high with major infectious diseases as diarrhea, hepatitis A, typhoid fever, leptospirosis, dengue fever, and malaria. However, great strides have been made in vaccination coverage with over 92% of infants covered by the immunization program including 10 vaccines (334). No cases of polio or measles has been reported since 1990 and 1997, respectively (335).

Nutrition status in Guatemala

Food availability in Guatemala is poor mainly due to environmental factors like droughts and floods, deforestation and soil erosion; and also to inadequate agricultural and economic policies. The food groups that sustain the population are cereals (mainly maize), sugars, and beans. These foods meet nearly 90% of energy requirements; the diet is deficient in total fats, proteins of animal origin, and micronutrients (36). Some 16% of the population have dietary intakes below the minimum level in 2004-2006 (336). Limited access to foods due to economic reasons is a problem for the majority of Guatemalans. The cost of the Basic Food Basket for a family is approximately half of the capita average for the population, and even more for people with official minimum wages. Access to portable water is also poor; only 67% of the homes had piped water (36), and 81% of population used an improved sanitation facility in 2008 (336).

Guatemala is among the countries with the highest prevalence of child malnutrition in the world (331). The prevalence of malnutrition in children under 5 years of age was very high in the 1960s, with 34.3% of children underweight, 75.9% stunted, and 1.6% wasted,

following from the WHO database (**Table 7.1**) (336). Then malnutrition rates gradually decreased overtime, to 17.7% underweight, 54.3% stunted, and 1.8% wasted in 2002. In 2007, approximately 49% of children under 5 years of age was stunted and 30% of pregnant women had nutritional deficits (37). Stunting is more common among the indigenous, rural population, those with a low level of education and those living in the North, Southwest and Northwest regions where the prevalence can be as high as 70% (36). Stunting in Guatemala is caused by several factors: stark income inequality, lack of education, higher price of beans and eggs, which are the only sources of high quality protein for villagers, poor infrastructure such as lack of running water and clean water, and diarrhea (333). In addition, Guatemala remains a dysfunctional society, damaged by a 36-year old civil war, a diversity of 23 different indigenous groups with different languages, and a wide disparity between wealthy and poor people. Therefore, Guatemala is the only Latin America country that has failed to decrease rates of malnutrition over the past decade, compared to others that were poorer, like Honduras and Nicaragua, or with worse income inequality, like Brazil (333).

Anemia is a common problem among women of reproductive age, pregnant women, and in children less than 5 years old (36). The prevalence of anemia (hemoglobin <11g/dL) was 26% in children less than 5 years of age in 1995 (337). Vitamin A deficiency is moderate; there were 15.8% of preschool-age children with subclinical vitamin A deficiency in 1995 (336). This deficiency is most likely affecting young girls and children under 2 years of age. Only 33% and 29% of children <5 years of age received the 1st and the 2nd doses of vitamin A supplement, respectively, in 2007 (336). Levels of iodine excretion in the urine suggest iodine adequacy, but the values vary from region to region because the iodine fortification is

not optimal; only 76% households consume iodized salt (336). Information of the prevalence of low serum zinc, ferritin, and copper levels is unknown.

The improvement reported in the prevalence of malnutrition can be attributed to the following factors: subscription of the 1996 Peace accords; stimulus to the municipalities and toward decentralization; extended education and health coverage with an increase in public spending for these sectors; and growth of the social participation of non-governmental organizations and of the civil society due to conciliation and democratization (36).

Institute of Nutrition of Central America and Panama (INCAP) in Guatemala

The Institute of Nutrition of Central America and Panama (INCAP) was created in 1949 under the supports of the Pan American Health Organization (PAHO), the Kellogg Foundation, the Rockefeller Foundation, and the governmental agreements of five countries in Central America and Panama (338). Guatemala provided a suitable building, the countries agreed to pay an annual quota of US$8500, and the Kellogg Foundation paid $15000 yearly to PAHO for its supports in the beginning. Its mission was to do following tasks: 1) determine the nutrition and related health problems of the countries of Central America and Panama; 2) through research, find practical solutions to these problems; and 3) to assist the member countries to apply these solutions through advisory services and training of personnel at all levels. In reality, INCAP's research finding and advanced training capacity have contributed tremendous scientific knowledge and experiences to Latin America and developing countries. For example, in 1989 there were 1507 articles in English, 1251 in Spanish, 144 theses, and 51 books (338). Some important practical research findings were contributions to epidemiology and treatment of kwashiorkor; development of Incaparina as

a low-cost, protein rich food; demonstration of the feasibility and effectiveness of adding vitamin A to sugar on a national scale; identification of iodine fortification with water-insoluble potassium iodate; reduction of Guatemala's prevalence of endemic goiter from 40% to less than 5% in 8 years; and demonstration of the bad impacts of iron deficiency on cognitive performance in children (339). The research studies were not only to pioneer effective technical approaches to eliminating kwashiorkor, iodine deficiency, and vitamin A deficiency, but also to convince the governments to enact necessary legislations and mechanisms for monitoring compliance.

Population and geography of Guatemala City

Guatemala City is the economic, governmental and cultural capital of Guatemala. Most activities of the country as transportation, communication, business, politics, and urban activity occur in its capital. In 2010, Guatemala City had 3.5 million inhabitants, including indigenous and many ethnic groups (340). Its population has increased rapidly since the 1970s with the influx of indigenous migrants from the outlying departments and a large influx of foreign groups. For this reasons and along with several others, Guatemala City has experienced some common problems, such as transportation saturation, unavailability of safe portable water, and increased crime (3).

Tables and Figures in 2.7.

Figure 2.7.1. Map of Guatemala. Reproduced from http://en.wikipedia.org/wiki/File:Guatemala-CIA_WFB_Map.png (3).

Table 2.7.1: Prevalence of underweight, stunting and wasting in 0- to 5-year old children in Guatemala from 1968 to 2007

Year	Sample	n	WAZ <-2 (%)	HAZ <-2 (%)	WHZ <-2 (%)
1968-69	Local	431	34.3	75.9	1.6
1972	Local	551	28.5	69.3	1.8
1979	Local	800	20.2	60.8	1
1987	National	2227	27.8	62.1	2.8
1995	National	8028	21.7	55.4	3.8
1998-99	National	3762	20.3	53.1	2.9
2002	National	5621	17.7	54.3	1.8

* Data Reprinted from WHO. Global database on child growth and malnutrition: Guatemala. Child malnutrition estimated by WHO child growth standards. 2009 [cited; Available from: http://www.who.int/nutgrowthdb/database/countries/who_standards/gtm.pdf] (336).

CHAPTER III

METHODS

This chapter describes the research objectives based on the preceding hypotheses, study design, study setting, study participants, data collection methods, and statistical analysis.

3.1. Research Objectives

Primary objectives: Corresponding to the hypotheses, we have the following objectives.

1. To investigate the association between serum C-reactive protein concentration and biochemical concentrations of zinc, iron, and copper status.

2. To investigate the pairwise correlations and agreements among zinc-related continuous and dichotomized variables (i.e., concentrations and levels of dietary zinc intake and serum zinc, alkaline phosphatase, albumin) in individuals.

3. To investigate the effect of zinc supplementation on serum zinc, ferritin, and copper concentrations and levels.

3.2. Study Design

We performed a randomized, controlled, double-blind trial in 5 public schools in a low-income community in Guatemala City (www.clinicaltrials.gov, ID no. NCT00283660). The study was a collaborative effort between the Hubert Department of Global Health at the Rollins School of Public Health (RSPH), Emory University, Atlanta, GA, and the

Institute of Nutrition of Central America and Panama (INCAP) in Guatemala City. The study protocol was reviewed and approved by the ethics committees of both institutions. The Data Safety Monitoring Committee (DSMC) included 2 nutritional epidemiologists from US-based universities and a pediatrician from a local hospital in Guatemala; none of the DSMC members were co-authors in any the publications of this study.

3.3. Study Setting and Participants

The study was conducted in public schools in San Jose la Comunidad, which is a low-income area in Guatemala City, the capital of Guatemala. Permission was obtained from the Municipality Office of the Ministry of Education and school directors; teachers were then informed of the study and requested to cooperate. We conducted informational meetings at each school. Interviewers scheduled an appointment for an initial screening with parents who expressed interest in having their child participate in the study. Recruitment occurred in January-March 2006. Inclusion criteria included all school children in grades 1-4 (ages 6-11 year). Exclusion criteria were children with any known severe illness affecting zinc status (e.g., sickle cell disease, cystic fibrosis, renal or liver disease, severe burns, or acrodermatitis enteropathica) (109). All children were screened for any known severe illness shown to affect zinc status; no children were identified as having any of these conditions. In addition, children were screened for any other severe or chronic illness not necessary linked to zinc status (e.g., cancer, diabetes, or seizures). If a child was eligible on the basis of the screening, we spoke to the parents at home to obtain their written consent as well as to the child at school to obtain his or her verbal assent. Multiple children within the same family were allowed to participate.

3.4. Randomization and Blinding

Blinding. Chewable zinc (10 mg zinc oxide) and placebo (10 mg glucose) tablets were developed, packaged, and donated by Laboratory Zerboni SA (Mexico City, Mexico). The supplement dosage of zinc was based on the criteria of both efficacy and safety from reviews of previous studies (58, 341-344), and was approved by the DSMC before beginning the intervention. The placebo was similar in appearance and taste to the zinc tablet. The zinc and the placebo tablets were divided into vials coded by 4 colors (2 were assigned to zinc; two colors were assigned to placebo) by a staff member at the INCAP who was not involved in the study.

Randomization. Children in each classroom were allocated into 2 groups: zinc and placebo. The randomization was assigned using a computer, which generated a list on the basis of a 1:1:1:1 allocation ratio without blocking constraints (345). The randomization permitted a balance between two groups in each classroom. Each child was assigned with a color; there were totally 4 colors: two colors for zinc group, and two colors for placebo group. In each classroom, investigators (n = 7) received a list of names of the participants with their assigned color group which were distributed to the children with their corresponding tablets. The tablets for each child were kept in a small plastic container with a label that had the child's name and a colored circle that correspond to the child's assigned color. All study participants and members of the study team were blinded to the treatment code, which was maintained in sealed envelopes at INCAP and the Rollins School of Public Health and was opened at the end of the study after preliminary data analyses had been completed.

3.5. Interventions

The first school began supplementation in late February 2006, and the fifth school began supplementation in early April 2006. Children were supplemented daily (5 days/week) for approximately 5.8 months (125 days) in their classrooms. The zinc supplement or placebo was given in the form of chewable tablets with a fruit flavoring during the morning hours (i.e., between 8:30 to 10:00) and was consumed in the presence of the study staff who administered the supplement and recorded this information. No food was given with the supplement. In cases where a child was expected to be absent from school the next day (e.g., school was not in session, or the child had an appointment), the person giving the supplement gave the child a tablet to be consumed at home the next day and followed up on the child's return to school to assess if the child had consumed the supplement; this information was recorded. Adherence was defined as the number of days the supplement was consumed, observed and un-observed, out of the total number of days of supplementation. The denominator for calculating adherence was based on the date the class began supplementation and the date of the last dose. The dates were the same for all children in a classroom after baseline measurements; the exact number of days varied slightly by class (range 100-128 days) but did not vary by individuals within the classroom.

3.6. Unanticipated and Concurring Intervention

At approximately the time our study began, the local government in the study area implemented a school-based fortified milk program. Children in 4 out of the 5 schools received 200 mL whole milk/day fortified with zinc amino acid chelate to a target concentration of 2.6 mg elemental Zn/200 mL. Analyses in our laboratory showed that the

actual concentration was 1.6 mg/200 mL. The milk also contained 4.7 mg iron (actual amount was 1.12 mg iron on the basis of laboratory analysis), 200 μg vitamin A, 0.8 μg vitamin B-12, and 133 μg folic acid. A tablet of folic acid was also distributed weekly at the schools, although not consistently. Children in the fifth school (n = 47) did not receive the fortified milk but received instead Incaparina (Alimentos SA, Guatemala City, Guatemala) daily, which is a cereal-based drink containing 2.1 mg zinc, 3 mg Fe, 35 μg folic acid, and 56 mg calcium [32]. We did not learn about this program until our fieldwork was well underway and thus, did not collect information on the actual consumption by the children.

3.7. Data-Collection Procedures and Study Variables

Data collection procedures. Baseline data collection occurred from February to early April 2006, supplementation occurred from late February to early October 2006, and follow-up occurred from August to early October 2006. During the 6-month intervention, highly trained workers distributed the supplement to children in the classroom in the early morning and reported the adherence. At the completion of the six-month supplementation, all variables were collected again using similar methods. Information from children and their parents were obtained by interviews at home. Trained interviewers visited each child's home and conducted an interview with the child's mother on her own psychological status, her child's socio-demographic characteristics, psychological and behavior symptoms, and dietary information. When the mother was not present, the information was based on the child's caregiver (only 7% of children at baseline; 6.7% of children at 6-month follow-up). Physical assessments of the children were conducted in 2 separate buildings in the community (one assessment for the health and nutrition data, and one for the psychological data). The buildings were a short driving distance from the study schools. There were several private,

enclosed spaces to conduct various assessments. Children were transported to the buildings. On the first day, children completed a series of psychological questionnaires and cognitive tests that lasted approximately 2 hours. The assessments were administered by trained research staff in a quiet, controlled environment. On the next day, a study physician examined each child for illness, a nurse conducted anthropometric measurements, and another nurse drew 7 mL venous blood using standard procedures into trace element-free Vacutainers (Bectons Dickinson, Franklin Lakes, NJ).

Socio-demographic information of children & mothers. The interview information collected from the primary caregiver included age, sex, school, and grade of the child; age, marital status, education (in years), employment, and language of the mother and father or partner; number of children in the household; and characteristics of the home where the child lives. Socioeconomic status score (SES) was estimated by using principal components' analysis to construct a wealth index on the basis of participants' household possessions (e.g., radio, iron, bicycle, microwave, refrigerator, and television) and housing construction materials (e.g., floor, roof, and walls) by using a method previously used in Guatemala (346).

Anthropometric measurements. The child's anthropometric measurements included weight, height; head, abdominal, and calf circumferences; triceps and sub-scapular skin folds; sitting height, knee-heel length and leg length. They were measured by trained nurses as the mean of 2 repeated times for each measure, using equipment with daily calibration and standardized techniques (347). Weights were measured with clothes to the nearest 0.1 kg by using an electrical scale (Tanita Scale, Tanita Corp, Arlington Heights, IL). Heights in standing and sitting positions were measured to the nearest 0.5 cm by using standard techniques with the Shorr stadiometer (Schorr Industry, Glen Burney, MD). Knee-heel

lengths were measured by a small hand held anthrop meter (Lafayette instrument, Lafayetter, IN) taking the mean of 5 consecutive measurements (resolution 0.01 mm). Lower leg lengths were measured by using mini-kneemometry. Head circumference was measured with a fiberglass tape in the line just above the supraorbital ridges and over the occipital prominence that gave the maximal circumference. Mid-upper arm circumference (MUAC) was measured at the midpoint of the upper left arm with a fiber glass insertion tape. Abdominal circumference and calf circumference were measured by ruler tape with standard techniques. Triceps skin fold (TSF) was measured to the nearest 0.5 mm on the left arm; subscapular skin fold was measured below and laterally to the angle of the left shoulder blade with the shoulder and arm relaxed; both used precision Lange caliper (Cambridge Scientific Industries Inc, Cambridge, MD). All duplicate anthropometric measurements had small within subject variations: technical errors of measurements within the reference values set by Frisancho (347), coefficients of reliability ranged from 0.97% to 1.0 (median 1), very small deattenuated fraction (maximal 0.01, median 0.01).

Standardized Z scores of height-for-age (HAZ), weight-for-age (WAZ), weight-for height (WHZ), and body mass index (BMIZ) were calculated based on weight, height, age in month, and gender, using the WHO AnthroPlus software of the World Health Organization for children 5-19 years of age (348).

Dietary assessments. Children's dietary intakes were reported by the mother or caregiver using a semi-quantitative food frequency questionnaire (FFQ). The FFQ included 104 food items. Daily nutrient intakes of energy, zinc, and other nutrients in the FFQ were calculated using the Institute of Nutrition of Central America and Panama (INCAP) food composition tables. Nutrient intakes by FFQ were adjusted for total energy intake using the

calorie residual method (169). The FFQ was validated in a separate study with 50 children, comparing results from the FFQ with three 24-hour dietary recalls. Correlation coefficients between dietary residual zinc intakes from FFQ intakes and average 24-hour recall intakes were 0.33 and 0.41; the slopes of residual zinc intakes from 24-hour recall method by FFQ method were 0.37 and 0.34 respectively, at baseline and 6-month follow-up.

Biochemical measurements. Trained nurses collected non-fasting peripheral venipuncture blood samples, a total of 7 ml, in the morning, approximately 2 hours after eating (between 9am and noon) in trace element-free vacutainers (Becktons Dickinson, Franklin Lakes, NJ), using standard procedures. One drop of blood was used to measure hemoglobin concentration on site. The remaining blood was quickly centrifuged at 2500 x g for 10 minutes at room temperature, aliquoted, and refrigerated at the school; frozen in trace element-free cryogenic vials at -20°C at the Institute of Nutrition in Central America and Panama (INCAP) in Guatemala City; then shipped in nitrogen liquid, stored at -70°C, and analyzed at the National Institute of Public Health (INSP) laboratories, Cuernavaca, Mexico.

Hemoglobin concentrations were measured with the use of the Hemocue® B-Hemoglobin machine (Hemocue Inc., Mission Viejo, CA). Serum C-reactive protein concentration was measured by a high-sensitive laser nephelometry with an antiserum specific for CRP (Behring Nephelometer 100 Analyzer, Behring Laboratories, Messer Grisheim Gmbh, Frankfurt, Germany). Serum zinc and copper concentrations were assessed with flame atomic absorption chromatography spectroscopy using a graphite furnace (Analyser 300, Perkin Elmer Co Norwalk, CT USA). Serum ferritin concentration was measured by an automatized immune assay (Dade Behring Inc., Newark, DE 19714, USA). Serum alkaline phosphatase activity was measured by the hydrolysis of p-nitrophenyl

phosphate at 37°C (CE Human, Bisbaden, Germany). Serum albumin concentration was analyzed by a colorimetric method using kits from Human Gesellschah fur Biochemica und Diagnostica, Wiesbaden, Germany, using a spectrophotometer Prestige 24i (Tokyo Boeki Medical System, Tokyo, Japan). The accuracy of the determination was assessed by certified material from the US National Institute of Standard and Technology and the UK National Institute of Biological Standard and Control. Precision was assessed through duplicated measurements on 20% of the sample. The between-assay coefficients of variations for serum CRP, zinc, ferritin, copper, albumin and phosphatase alkaline were 5.29%, 4.77%, 1.54%, 6.34%, 4.80% and 3.43%, respectively.

Psychological measurements. Psychological measures were multiple; including child self-reported measures of symptoms of depression and anxiety, and parental reports on a variety of behavioral symptoms. The Spanish versions of the measures were tested in a pilot study, and the items were tested in this community of children and parents in Guatemala. Measures of internal consistency (coefficient α) were calculated for the measures and subscales; all internal coefficients were adequate (ranged from 0.61 to 0.86). However, in order to focus on the objectives of this dissertation, they were not addressed.

3.8. Definitions of Categorical Variables

Continuous variables were categorized, mostly dichotomized, by specific cutoffs or by conventional definitions.

- Serum CRP concentration was categorized by CRP cutoffs of 0.5, 1, 3, 5, and 10 mg/L into six intervals as 0.5-1 mg/L, >1-3 mg/L, >3-5 mg/L, >5-10 mg/L, and >10

mg/L. Then, it was dichotomized into high (>CRP cutoff) versus low (≤CRP cutoff) levels corresponding to each CRP cutoff. The cutoffs were chosen based on previous studies and the distribution of CRP on healthy US children (186, 246, 266, 283, 294).

- Low serum zinc level was defined as serum zinc concentration < 65 μg/dL (58, 114, 349).

- Low serum ferritin levels were defined as serum ferritin concentration < 15 μg /L (35, 350).

- Low serum copper level was defined as serum copper < 90 μg/L (351).

- Anemia was defined as hemoglobin <115g/L (350).

- Low albumin was defined as albumin concentration <3.8 g/dL (352).

- Low dietary zinc intake (dietary zinc intake inadequacy) was defined as the energy-adjusted zinc intake less than the estimated average zinc requirement (EAR)-- 4 and 7mg/d for children 4-8 years and 9-13 years of age, respectively-- based on the IZiNCG recommendation for high-phytate, unrefined cereal-based diets (58).

- For serum ALP, albumin, and the zinc: albumin ratio, low levels were defined as less than the medians.

3.9. Sample Size

An initial sample size of 750 children was calculated for the power to detect differences between 2 groups in regard to psychological outcomes. In theory, sample size

calculations for comparing means are based on the expected effect size, power to detect desired differences, and level of significance.

$$n = 2\sigma^2 (z_\alpha + z_{\beta/2})^2 / d^2 = 2 (z_\alpha + z_{\beta/2})^2 / EF2 \quad (353)$$

Where n = sample size per group (2n for total sample size),

EF = effect size = difference in the means between treatment and placebo groups divided by the pooled standard deviation (SD) of the measure interest = d/σ

α = level of significance (e.g., 0.05, $z_{\alpha/2} = 1.96$)

β = power to detect desired difference (e.g., $\beta = 0.80$, $z_{\beta/2} = 1.28$)

For psychological outcomes, several studies of depression and anxiety reported effect sizes larger than 0.50 SD (354-356). A meta-analysis of studies on the efficacy of tricyclic drugs in treating children and adolescent depression noted a pooled effect size of 0.35 SD (356). A study examining effects of zinc supplementation on symptoms of depression and anxiety in patients with anorexia nervosa observed moderate or large effect size of 0.67 to 1.17 SD (357). However, most of the studies were performed with children referred for mood disorder or anxiety programs; thus, dealt with children at the more severe stage. In our healthy participants, the average severity of symptoms may be lower than in previous studies, the variability along the continuum of depression and anxious symptoms may be higher. Therefore, the expected effect size may be lower than the reported results, and may be expected as 0.25 SD. For an expected effect size of 0.25 SD, a 2-tail test at $\alpha = 0.05$, and a power of 0.8, the sample size would be 310 per group, or 620 children in total. With approximately 20% missing data, the initial recruited number was 750 children.

For the secondary biochemical outcomes in post-hoc power calculations, given the standard deviations of serum zinc, ferritin, and copper as 12, 25, 12 μg/dL, the remaining sample size of 720 children could detect differences between 2 groups as small as 1.5 μg/dL, 5.2 μg/L, and 2.5 μg/dL, respectively, for serum zinc, ferritin, and copper concentrations; with a 2-tailed alpha of 0.05, power of 0.80.

3.10. Statistical Methods

Missing data. Data were entered and checked for accuracy using Epi-Info 2004. Incorrect entries and missing data were checked and verified on a systematic basis using range checks for values beyond permissible values (outliers) and missing values. Outliers and missing values were set to missing. Particularly, the measurements of serum copper concentrations at baseline were not available for many blood samples due to technical problems. To deal with missing data, we used complete-case analyses, which included only individuals with no missing data on key outcomes depending on study hypotheses. Data available for the analyses are summarized in **Table 3.1**.

Flow chart of participants. The flow chart of participants was based on the CONSORT statement (7), and is presented in **Figure 3.1**. A total of 800 children were screened; of whom 30 (3.7%) children did not meet inclusion criteria and a further 20 (2.5%) children refused to participate. The remaining 750 eligible children, aged 6-11 years, at baseline were randomly assigned to the zinc or placebo groups. Of the 750 children, 30 (4.0%) children never received treatment or completed the baseline assessment. Of the 720 children who received at least one tablet, 6 (<1.0%) children were lost to follow-up (because of a change of address or parental refusal), which left a sample of 715 children, who finished the

intervention program and were present at the 6-month follow-up. However, biochemical outcomes were not collected for all the children because of either failures in obtaining blood samples or technical problems in measuring nutrients in the laboratory. For the analysis in paper 1 (chapter 4), key outcomes including serum CRP, zinc, ferritin, and copper concentrations at baseline were completed in 634 participants. For the analysis in paper 2 (chapter 5), key outcomes that consisted of serum zinc, alkaline phosphatase, albumin concentration and dietary zinc intake (by FFQ) were completed at baseline in 691 and at six-month follow-up in 659 participants. For the analysis of paper 3 (chapter 6), key outcomes that included serum zinc, ferritin, and copper at both baseline and 6-month follow-up were completed in 672 participants.

General statistical methods. Statistical analyses were performed using SAS for Window 9.2 (SAS Institude Inc., Cary, NC, USA). All continuous variables were tested for normality using the Kolmogorov-Smirnov test and plots of normality. Nutrient concentrations that were associated with serum CRP concentrations were adjusted for high CRP levels using correction factors that were ratios of geometric means of nutrients in the low CRP level by the high CRP level. Dietary zinc intakes measured by the food frequency questionnaire were adjusted for total calorie intakes by residual regression method following Willet (10). Distributions of variables were described by mean (standard deviation) for continuous variables with normal distribution, by median (25^{th} percentile, 75^{th} percentile) for continuous with non-normal distribution, and frequency (percentage) for discrete variables. In the stratified analysis, the outcomes were compared across subgroups. Differences of continuous variables were tested by Student's t test or Wilcoxon's sign ranked test for variables with normal or non-normal distributions, respectively. Differences with discrete

variables, mainly binary ones, were tested by chi-square test or Fisher's exact test (if frequencies < 5 in any cell of contingency tables). Associations between 2 continuous variables were measured by correlation coefficients using Pearson's or Spearman correlation for variables with normal or non-normal distributions, respectively. Associations between 2 binary variables were measured by odds ratios. Agreement between 2 binary variables was measured by Cohen's Kappa agreement. Adjustments for confounding effects by covariates were performed by multiple linear or logistic regressions. Covariates for the analysis were gender and age (in months) or age groups (<9 versus ≥9 years of age, based on the IZiNCG (58)). Mixed generalized linear models (PROC GLIMMIX in SAS) were used to adjust associations between the changes in specific nutrient concentrations and treatment groups for autocorrelations of repeated measurements and covariates. The estimates were furthermore adjusted for measurement errors using deattenuated factors for repeated measurements in biomarkers and the slope of linear regressions between dietary zinc intakes obtained by the food frequency questionnaires and those from three 24-hour recalls in validation studies (169, 358). Statistical significances were defined as P-value <0.05.

Specifically, statistical methods depended on specific hypotheses of each paper.

- Paper 1 (chapter 4): We assessed correlations between serum CRP concentration and nutrient concentrations using Spearman's correlation coefficients. Specific nutrients with significant correlations, mainly serum ferritin and copper concentrations, were further explored. First, we stratified nutrient concentrations across categories of CRP levels (i.e., 0-0.5, >0.5-1, >1-3, >3-5, >5-10, and >10 mg/L). Global differences in nutrient concentrations among all levels were assessed by Kruskall-Wallis tests. Pairwise differences in concentrations between the CRP 0-0.5 mg/L levels and higher CRP levels were assessed

by Wilcoxon tests. Next, for each CRP cutoff of 0.5, 1, 3, 5, and 10 mg/L, we defined a high CRP level and a low CRP level (≤CRP cutoff vs. >CRP cutoff) and assessed their differences in specific nutrient concentrations and the prevalence of low nutrient levels by Wilcoxon tests for continuous variables and Fisher's exact-tests for binary variables. Finally, we adjusted the specific nutrient concentrations using correction factors based on each CRP cutoffs, which were factors are ratios of geometric means of the nutrients in the reference group to those in the elevated group (246). Then we calculated the averages of nutrient concentrations and their low nutrient prevalence after adjustment and compared with those before adjustment or after exclusion of CRP >10 mg/L.

- Paper 2 (chapter 5): Crude associations between continuous variables were measured by Pearson's correlation coefficients. Crude associations between binary variables were assessed by Chi-square statistic tests. Agreement between binary variables was assessed by Cohen's Kappa statistic. Based on the Cohen's Kappa statistic, two binary variables were classified as having no (Kappa < 0), slight (Kappa = 0-0.2), fair (Kappa = 0.21-0.4), moderate (Kappa = 0.41-0.6), substantial (Kappa = 0.61-0.8) or almost perfect (Kappa = 0.81-1) agreement (359). The estimated coefficients were adjusted for age and gender, by multiple linear regression models for continuous outcomes, and multiple logistic regression models for binary outcomes. We checked for two-term interactions between each variable and age groups (<9 years vs. ≥9 years) and gender by linear or logistic regression models. Estimated coefficients of determinants were standardized for the sake of comparison. Furthermore, the crude and adjusted estimates (i.e., correlation coefficients and odds ratios) were adjusted for measurement errors using deattenuated factors for measurements of

biomarkers and slopes of linear regression in validation studies for measurements of dietary zinc intakes as described by Willett (169).

Paper 3 (chapter 6): To evaluate the effectiveness of randomization, we compared the zinc and control groups on baseline characteristics. To assess the effect of zinc supplementation, we compared the six-month changes (final - initial) of outcome variables between two treatment groups. Serum ferritin and copper concentrations related to inflammation were adjusted for high CRP levels (>5 mg/L versus ≤5 mg/L) using correction factors. In crude analysis, the comparisons of variables between treatment groups were performed by Student's t-test for continuous variables and Fisher's exact test for binary variables. In multivariate analysis, we adjusted for correlations of repeated outcomes using random effects models with generalized linear mixed regression (SAS GLIMIX procedure, SAS version 9.2, SAS Inc., Cary, NC) for both continuous and binary outcomes. Initially, we assessed the associations between the changes in biochemical concentrations or their low levels after supplementation and supplementation group. Then, given the occurrence of the unanticipated local school milk program in both groups, we assessed the associations between the changes in serum zinc concentrations after supplementation and the changes in other biochemical concentrations. All analyses were adjusted for age (in months) and gender.

Tables and Figures in Chapter III

Tables 3.1. Data availability of selected variables at baseline and 6-month follow-up (Total N = 720)

Variables	Baseline		Follow-up	
	Available	%	Available	%
Age	720	100.0%	720	100.0%
Gender	720	100.0%	720	100.0%
Intervened group	720	100.0%	720	100.0%
SES	717	99.6%	717	99.6%
Grade	720	100.0%	720	100.0%
Weight	720	100.0%	715	99.3%
Height	718	99.7%	713	99.0%
Dietary zinc intake	704	97.8%	671	93.2%
Serum CRP	710	98.6%	677	94.0%
Serum zinc	710	98.6%	677	94.0%
Serum ferritin	710	98.6%	676	93.9%
Serum copper	634	88.1%	635	88.2%
Serum ALP	709	98.5%	676	93.9%
Serum albumin	708	98.3%	677	94.0%
Hemoglobin	711	98.8%	678	94.2%

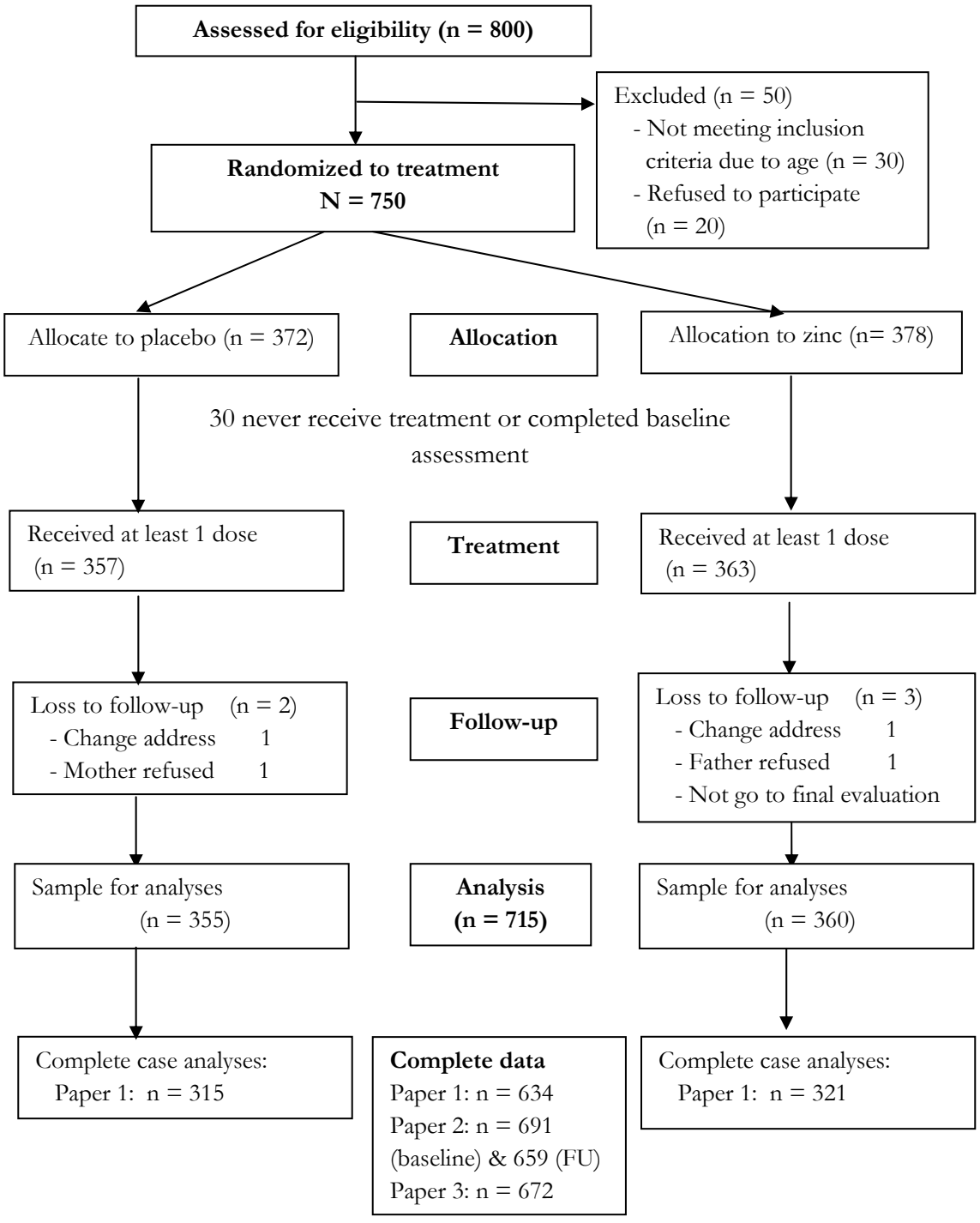

Figure 3.1. Participant flow (on the basis of guidelines for randomized controlled trial of the CONSORT (Consolidated Statement of Reporting Trials) (7)

CHAPTER IV

MEASUREMENTS OF SERUM FERRITIN AND COPPER, BUT NOT ZINC, ARE INFLUENCED BY C-REACTIVE PROTEIN LEVELS IN GUATEMALAN SCHOOL CHILDREN

Measurements of Serum Ferritin and Copper, but Not Zinc, Are Influenced by C-Reactive Protein Levels in Guatemalan School Children

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[c] Abbreviations: CRP, C-reactive protein; hs-CRP, high-sensitive C-reactive protein; AGP, alpha-1 acid glycoprotein.

ABSTRACT

Background: Inflammation may affect biomarkers of trace element status but its effect in healthy children are inconclusive. We assessed the association between serum C-reactive protein and biomarkers of zinc, iron, and copper; and estimated the magnitude of the bias for significant associations. **Methods:** We measured serum CRP (using a high-sensitive method with detection limit of 0.1 mg/L), zinc, alkaline phosphatase, albumin, ferritin, copper, and hemoglobin in 634 healthy 6-11 year old Guatemalan school children. We examined Spearman's correlations between CRP and nutrient concentrations. For significant associations, we stratified nutrient concentrations across categories of CRP, compared their concentrations above and below several CRP cutoff points of 0.5, 1, 3, 5, and 10 mg/L, and adjusted them using correction factors as ratio of geometric means. **Results:** The prevalence of low serum zinc < 65 μg/dL, ferritin <15 μg/L, and copper <90 μg/dL were 21%, 2.1%, and 23.8%. Serum CRP median (25^{th}, 75^{th} percentiles) was 0.56 (0.26, 1.54) mg/L. CRP concentration was positively associated with ferritin and copper concentrations ($r = 0.23$ and 0.29, respectively; $p < 0.0001$); but not with serum zinc and other biomarkers ($p > 0.05$). Regardless of CRP cutoffs, high (>cutoff) vs. low (≤cutoff) CRP levels had higher ferritin concentrations, higher copper concentrations, and lower prevalence of copper deficiency <90 μg/dL ($p < 0.05$). Adjustment for inflammation had the greatest influence on recalculated prevalence in the case of the CRP 0.5 mg/L cutoff. The low ferritin prevalence hardly changed (from 2.1% to 2.5%) while the low copper prevalence changed more (from 23.8% to 31.2%).

Conclusions: Serum CRP was positively associated with ferritin and copper, but not with zinc. Given the very low prevalence of iron deficiency, the adjustment had little effect on low ferritin prevalence, but large impact on low copper prevalence.

INTRODUCTION

Inflammation induces transient changes in the biomarker concentrations of zinc, iron, and copper status (1, 2). Even apparently healthy children may have elevated inflammatory markers (3, 4). However, the influences of inflammation on those biomarkers in healthy children are heterogeneous and inconclusive. The impact of inflammation on serum ferritin is very strong and consistently demonstrated in many studies because it is an acute phase protein (APP) of the inflammation process (5-8). The influence of inflammation on serum zinc and copper concentrations may be weaker (9). Some studies found significant associations between inflammation and serum zinc concentrations in community-based populations (5, 10), while other studies reported no association (6, 11-13). Some studies investigated the association between inflammation and serum copper concentration in hospitalized patients and adult volunteers (14-17); but, to our knowledge, no study has assessed the association in apparently healthy children.

In addition, evaluating the magnitude of inflammation on specific nutrient biomarkers, especially serum ferritin, in apparent healthy children is difficult. The WHO/CDC recommends the measurement of one or many APPs, such as CRP, alpha-1-acid glycoprotein (AGP), and alpha-1-antichymotrypsin, together with serum ferritin (18). Several community-based studies measure CRP alone with different cutoff points (8). Traditional methods can detect only CRP concentrations >3 mg/L (19). Most studies consider CRP >10 mg/L as inflamed (5, 6), which corresponds to the fact that 99% of healthy adults have CRP <10 mg/L (20). Thurnham *et al.* suggested using the CRP cutoff of 5 mg/L because CRP values >5 to 10 mg/L probably indicate mild inflammation (21). The American Heart Association defined the risk of inflammation on cardio-vascular diseases as

average (CRP 1 to 3 mg/L) and high (CRP >3 mg/L) (22). Abraham *et al* demonstrated that inflammation may influence nutrient concentrations at levels as low as CRP >0.6 mg/L (75^{th} percentile) (7). The 60^{th} and 75^{th} percentiles of healthy US children and young adults were 0.5 mg/L and 1 mg/L, respectively (23). Therefore, the use of lower CRP cutoffs may yield a better reference group of healthy individuals and improve the adjustment of nutrient concentrations for inflammation.

This paper aims to investigate associations between serum CRP and biomarkers of zinc, iron, and copper, and to assess effect of inflammation on nutrient concentrations and prevalence of low status where the associations are significant. We measured CRP concentration using a high-sensitive method with a detection limit as low as 0.1 mg/L (24), which permits the use of a range of CRP cutoffs from 0.5, 1, 3, 5, to 10 mg/L to adjust for inflammation and to assess the magnitudes of bias on specific nutrient biomarkers.

METHODS

Study design and population

We analyzed data collected from school-aged children in a low-income community in Guatemala City, Guatemala in 2006 (25). The study was a collaborative effort between the Hubert Department of Global Health at the Rollins School of Public Health (RSPH), Emory University, Atlanta, GA, and the Institute of Nutrition of Central America and Panama (INCAP), Guatemala City. The study protocol was reviewed and approved by the ethics committees of both institutions.

We recruited children in grades 1-4 (6-11 years old) from 5 public schools in San Jose la Comunidad, a low-income area in Guatemala City, Guatemala. Exclusion criteria

included children with any known severe illness affecting zinc status (e.g., sickle cell disease, cystic fibrosis, renal or liver disease, severe burns, or acrodermatitis enteropathica) (26), and other severe or chronic illness (e.g. cancer, diabetes, or seizures). Parental informed consent and child assent were obtained before data collection.

Data collection procedures

Data were collected from February to April 2006. Blood samples were obtained between 9am and noon, approximately 2 hours after eating. Following standard procedures, a nurse drew 7 mL venous blood into trace element-free Vacutainers (Bectons Dickinson, Franklin Lakes, NJ). One drop of blood was used to measure hemoglobin concentration with the use of the HemoCue® B-Hemoglobin machine (Hemocue Inc., Mission Viejo, CA). The remaining blood was quickly centrifuged at 2500 x g for 10 minutes at room temperature, aliquoted, refrigerated at the school; frozen in trace element-free cryogenic vials at -20°C at the Institute of Nutrition in Central America and Panama (INCAP) in Guatemala City; then shipped in liquid nitrogen, stored at -70°C, and analyzed at the National Institute of Public Health (INSP) laboratories, Cuernavaca, Mexico.

Serum C-reactive protein was measured by a high-sensitive laser nephelometry with an antiserum specific for CRP (Behring Nephelometer 100 Analyzer, Behring Laboratories, Messer Grisheim Gmbh, Frankfurt, Germany). Serum zinc and copper concentrations were assessed with flame atomic absorption chromatography, using graphite furnace (Analyser 300, Perkin Elmer Co Norwalk, CT USA). Serum ferritin concentration was measured by an automatized immune assay (Dade Behring Inc, Newark, DE 19714, USA). Serum alkaline phosphatase activity was measured by the hydrolysis of p-nitrophenyl phosphate at 37°C

(CE Human, Bisbaden, Germany). Serum albumin concentration was analyzed by a colorimetric method using kits from Human Gesellschah fur Biochemica und Diagnostica, Wiesbaden, Germany, using a spectrophotometer Prestige 24i (Tokyo Boeki Medical System, Tokyo, Japan). The accuracy of the determination was assessed by using certified material from the US National Institute of Standard and Technology and the UK National Institute of Biological Standard and Control. Precision was assessed through duplicate measurements on 20% of the sample. The between-assay coefficients of variation for serum CRP, zinc, ferritin, copper, albumin and phosphatase alkaline were 5.29%, 4.77%, 1.54%, 6.34%, 4.80% and 3.43%, respectively.

Definitions of categorical variables

Continuous variables were categorized using study-specific cutoffs or conventional definitions. Serum CRP concentrations were categorized into six intervals using cutoff points: 0.5, 1, 3, 5, and 10 mg/L. The values were based on previously suggested CRP cutoffs and the range of CRP values in normal healthy US children and young adults (5, 7, 21-23). Low serum zinc level was defined as serum zinc concentration <65 μg/dL (27-29). Low serum ferritin level was defined as serum ferritin concentration <15 μg/L as suggested by the WHO for the assessment of iron deficiency in children >5 years of age (18). Low serum copper level was defined as serum copper concentration <90 μg/L (30). Anemia was defined as <115 mg/L (18).

Statistical analyses

Only data from children with complete measurements for serum CRP and key outcome variables (i.e., serum zinc, ferritin, and copper concentrations) were analyzed. We

assessed normality using the Kolmogorov-Smirnov test. Serum CRP and ferritin were not normally distributed. Continuous variables were reported as means (standard deviations) and medians with interquartile ranges (25^{th}, 75^{th} quartiles) for normal and non-normal distributions. Categorized variables were described as frequencies (percent). We assessed correlations between serum CRP and nutrient concentrations using Spearman's correlation coefficients. Significant correlations (i.e., serum CRP-ferritin concentrations and serum CRP-copper concentrations) were further explored. First, we stratified nutrient concentrations across categories of CRP levels (i.e., 0-0.5, >0.5-1, >1-3, >3-5, >5-10, and >10 mg/L). Global differences in nutrient concentrations among all levels were assessed by Kruskall-Wallis tests. Pairwise differences in concentrations between the CRP 0-0.5 mg/L levels and higher CRP levels were assessed by Wilcoxon tests. Next, for each CRP cutoff of 0.5, 1, 3, 5, and 10 mg/L, we defined a high CRP level and a low CRP level (≤CRP cutoff vs. >CRP cutoff) and assessed differences in specific nutrient concentrations and the prevalence of low nutrient levels by Wilcoxon tests for continuous variables and Fisher's exact-tests for binary variables. Finally, we adjusted the specific nutrient concentrations using correction factors based on each CRP cutoffs, which are ratios of geometric means of the nutrients in the reference group to those in the elevated group (21). We then calculated the averages of nutrient concentrations and their low nutrient prevalence using correction factors and compared them with those before adjustment or after exclusion of cases with CRP >10 mg/L.

SAS version 9.2 (SAS institute) was used for all analyses. We used $p < 0.05$ as the criterion of statistical significance. The resulting sample of 634 children allowed us to detect

a correlation as low as 0.11 between CRP and any nutrient indicator, given a type-1 error α of 0.05, a power of 0.80, using POWER procedure in SAS 9.2 version.

RESULTS

Characteristics of the study population

Six hundred and thirty four children had complete data on serum CRP, zinc, ferritin, and copper and were included in analyses. Half of the children (51%) were males and the mean (SD) age was 9.0 (1.2) years (**Table 4.1**). The prevalence of stunting was 15% (n = 96) and the prevalence of serum zinc <65 μg/dL, ferritin <15 μg/dL, and copper <90 μg/dL was 21.0%, 2.1%, and 23.8%, respectively. Only 2 (0.3%) children had hemoglobin <11.5 g/dL, and none had serum albumin <3.5 g/dL. The median (25^{th}, 75^{th} quartiles) serum CRP was 0.57 (0.26, 1.59) mg/L. There were 17 (2.7%) and 47 (7.4%) children with CRP concentrations <10 and <5 mg/L, respectively.

Association between serum CRP concentration and biochemical concentrations

Serum CRP concentration was not associated with serum zinc, alkaline phosphatase, albumin, and hemoglobin concentrations (p >0.05) (**Table 4.2**). Serum CRP concentration, however, was associated with serum ferritin and copper concentrations (r = 0.23 and 0.29, respectively; both p <0.0001).

Associations of serum ferritin and copper concentrations/levels with CRP levels

Stratified by CRP intervals, ferritin and copper concentrations gradually increased from the lowest to the highest CRP intervals (**Table 4.3**). Concentrations varied across CRP intervals (p<0.0001); higher CRP intervals had ferritin and copper concentrations higher

than the lowest CRP interval (0-0.5 mg/L). Categorized in to low and high CRP levels (≤cuttof vs. >cutoff), high CRP levels had increased ferritin and copper concentrations compared to low CRP levels for any CRP cutoff from 0.5 mg/L to 10 mg/L ($p < 0.001$) (**Table 4.4**).

Similar analyses using the prevalence of serum copper <90 μg/dL as the outcome also showed significant differences between high and low CRP levels ($p < 0.05$), except for CRP 10 mg/L (**Table 4.5**). However, there were no significant difference when the outcome was low serum ferritin prevalence <15 μg/L ($p > 0.05$).

Adjustment of ferritin and copper measurements by correction factors

We compared the medians and prevalence of serum ferritin and copper before and after adjustment for inflammation using different adjustment methods and CRP cutoffs (**Table 4.6**). Before any adjustment, the median concentrations of serum ferritin and copper were 47.4 μg/L and 101 μg/dL, respectively; the prevalence of ferritin <15 μg/L and copper <90 μg/dL were 2.1% and 23.8%, respectively. Exclusion of cases with extreme CRP values >10 mg/L resulted in small changes (medians 46.6 μg/L and 100.8 μg/dL, prevalence 2.1% and 24.2%, respectively for ferritin and copper). Adjustment by correction factors had a larger impact compared to the exclusion method; lower CRP cutoffs resulted in greater changes than higher CRP ones. The changes were maximal with the CRP 0.5 mg/L cutoff. Overall, adjustment for inflammation was negligible to iron status (low ferritin prevalence changed from 2.1% to 2.5%), but was substantial for copper status (low copper prevalence changed from 23.8% to 32.1%).

DISCUSSION

Summary. The children in this study had low levels of inflammation, good iron status, but high prevalence of low serum zinc. We did not find any significant association between serum CRP and biomarkers of zinc status, but demonstrated significant and positive associations of serum CRP with ferritin and copper concentrations. Subsequently, for any CRP cutoff, high CRP levels had higher serum ferritin, higher copper concentrations, and lower prevalence of low copper than low CRP levels. The adjustment for inflammation achieved maximal effects with the use of CRP cutoff of 0.5 mg/L. Adjustment for inflammation had small effect on the prevalence of low ferritin, but a greater effect impact on the prevalence of low copper.

Low prevalence of inflammation. Only 2.7% and 7.4% of the children had elevated CRP concentrations (>10 mg/L and 5 mg/L). This may reflect a low risk of acute infections in the sample of healthy 6-11 year old urban children. Previous studies about inflammation recruited participants from populations with very high risks of infections. For examples, some studies indicated 14.6% of Indonesian infants with CRP >10 mg/L, 13.9% of Bangladeshi 3-7 year old children, 23% of Peruvian 11-19 months old children, and 50% of HIV-1 seropositive Kenyan adults (5, 31, 32). These studies demonstrate an important effect of inflammation on average concentrations or prevalence of nutrient status, particularly serum ferritin (5, 6, 33).

Problems with the assessment of serum CRP-nutrient associations. Even with high prevalence of elevated CRP levels, these studies have not detected significant linear associations (i.e., Pearson or Spearman correlations) between serum CRP concentration and any trace nutrient

concentrations (5, 6, 32, 33). Few studies investigated the association between serum CRP concentration and nutrient concentrations (13). One study reported non-significant correlations between serum CRP concentrations and ferritin or zinc concentrations in samples of children with a very high prevalence of CRP >10 mg/L (72%) (13). Most studies investigated the associations between high CRP levels (e.g., >5, >10 mg/L), not serum CRP concentrations, and the concentrations of serum zinc, ferritin, or copper (5, 6, 15, 21).

Lack of evidence for significant linear associations between serum CRP concentration and nutrient concentration in spite of significant associations between serum CRP levels and nutrient concentrations (e.g., ferritin) may be related to poor measurements of CRP concentrations. Two recent studies that reported significant correlations between plasma CRP and nutrient concentrations in infants and children used high-sensitive CRP methods (34, 35). Traditional methods have low sensitivity and can detect only CRP concentrations >3 mg/L (19). Therefore, using a high-sensitive CRP method which can measure CRP concentration as low as 0.1 mg/L (36), one can test associations between CRP and biomarkers of nutrients with greater precision.

Associations of CRP with nutrient concentrations. In general, our findings agree with the previous studies. We found a null association between serum CRP and zinc concentrations. In healthy children, previous studies observed that serum zinc concentrations were not different in Peruvian young children with signs, compared with no signs, of clinical infections (6); in Guatemalan children with elevated white blood cells or sedimentation rate, compared with healthy children (11); and in Zimbabwean school children with high CRP levels, compared with low CRP levels (12). There was no association between serum CRP and zinc concentrations in 72 low-income US children, although 72% had elevated CRP

(>10 mg/L) (13). Previous studies consistently observed high ferritin concentrations in high CRP levels among healthy children and adults (5-7, 37). Two studies reported significant correlations between plasma CRP and ferritin concentrations in infants and children (34, 35). The positive association between serum CRP levels and copper concentrations has been demonstrated in many human populations, such as hospitalized patients (16, 17), adult volunteers (9, 38), and healthy adults (14, 15), and children with clinical signs of infection (6). However, the present study was the first to investigate the effect of inflammation on serum copper using high-sensitive CRP methods and to show a positive association between serum CRP and copper concentrations in healthy children.

While studies measuring serum CRP by traditional CRP methods observed differences in ferritin concentrations between binary CRP levels dichotomized by CRP cutoffs of 10 mg/L, 5 mg/L or 3 mg/L (5, 21, 31, 37), we observed significant differences in serum ferritin concentrations and copper concentrations between high CRP levels and low CRP levels, as low as 0.5 mg/L. Subsequently, we observed significant differences in low copper prevalence, but not in low ferritin prevalence between CRP levels, probably due to high prevalence of low copper levels in children (23.8% before adjustments) but very low prevalence of low ferritin levels (only 2.1% before adjustment). Therefore, in this population, only the prevalence of low copper needed adjustment.

Implications. Our findings may have important implications in the assessments of iron and copper status. The use of low CRP cutoffs (e.g., 0.5 mg/L) may increase the ability to adjust for inflammation in apparent healthy children, especially when CRP is used as the only biomarker for inflammation. Thurnham *et al.* performed a meta-analysis to adjust ferritin concentrations for inflammation on 32 studies among children and adults (n = 8796

individuals) (21). The authors suggested that compared to the reference subgroup with normal CRP and AGP (i.e., CRP ≤5 mg and/or AGP ≤1 g/L), the prevalence of iron deficiency was underestimated by 14% if no adjustment, 5% if adjustment for CRP > 5 mg/L, and 9% if adjustment for AGP >1 g/L (21). They concluded that CRP had more impact than AGP in adjustment for inflammation (21). The use of lower CRP cutoffs (e.g., 0.5 mg/L) to define reference CRP subgroups can improve the estimation of prevalence of low ferritin levels. Because low reference CRP subgroups may not be "completely normal" with some inflamed individuals, the ferritin concentrations in those subgroups are elevated and therefore, the adjusted prevalence of iron deficiency is still underestimated.

Limitations. This study may have several limitations. First, there is no consensus on how to adjust for inflammation. Therefore, the true prevalence of iron deficiency is unknown and our estimations are assumptive. Second, there are no agreements on cutoff points of CRP or APPs for inflammation in the assessments of ferritin levels. Therefore, we need to compare the estimates based on the adjustment with low CRP cutoffs with those using other APPs. Third, we used CRP as the only biomarker of inflammation, which are less sensitive in chronic infections (1). Adjustment using one biomarker of inflammation may provide lower precision compared to using 2 biomarkers such as CRP and AGP. Fourth, because serum copper is not a sensitive biomarker for copper status in marginal copper deficiency (39-41), the adjustment of serum copper concentration by inflammation may have limited impact in the assessment of copper status.

In conclusion, CRP concentration was not associated with biomarker concentrations of zinc status (i.e., serum zinc, ALP, and albumin), but was related with serum ferritin and copper concentrations in healthy school children. High CRP levels, defined by low cutoff

points as low as 0.5 mg/L, can influence ferritin and copper concentrations. With very low risk of iron deficiency in participants, the adjustment for inflammation had little influence on the prevalence of iron deficiency. The adjustment had a strong impact on the prevalence of low copper levels. Further studies are required to compare the accuracy of using low CRP cutoffs with other methods, combining several APPs in the adjustment for inflammation.

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Table 4.1: Demographic, nutritional, and biochemical characteristics of school children in Guatemala 2006 (n=634)[1]

Age, year	9.0 (1.2)
Gender	
Boy	322 (50.8%)
Girl	312 (49.2%)
Height-for-age Z-score	-0.64 (-1.25, 0.07)
Serum CRP mg/L	0.56 (0.26, 1.54)
CRP <10 mg/L	17 (2.7%)
CRP <5 mg/L	47(7.4%)
Serum zinc, μg/dL	74.5 (66.5, 83.5)
Zinc < 65 μg/dL	133 (21.0%)
Serum ferritin, μg/L	47.37 (33.84, 63.05)
Ferritin < 15 μg/L	13 (2.1%)
Serum copper, μg/dL	101.04 (90.98, 110.81)
Copper < 90 μg/dL	151 (23.8%)
Serum alkaline phosphatase, IU/L	555.5 (469.0, 625.7)
Serum albumin, g/L	58.9 (56.7, 61.8)
Hemoglobin, g/L	137.5 (132.5, 143.0)

[1] Values are mean (SD), median (25th, 75th), or number (%).

Table 4.2: Spearman's correlations between serum C-reactive protein and biochemical variables (n = 634)

Pair of variables	Spearman coefficient	p
CRP-zinc	0.05	0.23
CRP-ferritin	0.23	<0.0001
CRP-copper	0.29	<0.0001
CRP-alkaline phosphatase	0.03	0.5
CRP-albumin	-0.01	0.77
CRP-hemoglobin	0	1

Table 4.3: Serum ferritin and copper concentrations within CRP intervals (n = 634)

CRP intervals	n	Ferritin µg/L	Copper µg/dL
0-0.5 mg/L	285	44.5 (31.7, 57.4) [2]	96.4 (86.8, 105.6) [2]
>0.5-1mg/L	137	44.9 (32.8, 64.0)	103.4 (92.5, 113.1) [c]
>1-3 mg/L	116	49.2 (34.9, 67.6) [a]	104.0 (95.4, 113.4) [c]
>3-5 mg/L	49	51.0 (40.6, 66.8) [b]	105.0 (93.5, 112.4) [c]
>5-10 mg/L	30	56.3 (40.4, 98.1) [c]	106.9 (98.3, 113.1) [c]
>10 mg/L	17	81.1 (62.7, 100.4) [c]	111.2 (104.2, 119.0) [c]
p[3]		<0.0001	<0.0001

[1] Values are median (25th, 75th percentiles).

[2] Reference group for pairwise comparison of ferritin and copper concentrations.

[3] P-values by Kruskall Wallis for global comparisons.

[a, b, c] P-values by two-sample Wilcoxon tests are <0.05, <0.01, and <0.0001.

Table 4.4: Serum ferritin and copper concentrations within groups defined by binary CRP levels (n = 634)

	CRP levels	Low level		High level		p[1]
		n	Median (25th, 75th)	n	Median (25th, 75th)	
Ferritin, μg/L	≤0.5 vs. >0.5	285	44.5 (31.7, 57.4)	349	49.9 (36.4, 70.6)	<0.0001
	≤1 vs. >1	422	44.6 (31.9, 59.9)	212	51.8 (39.3, 73.0)	<0.0001
	≤3 vs. >3	538	45.7 (32.5, 60.7)	96	58.3 (42.0, 77.2)	<0.0001
	≤5 vs. >5	587	46.5 (33.0, 61.4)	47	64.1 (43.5, 100.4)	<0.0001
	≤10 vs. >10	617	46.6 (33.3, 61.9)	17	81.1 (62.7, 100.4)	<0.0001
Copper, μg/dL	≤0.5 vs. >0.5	285	96.4 (86.8, 105.6)	349	104.8 (94.7, 113.2)	<0.0001
	≤1 vs. >1	422	98.9 (88.1, 107.6)	212	106.2 (95.6, 113.4)	<0.0001
	≤3 vs. >3	538	100.3 (89.7, 109.4)	96	107.4 (95.9, 113.7)	0.0002
	≤5 vs. >5	587	100.7 (90.1, 109.8)	47	108.4 (98.3, 114.1)	0.0003
	≤10 vs. >10	617	100.8 (90.3, 110.1)	17	111.2 (104.2, 119.0)	0.004

[1] P-values by Wilcoxon test for pairwise comparisons.

Table 4.5: Prevalence of low serum ferritin and copper levels within groups defined by binary CRP levels (n = 634)

	CRP levels	Low CRP level		High CRP level		p[1]
	(mg/L)	n total	n (%)	n total	n (%)	
Ferritin						
<15 μg/L	≤0.5 vs. >0.5	285	6 (2.1)	349	7 (2.0)	1
	≤1 vs. >1	422	12 (2.8)	212	1 (0.5)	0.07
	≤3 vs. >3	538	13 (2.4)	96	0 (0)	0.23
	≤5 vs. >5	587	15 (2.2)	47	0 (0)	0.36
	≤10 vs. >10	617	13 (2.1)	17	0 (0)	0.7
Copper						
<90 μg/dL	≤0.5 vs. >0.5	285	96 (33.7)	349	55 (15.8)	<0.0001
	≤1 vs. >1	422	121 (28.7)	212	30 (14.2)	<0.0001
	≤3 vs. >3	538	137 (25.5)	96	14 (14.6)	0.02
	≤5 vs. >5	587	146 (24.9)	47	5 (10.6)	0.03
	≤10 vs. >10	617	149 (24.2)	17	2 (11.8)	0.39

[1] P-values by Fisher-exact tests.

Table 4.6: Concentrations of serum ferritin and copper and the prevalence of their low levels before and after adjustment (n = 634)

	Ferritin (µg/L)				Copper (µg/dL)			
	CF[1]	Media n	GMC[2]	% <15 µg/L	CF[1]	Media n	GMC[2]	% <90 µg/dL
Adjustment using CRP cutoff [1]								
0.5 mg/L	0.85	43.0	42.4	2.5	0.93	97.0	95.7	31.2
1 mg/L	0.82	43.8	43.4	2.4	0.94	99.1	97.6	27.3
3 mg/L	0.74	45.3	44.4	2.2	0.95	100.5	98.7	25.1
5 mg/L	0.67	46.4	45.1	2.1	0.93	100.7	99.0	24.6
10 mg/L	0.54	46.5	45.7	2.1	0.91	100.9	99.3	24.1
Excluding CRP >10 mg/L		46.6	45.7	2.1		100.8	99.3	24.2
No adjustment		47.4	46.9	2.1		101.0	99.5	23.8

[1] Adjustment by multiplying the measures in higher CRP levels with correction factor (CF = ratio of the geometric means of lower CRP level by higher CRP level) for each CRP cutoff.

[2] Geometric mean concentration.

CHAPTER V

DIETARY ZINC INTAKE IS CONSISTENTLY ASSOCIATED WITH SERUM ZINC CONCENTRATION IN CROSS-SECTIONAL ANALYSES IN HEALTHY GUATEMALAN SCHOOL CHILDREN

Dietary Zinc Intake is Consistently Associated with Serum Zinc Concentration in Cross-Sectional Analyses in Healthy Guatemalan School Children

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Running head: Associations among zinc-related variables in school-aged children.

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ABSTRACT

Background: The associations among dietary zinc intakes and biomarkers are unknown in healthy children with high risk of zinc deficiency.

Methods: We performed two cross-sectional analyses, one before and one after receiving six-months of zinc supplementation and fortified milk, in healthy Guatemalan school children to assess associations among dietary zinc intake (measured by food frequency questionnaires), serum zinc, alkaline phosphatase (ALP), and albumin.

Results: The children (n = 691) were 9 ± 1.2 years old at baseline. Low serum zinc (<65 μg/dL) and low dietary zinc intake (estimated average requirements <4 mg/d and <7 mg/d in 4-8 and 9-13 year-olds, respectively) were observed in 21.6% and 39.4% children. Serum zinc concentration was weakly associated with dietary zinc intake, serum ALP, and albumin ($r = 0.20, 0.15, 0.07$; $p < 0.05, < 0.0001$, and $= 0.08$; respectively). At six-month follow-up, 659 children had complete data. Low serum zinc and low dietary zinc intakes were observed in 2.6% and 16.8% children. Serum zinc concentration was associated with only dietary zinc intake ($r = 0.21$, $p = 0.06$).

Conclusions: Dietary zinc intake was consistently associated with serum zinc concentration before and after zinc supplementation and fortification. Therefore, serum zinc concentration is a zinc biomarker for individuals with low or high zinc status. Serum zinc concentration was associated with serum ALP and albumin prior to, but not after, zinc supplementation.

INTRODUCTION

Zinc deficiency continues to be a problem in children and adolescents in developing countries. It was ranked 11th among global risk factors for mortality and 12th for burden of disease in 2001(1). The assessment of zinc deficiency presents a challenge, especially for individuals with a marginal deficient status.

Plasma (or serum) zinc concentration may be one of the most sensitive indicators of zinc status in individuals. Systematic review by Lowe *et al.*, on 32 potential biomarkers from 48 studies of randomized controlled trials, controlled clinical trials, and before-and-after studies in healthy individuals, confirmed that plasma, urinary, and hair zinc were reliable biomarkers of zinc status in healthy individuals (2). Serum alkaline phosphatase (ALP) activity and albumin concentration decrease in severe zinc deficiency in clinical studies among volunteers receiving zinc or hospitalized patients with diseases affecting zinc status (3-5). Approximately 20% of the world's population is zinc deficient based on food intake patterns and dietary zinc intakes (6).

However, markers of zinc status may not be sensitive in marginal zinc deficiency. Clinical studies show that when dietary zinc intakes decrease, zinc balance remains positive for a period without significant changes in circulating plasma zinc (7-10). When the whole body zinc content is reduced by approximately 30% after prolonged zinc restriction, zinc concentrations may drop in plasma but remain constant in some other tissues such as skeletal muscle and skin (11). At this stage, plasma serum zinc concentration is the most useful biomarker of zinc status (11). Other zinc-related biomarkers may be reduced in zinc deficiency, but have been rarely investigated in randomized control trials and repletion studies (2). The review of Lowe *et al.* included 50 studies that reported plasma or serum zinc

measurement, but only 6 studies that measured plasma alkaline phosphatase, and no study that assessed serum albumin (2). Combining several zinc-related biomarkers may enhance the ability to detect mild or more moderate zinc deficiency in otherwise healthy individuals, but there have been few studies investigating associations among zinc-related markers in children.

The purpose of this study was to assess associations among zinc-related variables (concentrations and low levels of dietary zinc intakes, serum zinc concentration, ALP activity and albumin) in healthy school-aged children at baseline and a six-month follow-up after receiving zinc supplementation and fortification. Those occasions represented chronic zinc deficiency and zinc repletion, respectively.

SUBJECTS AND METHODS

Study design and population

In this paper, we performed two cross-sectional analyses using data at baseline and six-month follow-up from a randomized controlled trial of 10-mg zinc oxide versus placebo supplementation in school-aged children from a low-income community in Guatemala City, Guatemala in 2006 (12). At the same time the children received the supplementation, all the children received daily 200 ml fortified milk that contained 1.6 mg zinc, except for 47 children who received a cereal-based drink instead containing 2.1 mg zinc. The study was a collaborative effort between the Hubert Department of Global Health at the Rollins School of Public Health (RSPH), Emory University, Atlanta, GA, and the Institute of Nutrition of Central America and Panama (INCAP) in Guatemala City. Grades 1-4 (6-11 years old) children were recruited from 5 public schools in San Jose la Comunidad, a low-income area

of Guatemala City, Guatemala. Exclusion criteria included children with any known severe illness affecting zinc status (e.g., sickle cell disease, cystic fibrosis, renal or liver disease, severe burns, or acrodermatitis enteropathica) (13), and other severe or chronic illness (e.g. cancer, diabetes, or seizures). Parental informed consent and child assent were obtained before data collection.

Data collection procedures

Baseline data were collected from February to April 2006, the six-month follow-up data from August to October 2006. Zinc-related variables included dietary zinc intakes, serum zinc, alkaline phosphatase, and albumin. Age and gender were included as covariates. Investigators visited and interviewed the child's mother or care-giver within the child's home to obtain information on demographic variables and dietary intakes. Blood samples were collected in the schools.

Dietary assessments

Children's dietary intakes were reported by the mothers or caregivers using a semi-quantitative food frequency questionnaire (FFQ), which included 104 food items. Daily nutrient intakes of energy, zinc, and other nutrients in the FFQ were calculated using the Institute of Nutrition of Central America and Panama (INCAP) food composition tables (14). Nutrient intakes by FFQ were adjusted for the total energy intake using the calorie residual method (15). The FFQ was validated in a separate study with 50 children, comparing results from the FFQ with three 24-hour dietary recalls. Correlation coefficients between dietary residual zinc intakes from FFQ intakes and average 24-hour recall intakes

were 0.33 and 0.41; the slopes of residual zinc intakes from 24-hour recall method by FFQ method were 0.37 and 0.34 respectively, at baseline and six-month follow-up.

Biochemical measurements

A registered nurse collected blood samples of 7ml from each of the children, approximately 2 hours after eating between 9am and 12pm, using the standard procedures, into trace element-free Vacutainers (Bectons Dickinson, Franklin Lakes, NJ). The blood was centrifuged at 2500 x g for 10 minutes at room temperature, then aliquoted, and refrigerated at the school. It was then frozen in trace element-free cryogenic vials at -20ºC at INCAP, and shipped in nitrogen liquid before being stored at -70°C. It was later analyzed at the National Institute of Public Health laboratories, Cuernavaca, Mexico.

Serum zinc concentration was assessed with atomic absorption chromatography, using a graphite furnace (Analyser 300, Perkin Elmer Co Norwalk, CT USA). Serum alkaline phosphatase (ALP) activity was measured by the hydrolysis of p-nitrophenyl phosphate at 37°C (CE Human, Bisbaden, Germany). Serum albumin was analyzed by a colorimetric method using kits from Human Gesellschah fur Biochemica und Diagnostica, Wiesbaden, Germany, and a spectrophotometer Prestige 24i (Tokyo Boeki Medical System, Tokyo, Japan). The accuracy of the determination was assessed by using certified material from the US National Institute of Standard and Technology and the UK National Institute of Biological Standard and Control. Precision was assessed through duplicate measurements on 20% of the sample. The between-assay coefficients of variations for serum zinc, ALP, and albumin were 4.77%, 3.43%, and 4.80%, respectively.

Definitions of categorical variables

Continuous variables were categorized using study-specific cutoffs or conventional definitions (16-18). Low dietary zinc intake was defined as the energy-adjusted zinc intake less than the estimated average zinc requirements (EAR), which were 4 mg/d and 7 mg/d for children 4-8 years and 9-13 years of age, respectively. This was based on the International Zinc Nutrition Collaborative Group (IZiNCG) recommendation for high-phytate, unrefined cereal-based diets (16). The low serum zinc level was defined as serum zinc concentration <65 μg/dL (9.9 mmol/L) (16-18). Serum ALP and albumin distributions were dichotomized at their respective medians (19, 20). Age was dichotomized into two groups of 6-<9 years and ≥9 years based on conventional age groups defined by the IZiNCG (16).

Statistical analyses

Only data from children with complete measurements of outcome variables (i.e., dietary zinc intakes, serum zinc, ALP, and albumin) at each time point were analyzed. Covariates related to these variables were age and gender. Serum CRP concentration was not considered because it was not associated with serum zinc, ALP, and albumin concentrations in healthy children as demonstrated in previous studies (21-25). The resulting sample of 691 children at baseline and 659 children at six-month follow-up allowed the detection of correlations as low as 0.11, given a type-1 error α of 0.05, a power of 0.80, using the POWER procedure in SAS 9.2 version. Normal distribution of variables was assessed using the Kolmogorov-Smirnov test. Summary statistics were described as means (standard deviations, SDs) for continuous variables and frequencies (proportion) for binary variables. Crude associations between continuous variables were measured by Pearson's correlation coefficients. Crude associations between binary variables were assessed by the Chi-square

statistic test. Agreement between binary variables was assessed by Cohen's Kappa statistic. Based on the Cohen's Kappa statistic, two binary variables were classified as having no (Kappa < 0), slight (Kappa = 0-0.2), fair (Kappa = 0.21-0.4), moderate (Kappa = 0.41-0.6), substantial (Kappa = 0.61-0.8), or almost perfect (Kappa = 0.81-1) agreement (26). The estimates were adjusted for age (<9 years vs. ≥9 years) and gender by multiple linear regression models and multiple logistic regression models for continuous and binary outcomes, respectively. Furthermore, correlation coefficients were adjusted for measurement errors using deattenuated factors and the slopes of the linear associations between dietary zinc intakes from the food frequency questionnaire and the three 24-hour recalls, as described by Willett (15, 27). All statistical tests were two-sided, with p-value significance defined as < 0.05. All analyses were conducted using SAS version 9.2 (SAS Institute Inc, Cary, North Carolina).

RESULTS

Characteristics of the study population

At baseline 691 children had complete data on zinc-related variables and were included in the analyses. The participants showed a mean (SD) age of 9.0 (1.2) years, and boys and girls were equally represented (**Table 5.1**). Stunting was present in 102 (15.4%) children, while 272 (39.4%) children had low dietary zinc intakes, and 149 (21.6%) children had low serum zinc <65 μg/dL. None of the children had low ALP activity <54 U/L (2.5^{th} percentile) or low albumin <3.9 mg/dL (2.5^{th} percentile). Only 659 children had complete data at six-month follow-up. Dietary zinc intakes, serum zinc concentration, and serum

alkaline phosphatase activity at the follow-up were higher than at baseline; 16.8% children showed low dietary zinc intakes and 2.6% children showed low serum zinc levels.

Associations among continuous variables

At baseline, correlations among the 4 continuous zinc-related variables were weak (**Table 5.2**). Serum zinc concentration was correlated with dietary zinc intakes ($r = 0.18$, $p = 0.05$), and serum ALP activity ($r = 0.15$, $p < 0.0001$). At six-month follow-up, correlations among the 4 continuous zinc-related variables were even weaker (**Table 5.3**). The correlation between serum zinc concentration and dietary zinc intake was similar to baseline ($r = 0.21$, $p = 0.06$). Other correlations were not significant. Serum ALP was correlated with serum albumin at both baseline and follow-up ($r = 0.16$, $p < 0.0001$).

Associations and agreements among binary variables

At baseline, low serum zinc level was associated with low ALP and low albumin levels ($p < 0.05$) (**Table 5.4**). There were slight agreements in the status of children with low zinc levels and low ALP or low albumin levels (Cohen's Kappa = 0.08 and 0.07, respectively). At six-month follow-up, low serum zinc level was not associated with any level of other zinc-related variables ($p > 0.05$) (**Table 5.5**). Low ALP level was associated with low albumin both before and after interventions ($p < 0.01$, kappa = 0.12 and 0.10, respectively).

DISCUSSION

Summary. This study showed that the associations among variables related to zinc status were weak in a sample of Guatemalan healthy school children. At baseline, participant samples had high risk of zinc deficiency based on the high prevalence of both low dietary

zinc intake and low serum zinc (21.6% and 39.4%, respectively) (28). Dietary zinc intake was associated with only biomarker serum zinc concentration ($r = 0.20$, $p < 0.05$), which was also associated with serum ALP ($r = 0.15$, $p < 0.0001$). At six-month follow-up, zinc status was improved; only 2.6% of the children had low serum zinc and 16.8% had low dietary zinc intake. Dietary zinc intake was still associated with zinc concentration ($r = 0.21$, $p = 0.06$).

Zinc status of participants was different between baseline and follow-up. The children had high risk of zinc deficiency at baseline and their zinc status was improved after zinc supplementation and fortified-milk in both zinc and placebo groups. The improvement of zinc status in the placebo group may be related to zinc-fortified milk or seasonal changes in dietary zinc intakes between baseline and six-month follow-up.

Dietary zinc intakes and biomarkers. The results showed that among the three zinc biomarker concentrations, only serum zinc concentration was associated with dietary zinc intakes at both time points. The associations between dietary zinc intake and serum zinc concentrations in this study at baseline and at six-month follow-up were consistent and weak ($r = 0.20$, $p = 0.05$ and $r = 0.21$, $p = 0.06$, respectively). The results in this study indicate that serum zinc is a reliable biomarker of zinc status in individuals with either low or adequate zinc levels (2, 29). Previous studies using the 24-hour recall method did not show a significant association between dietary zinc intakes and serum zinc concentrations among Guatemalan school children aged 7.0 ± 0.5 years and among Iranian adolescents aged 13.2 ± 1.0 years (30, 31). Using the FFQ method, a study in adults in Western India observed a positive association between dietary zinc intakes and plasma zinc (32). The variations in these results may largely depend on the instruments used to measure dietary zinc intakes.

The FFQ method may be better than the 24-hour recall in estimation of long-term dietary intakes (33).

In reality, even accurate and available dietary zinc intakes may provide little help to the assessment of individual zinc status due to the ability of the body to adapt to a very low or very high zinc intakes in healthy people, except if the reduced dietary zinc intakes occurred for a long duration (34). A healthy adult with an average weight of 50 kg having zinc intakes ranging from 107 to 231 μmol/d, equivalent to 14- 30 mg/kg, will show unchanged serum zinc concentration even when zinc intakes are as little as 22 μmol/d (2.8 mg/kg) or as much as 306 μmol/d (40 mg/kg) (7). Therefore, dietary zinc intakes may have a limited role in the assessment of marginal zinc deficiency in individuals.

Serum zinc concentration and other zinc biomarkers. This study showed that serum zinc concentration was associated with serum ALP activity at baseline but not at six-month follow-up ($r = 0.5$, $p < 0.0001$ and $r = 0.05$, $p > 0.05$, respectively). The association between serum zinc concentration and other biomarkers may be stronger in marginal zinc deficient status than in zinc-supplemented status. When serum zinc concentration drops, other zinc biomarkers will subsequently decrease. When individuals are given high zinc intakes, their serum zinc concentrations increases, but the production of other zinc biomarkers in specific tissues is either saturated or weakened. Previous studies reported significant correlations between serum or plasma ALP activity and zinc concentration in Guatemalan school children aged 7 ± 0.5 years ($r = 0.16$, $p < 0.05$) (30). Many other studies measured serum ALP and serum zinc concentrations, but did not address their association at baseline (35, 36).

Serum zinc concentration was not associated with serum albumin at baseline and six-month follow-up ($r = 0.07$, $p > 0.05$ and $r = 0.05$, $p > 0.05$, respectively). Several studies demonstrated a positive correlation between serum zinc and serum protein concentrations in patients with severe zinc deficiency (3, 4, 37). In healthy populations, serum albumin was reported to be associated with serum zinc in Guatemalan school children ($r = 0.3$, $p < 0.0001$) and in healthy adults participating in the United States National Health and Nutrition Examination Survey (NHANES) 2005-2006 ($r = -0.15$, $p < 0.001$) (30, 38). The differences in the associations from our study and that of previous studies may be related to differences in baseline characteristics of the participants. Baseline albumin concentrations were higher in our study (mean 5.99 g/dL) compared to those in the previous studies in Guatemala (means of 4.46 and 4.61 g/dL in males and females) and in the U.S. (means 4.20 g/dL) (30, 38). This suggests that serum zinc concentration is closely associated with serum albumin in individuals with low albumin concentrations.

Limitations. This study may present some limitations. The results are based on an observational analysis performed in a sample of selected participants in a randomized controlled trial. The results are probably not appropriate to school-aged children living in other areas or to populations with different characteristics, such as younger or more stunted children. The estimation of children's dietary zinc intakes by FFQs was based on information provided by the mother or caregiver which could result in high measurement errors and low precision. Although many previous studies suggest high phytate-zinc ratios in diets of Guatemalan children and women (39, 40), phytate intakes were not measured due to lack of phytate information in the Guatemalan food composition. This may affect the classification of children's diets as unrefined, cereal-based diets with high phytate. Finally,

serum ALP activities in children have wide variations and depend on ages (19). Therefore, the results of this study should be cautiously interpreted in younger children or adolescents.

Conclusions. Dietary zinc intake was consistently associated with serum zinc concentration before and after zinc supplementation. Serum zinc concentration was associated with serum ALP before, but not after, zinc supplementation. All associations, including those that were significant, were weak. The results suggest that in healthy individuals, serum zinc is a reliable biomarker of zinc status. Serum ALP may only be useful in advanced stages of zinc deficiency but this should be confirmed in future research.

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Table 5.1: Descriptive characteristics of age, gender, and zinc-related variables at baseline and six-month follow-up

Variables	Baseline[1] n = 691	Follow-up[1] n = 659
Age, years	9.0 (1.2)	
6- < 9 yr	350 (50.7%)	
≥ 9 yr	341 (49.4%)	
Gender		
Male	350 (50.7%)	
Female	341 (49.3%)	
HAZ	-1.2 (0.9)	
HAZ < -2	102 (14.8%)	
Serum zinc, μg/dL	75.3 (12.9)	108.9 (31.2)
Zinc < 65μg/dL	149 (21.6%)	17 (2.6%)
Dietary zinc intake, mg/day[2]	6.2 (1.5)	12.7 (6.3)
Dietary Zn intake <EAR[3]	272 (39.4%)	111 (16.8%)
Serum ALP, IU/L	552.9 (119.7)	660.2 (150.4)
Serum albumin, g/dL	6.0 (0.5)	5.8 (0.3)

[1] Values are mean (SD) or frequency (%).

[2] Dietary zinc intakes by food frequency questionnaire were adjusted for calorie intake by residual methods following Willet (15).

[3]Estimated Average Requirements (EAR) for zinc are 4 and 7 mg/d for children 4-8 years and 9-13 years of age, respectively (16).

Table 5.2: Pearson's correlation among continuous zinc-related variables at baseline (n = 691)[1]

	Serum Zinc	Zinc intakes	ALP	Albumin
Zinc intakes [2]	**0.20** [3, a]	1		
ALP	**0.15** [b]	-0.13 [3]	1	
Albumin	**0.07** [c]	-0.12 [3]	**0.20** [b]	1

[1] Adjustment for age and gender did not change the significances of the crude correlations.

[2] Dietary zinc intakes adjusted for total calorie intakes following Willett.

[3] After adjustments for measurement error using linear correlation between dietary zinc intakes from FFQ and 24-hour records.

[a, b] P-values from Pearson's correlation analysis are <0.05 and <0.0001, respectively.

[c] P-value from Pearson's correlation analysis is 0.08.

Table 5.3: Pearson's correlation coefficients among zinc-related variables at the six-month follow-up (n = 659)[1]

	Serum Zinc	Zinc intakes	ALP	Albumin
Zinc intakes	**0.21** [2,a]	1.00		
ALP	0.05	0.10 [2]	1.00	
Albumin	0.05	0.03 [2]	**0.16**[b]	1.00

[1] Adjustment for age and gender did not change the significances of the crude correlations.

[2] After adjustments for measurement error using correlation between dietary zinc intakes from FFQ and 24-hour records.

[a] P-value from Pearson's correlation analysis is 0.06.

[b] P-value from Pearson's correlation analysis <0.0001.

Table 5.4: Cohen's Kappa agreements between zinc-related binary variables at baseline (n = 691)

	Zinc < 65 µg/dL	**Zinc intake < EAR[1]**	**ALP < median[2]**
Zinc intake < EAR	-0.02	-	
ALP < median[2]	**0.08[a]**	-0.17[c]	-
Albumin < median[3]	**0.07[a]**	0.00	**0.12[b]**

[1] Dietary zinc intakes were adjusted for total calorie intake by the residual method following Willett (15). EAR: Estimated Average Requirements for zinc are 4 and 7 mg/d for children 4-8 years and 9-13 years of age, respectively (16).

[2] Low serum ALP was < median (556 IU/L).

[3] Low serum albumin was defined as < median (5.91 g/dL).

[a, b, c] P-values from Chi-square tests are <0.05, <0.001, and <0.0001, respectively. Multiple logistic regression models adjusted for age and gender do not change the significances.

Table 5.5: Agreements among zinc-related binary variables at six-month follow-up (n = 695)[1]

	Zinc < 65μg/dL	**Zinc intake < EAR[2]**	**ALP < median[3]**
Zinc intake < EAR	0.00	1	
ALP < median[2]	-0.02	-0.03	1
Albumin < median[3]	0.01	-0.03	**0.1[a]**

[1] Values are Cohen's kappa agreement.

[2] Dietary zinc intakes were adjusted for total calorie intake by the residual method following Willett (15). EAR: Estimated Average Requirements for zinc are 4 and 7 mg/d for children 4-8 years and 9-13 years of age, respectively (16).

[3] Low serum ALP was < median = 645 IU/L.

[4] Low serum albumin was defined as < median = 5.77 g/dL.

[a] Chi-square p-value <0.01.

Multiple logistic regression models adjusted for age and gender do not change the significances.

CHAPTER VI

THE EFFECT OF ZINC SUPPLEMENTATION ON BIOMARKERS OF ZINC, IRON, AND COPPER STATUS IN SCHOOL CHILDREN: A RANDOMIZED CONTROLLED TRIAL IN GUATEMALA

The Effect of Zinc Supplementation on Biomarkers of Zinc, Iron, and Copper Status in School Children: a Randomized Controlled Trial in Guatemala

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[6] Abbreviations: ALP, alkaline phosphatase; RDA, recommended dietary allowance; IZiNCG, International Zinc Nutritional Collaborative Group.

ABSTRACT

Background: Zinc supplementation improved zinc status but its effect on iron, copper status and serum ALP activity are inconclusive in healthy children. The aim of this study was to assess the effect of zinc supplementation on serum ALP and other biomarkers of zinc, iron and copper status.

Methods: In a randomized controlled trial in Guatemala from January to October 2006, school-aged children (6-11 yrs) were selected to receive daily 10mg zinc oxide versus placebo for 6 months.

Results: A total of 672 children participated in the study and completed data for key variables (zinc 337, placebo 335). At baseline, the mean (SD) age was 9.0 ± 1.0 years, 5.5% were underweight, 14.7% stunted, and 21.5% had serum zinc levels < 65 μg/dL; no differences were observed between groups at baseline. After 6 months, compared to the placebo, the zinc group showed larger increases in serum zinc concentration and ALP activity (both $p = 0.03$), a smaller risk of low serum zinc ($p = 0.02$), but no differences in copper, albumin, hemoglobin, and ferritin concentrations ($p > 0.05$). Changes in serum zinc concentration were not associated with changes in serum ALP, albumin, hemoglobin, ferritin, and copper ($p > 0.05$). Compared to baseline, the 6-month follow-up presented higher serum zinc and ALP concentrations, but lower hemoglobin, serum ferritin, copper, and albumin concentrations in both treatment groups ($p < 0.001$).

Conclusion: Six-month zinc supplementation increased serum ALP activity and zinc status, but had no effect on iron and copper indicators among school-aged children.

INTRODUCTION

Zinc deficiency is a common and an important problem for children in developing countries with an estimated prevalence of 20.5% (based on dietary patterns), and is responsible for 4.4% of all childhood deaths (1, 2). Current strategy recommends using a 10- to 14- day course of zinc supplementation (20 mg/day and 10 mg/day for ≥ and <6 months of age, respectively) in addition to oral rehydration for the treatment of acute diarrhea (3, 4). Preventive zinc supplementation has proven to have many benefits in children, such as reduction of the incidences of diarrhea and acute lower respiratory tract infections (by 20% and 15%, respectively), and reduction of overall mortality (by 6%) (5, 6). The beneficial effect of zinc supplementation on growth is inconclusive since it was observed in some previous reviews (5-7), but was not demonstrated in a recent meta-analysis (8).

The results of long-term use of preventive zinc supplementation in children presents several problems, such as compliance with daily regimens, concurrent micronutrient deficiencies, and reduced absorption of other minerals (9). Specifically, prolonged zinc supplementation may be associated with impaired iron status and copper deficiency (i.e., lower serum copper and decreased copper-dependent enzymes) (10-13). Therefore, these adverse effects of zinc supplementation should be comprehensively studied before long-term zinc supplementation is implemented.

The effect of preventive zinc supplementation on serum alkaline phosphatase (ALP) activity, a zinc-containing enzyme, is poorly studied in children. Although many reviews suggest that serum ALP remains unchanged in randomized controlled trials with zinc supplementation or depletion/repletion studies (using zinc-restricted or zinc-enriched diets),

most of the reviewed studies focused on adults (14, 15). In studies focusing on healthy children, the results were inconsistent. A study with Thai school children observed significant increases in serum ALP after zinc supplementation (16), but another study with Guatemalan school children did not detect an effect (17). The effect of zinc supplementation on serum ALP therefore, needs to be further investigated in healthy children.

In this study, we assessed whether zinc supplementation affects biomarkers of zinc, ion, and copper status in children using data from a randomized controlled trial of zinc supplementation in school-aged children in Guatemala.

SUBJECTS AND METHODS

Study design and population

We performed a randomized controlled trial with double-blind interventions of zinc versus placebo supplementation among school-aged children in 5 public schools in a low-income area of Guatemala City, Guatemala in 2006. The study was a collaborative effort between the Hubert Department of Global Health at the Rollins School of Public Health (RSPH), Emory University, Atlanta, GA, and the Institute of Nutrition of Central America and Panama (INCAP) in Guatemala City, Guatemala. The study protocol was reviewed and approved by the ethics committees of both institutions. Details of the study's methods and population were described in a previous publication (18).

We recruited children from 5 public schools in San Jose la Comunidad, a low-income area in Guatemala City, Guatemala. Inclusion criteria involved all school children in grades 1-4 (6-11 years old). Exclusion criteria were children with any known severe illness affecting zinc status (e.g., sickle cell disease, cystic fibrosis, renal or liver disease, severe burns, or

acrodermatitis enteropathica) (19), and any other severe or chronic illness (e.g. cancer, diabetes, or seizures). Parental informed consent and child assent were obtained before the child's participation.

Randomization, blinding, and intervention

Individuals in each classroom were randomly selected into two groups: zinc versus placebo. The groups were randomly allocated by using statistical methods at Emory University, which generated a list on the basis of a 1:1:1:1 allocation ratio without block constraints (20), corresponding to a four-color coding to ensure a balance between the zinc and the placebo groups within the classroom (each group had 2 coded colors). The study staff who administered the supplements received a list of all children in their classrooms enrolled in the study and their assigned color groups.

Chewable zinc (10 mg zinc oxide) and placebo (10 mg glucose) tablets were developed, packaged, and donated by Laboratorio Zerboni SA (Mexico City, Mexico). The supplement dosage of zinc was based on both efficacy and safety reviews of previous studies (21-25). The placebo was similar in appearance and taste as the zinc tablet. The zinc and placebo tablets were divided into 4-color coded vials by a staff member at INCAP not involved in the study. All the tablets for each child were kept in a small plastic container, labeled with the child's name and a colored circle corresponding to the child's assigned group. All study participants and staff were blinded to the treatment code, which was maintained in sealed envelopes at INCAP and Rollins School of Public Health, until the end of the study.

Five schools started the supplementation from late February 2006 to early April 2006. During the six-month intervention period (weekend and holidays not included), the participating child received a chewable tablet daily, five days a week, in their classroom between 8:30am to 10:30am. The tablets were consumed in the presence of the administering study staff who recorded the completion status. No food was given with the supplement. When it was known that a child would be absent from school the following day, a tablet was given to the child by the staff on that day, to be consumed at home the following day, and it was recorded whether or not the child had consumed the supplement. Adherence was calculated using the number of days the supplement was consumed, divided by the total number of days of supplementation (based on the first and the last dates when the class received the supplementation). The average duration in which the children took supplements was approximately 125 days (5.8 months).

Unanticipated and concurrent intervention

At approximately the time when this study began, the local government in the study area implemented a school-based fortified milk program. Children in 4 out of the 5 schools received 200 mL of whole milk per day, fortified with zinc amino chelate to a target concentration of 2.6 mg elemental Zn/200 mL. Analyses in the laboratory showed that the actual concentration was 1.6 mg/200 mL. The milk also contained 4.7 mg iron (the actual amount was 1.12 mg iron from laboratory analysis), 200 μg vitamin A, 0.8 μg vitamin B-12, and 133 μg folic acid. A folic acid tablet was also distributed weekly at the schools, although not consistently. Children in the fifth school (n = 47) did not receive the fortified milk, but instead received Incaparina (Alimentos SA, Guatemala City, Guatemala) daily. Incaparina is a cereal-based drink containing 2.1 mg zinc, 3 mg Fe, 35 μg folic acid, and 56 mg calcium (32).

We were not aware of this program until the fieldwork was well underway and thus, information was not collected on the actual consumption of the fortified milk or Incaparina among the children.

Data collection procedures

Baseline data collection occurred from February to April 2006, supplementation from late February to October 2006, and follow-up from August to October 2006. At the completion of six-month supplementation, all variables were collected again using similar methods. Demographic characteristics of children were obtained by home interviews. Blood collections for the children were conducted in buildings near the schools.

Blood sample collections. Trained nurses collected non-fasting peripheral venipuncture blood samples, a total of 7 ml, between 9am and noon, approximately 2 hours after eating, into trace element-free Vacutainers (Bectons Dickinson, Franklin Lakes, NJ). One drop of blood was used to measure hemoglobin concentration on site. The remaining blood was quickly centrifuged at 2500 x g for 10 minutes at room temperature, aliquoted, and refrigerated at the school; frozen in trace element-free cryogenic vials at -20°C at the Institute of Nutrition in Central America and Panama (INCAP) in Guatemala City; then shipped in nitrogen liquid, stored at -70°C, and analyzed at the National Institute of Public Health (INSP) laboratories, Cuernavaca, Mexico.

Biochemical measurements. Hemoglobin concentrations were measured with the use of the HemoCue® B-Hemoglobin machine (Hemocue Inc., Mission Viejo, CA). Serum C-reactive protein concentration was measured by a high-sensitive laser nephelometry with an antiserum specific for CRP (Behring Nephelometer 100 Analyzer, Behring Laboratories,

Messer Grisheim Gmbh, Frankfurt, Germany). Serum zinc and copper concentrations were assessed with flame atomic absorption chromatography spectroscopy using a graphite furnace (Analyser 300, Perkin Elmer Co Norwalk, CT USA). Serum ferritin concentration was measured by an automatized immune assay (Dade Behring Inc., Newark, DE 19714, USA). Serum alkaline phosphatase activity was measured by the hydrolysis of p-nitrophenyl phosphate at 37°C (CE Human, Bisbaden, Germany). Serum albumin concentration was analyzed by a colorimetric method using kits from Human Gesellschah fur Biochemica und Diagnostica, Wiesbaden, Germany, using a spectrophotometer Prestige 24i (Tokyo Boeki Medical System, Tokyo, Japan). The accuracy of the determination was assessed by certified material from the US National Institute of Standard and Technology and the UK National Institute of Biological Standard and Control. Precision was assessed through repeated measurements on 20% of the sample. The between-assay coefficients of variations for serum CRP, zinc, ferritin, copper, albumin and phosphatase alkaline were 5.29%, 4.77%, 1.54%, 6.34%, 4.80% and 3.43%, respectively.

Study variables

Outcomes. Outcome variables were concentrations of biomarkers of zinc, iron, and copper status, including serum zinc, alkaline phosphatase, albumin, ferritin, copper, and hemoglobin; and the prevalence of their low status. Low serum zinc level was defined as serum zinc concentration <65 μg/dL (25-27), low serum ferritin level as serum ferritin concentration <15 μg/L following the WHO definition for children >5 years of age (28, 29), and low serum copper level as serum copper <90 μg /L (30). Anemia was defined as hemoglobin <11.5 mg/dL (28, 31).

Covariates. The children's age (in years) and gender were used in the analyses as covariates. Previous community-based studies show that serum CRP concentration is associated with serum ferritin and copper, but not zinc concentrations (32-34). For this analysis, both serum ferritin and copper concentrations were adjusted for high versus CRP levels (>5 mg/L versus ≤5 mg/L) at baseline and 6-month follow-up using correction factors following Thurnham *et al.* (35).

Statistical methods

Only data from participants with complete measurements of outcome variables at baseline and six-month follow-up were used. Continuous variables were checked for normality using Kolmogorov-Smirnov tests and plots of normality. Serum ferritin and copper concentrations in high CRP level (>5 mg/L) were adjusted for inflammation by multiplying their concentrations with correction factors, as the ratios of nutrient geometric means of nutrients in low (≤5 mg/L) CRP levels by high (>5 mg/L) CRP levels (35). Adjusted serum ferritin concentrations were skewed and normalized by log-transformation. Summary statistics were described as means (standard deviations) or median (25^{th}, 75^{th} percentiles) for continuous variables with normal and non-normal distribution, respectively; and frequencies (proportion) for categorical variables. To evaluate the effectiveness of randomization, we compared the zinc and control groups on baseline characteristics. To assess the effect of zinc supplementation, we compared the six-month changes (final vs. initial) of outcome variables between two treatment groups. To assess the effect of fortified milk program we compared the outcomes between baseline and 6-month follow-up in each treatment group. We also assessed associations between the changes in serum zinc concentrations and the changes in other biomarkers. In crude analyses, the comparisons of

variables between treatment groups were performed by Student's t-test for continuous variables and Fisher's exact test for binary variables. In multivariate analyses, we adjusted for age, gender, and autocorrelations of repeated outcomes using random effects models with generalized linear mixed regression (SAS GLIMMIX procedure, SAS version 9.2, SAS Inc., Cary, NC) for both continuous and binary outcomes. In addition, we assessed associations between the changes in serum zinc concentrations and the changes in other biomarkers. Crude analyses used simple linear regression; multiple analyses used the generalized linear mixed models to adjust for age, gender, and treatment group. In post-hoc power analysis, it determined that the sample size of 672 children could detect differences in concentrations between 2 groups as little as 2.8 μg/dL, 6.0 μg/L, and 2.8 μg/dL for serum zinc, ferritin, and copper concentrations, respectively, assuming that the standard deviations for serum zinc, ferritin, and copper were 13, 28, and 13 μg/dL, respectively, with a 2-tailed alpha of 0.05 and a power of 0.8. P-value <0.05 was used as the criterion of statistical significance. SAS version 9.2 (SAS Institute) was used for all analyses.

RESULTS

Baseline characteristics of participants

A flow chart of participants was presented in a previous study (**Figure 6.1**) (18). From 800 eligible children, 30 were excluded because of age and 20 refused to participate, leaving 750 children to be randomized. Another 30 children never received nor completed the baseline assessment. Thus, 720 children received at least one tablet and completed the baseline assessment. Of the 720 participants, 5 were lost to follow-up because of relocation (n = 2), parental refusal (n = 2), absence at the final examination (n = 1) and 43 did not have

complete data on the key outcomes (i.e., serum zinc, alkaline phosphatase, albumin, ferritin, and hemoglobin). The final sample for analysis included 672 children with complete data (though copper analysis was included for only 562 children due to measurement problems with serum copper at baseline). The children with missing data (n = 48 or 6.7% of participants) were younger in age and had higher proportions of stunting than those with completed data (P <0.05); no other differences in baseline characteristics were found (data not shown).

Among 672 participants with completed data, 337 received zinc supplements and 335 received placebo. The mean age (standard deviation) of the children was 9.0 (1.2) years and 48.7% were male (**Table 6.1**). Low serum zinc (<65 μg/dL), ferritin (<15 μg/L), and copper (<90 μg/dL) occurred in 21.5%, 2.2%, and 20.8% of children, respectively. Anemia occurred only in 2 children (0.3%) in the zinc group. Low albumin was not present in any of the children. There were no significant differences in the distributions of baseline characteristics between the two groups. In addition, serum C-reactive protein had median (25^{th}, 75^{th} percentiles) of 0.57 (0.26, 1.56) mg/L at baseline and 0.55 (0.22, 1.47) mg/L at 6-month follow-up. The prevalence of high CRP level (>5 mg/L) was 7.1% at baseline and 7.4% at follow-up.

Effect of fortified milk program on biochemical outcomes

Compared with baseline, significant changes were observed in all nutrient concentrations in both treatment groups at follow-up (Table 6.2). In the placebo group, the concentrations of serum zinc and ALP increased, but the concentrations of serum ferritin, copper, albumin, and hemoglobin decreased (all p <0.0001). Consequently, the prevalence of

low zinc level decreased and the prevalence of low ferritin and low copper levels increased at the 6-month follow-up in the placebo group (all $p < 0.01$). In the zinc group, the changes were similar (all $p < 0.05$), except for the prevalence of low ferritin, which did not differ between two time-points ($p = 0.30$).

Effect of zinc supplementation on biochemical outcomes

Compared to the placebo group, the change in serum zinc concentration in the zinc group was higher by 5.6 μg/dL (95% CI: 0.6-10.7 μg/dL) and the change in serum alkaline phosphatase activity was higher by 20.6 IU/L (95% CI: 1.8-39.4 IU/L) (**Table 6.2**). Consequently, the risk of low serum zinc in the zinc group was reduced by 72% compared to the placebo group (OR 0.28, 95% CI: 0.09, 0.85) (**Table 6.3**). Inversely, the changes in the concentrations of hemoglobin, serum albumin, ferritin, and copper, and the prevalence of low ferritin or low copper did not differ between the two groups (all $p > 0.05$) (**Table 6.2 and 6.3**).

Associations between changes in serum zinc concentrations and changes in other biochemical outcomes

The change in serum zinc concentration from baseline to follow-up was not associated with changes over the same period in serum ALP activity, serum ferritin, and serum copper concentrations (all $p > 0.05$) (**Table 6.4**).

DISCUSSION

Summary. We examined the effects of zinc supplementation on biochemical indicators of zinc, iron, and copper status in school children with high prevalence of zinc

deficiency. Zinc supplementation increased serum zinc and ALP activity, but had no effect on biomarkers for iron and copper status. Our results are consistent with findings from a recent review of Brown *et al.* on the effect of preventive zinc supplementation on biomarkers of zinc, iron, and copper (5).

Effect of the fortified milk program. The schools provided fortified milk during the period of zinc supplementation and influenced the results. Zinc and iron contents of the fortified milk may have altered the effects of zinc supplementation on zinc, iron, and copper status. The 200 mL of fortified milk supplied a daily amount of 1.6 mg Zn and 1.12 mg iron. Compared to the Recommended Dietary Allowance (RDA) for zinc and iron (5 mg and 8 mg of zinc for 4-8 years and 9-13 years, respectively; and 10 mg and 8 mg of iron for 4-8 years and 9-13 years, respectively), the amounts of zinc and iron in fortified milk were relatively small and may have some but not substantial effects on zinc and iron status of participants in both the intervention and control groups during the intervention period.

Effect of zinc supplementation on serum zinc concentration. The positive effect of zinc supplementation on the change in serum zinc concentration in this study is consistent with that of previous studies (5). The dosage of zinc oxide in chewable tablets is 10 mg daily from the IZiNCG recommendation for children aged 4-18 years (25). In a meta-analysis in 2009 of 22 studies comparing 30 pairwise groups, zinc supplementation increased serum zinc concentration by an estimated pooled effect size of 60.2% (95% CI: 43.9-76.6; P <0.0001) (5). The effect size of zinc supplementation on serum zinc in our study is 16.8%, smaller than the pooled effect size of the meta-analysis, probably because of the additional effect of the zinc fortified milk.

Effects of zinc supplementation on serum ALP. Zinc supplementation in this study also increased serum ALP concentration in our study. In the reviewed literature, serum ALP was not considered as an effective biomarker for zinc status because of inconsistent results (15). In a RCT study of 133 children aged 6-13 years in Thailand, ALP activity increased significantly after zinc supplementation (16). However, in another RCT study of 162 children aged 7 ± 0.5 years in Guatemala, there was no recorded change in ALP activity (36). Differences between studies may be explained by differences in serum ALP activities across age groups. Serum ALP activity in children strongly depends on their ages. The activity of bone ALP depends on osteoblastic activity of bone growth with two peaks during childhood: one during infancy, and one during puberty (37, 38). Therefore, the strong influence of puberty on ALP activity may mask differences due to zinc supplementation.

Effects of zinc supplementation on biomarkers of iron and copper status. In this study, there were no differences in concentrations of serum ferritin, copper, albumin, hemoglobin, and the prevalence of low ferritin and copper levels. We examined the associations between changes in serum zinc concentration and changes in other biomarkers. The changes in serum zinc concentrations were not associated with changes in serum ferritin, copper, and other biomarker concentrations (all $p > 0.05$). Therefore, zinc supplementation may not have influenced iron and copper status. The staff did not record any clinical adverse events related with the interventions or school milk program among the participants. Previous studies of zinc supplementation using a dose corresponding to the RDA have not showed an overall effect on changes in hemoglobin, serum ferritin, and serum copper concentrations (5). Overall, adverse effects of zinc supplementation on biomarkers may be minimal in children.

Effects of fortified milk on biomarkers. Effects of fortified milk on biomarkers, separated from zinc supplementation, can be demonstrated in their changes in the placebo group. Children in the placebo group without zinc supplementation showed improved zinc status and diminished iron and copper status. While fortified milk supplied additional zinc, iron, and other nutrients, the decreases in hemoglobin, serum ferritin, and copper concentrations were unusual. A recent review of literature of zinc fortification including 7 RCTs using zinc-fortified milk and 14 using zinc-fortified cereal products concluded that zinc fortification did not adversely affect the absorption of iron and copper (39). All the 7 studies assessed the effects of zinc-fortified versus non-fortified infant formulas and observed no changes in hemoglobin, serum ferritin, and copper concentrations. There was no study using zinc-fortified milk in this review. Three previous studies in children 1-4 years of age showed that zinc- and iron-fortified whole milk (as reconstituted milk or milk powder), compared with non-fortified milk, benefited iron status measured by hemoglobin (3/3 studies), serum ferritin concentration (1/3 studies), or serum transferritin soluble receptor (1/3 studies) (40-42). A study of school children in Vietnam comparing fortified milk, regular milk, and control (no supplements) with school children, observed increases in hemoglobin, serum ferritin, and zinc concentrations after 6 month of intervention in all 3 groups, with the highest changes in the milk groups compared to the placebo group (43). In a study in Kenya where 555 school children 5-14 years of age were randomly selected to receive either meat, milk, energy, or no supplemental food from September 1998 to July 1999, compared to the baseline, plasma ferritin, serum zinc, and copper concentrations decreased in the end of this study in all four groups. There were no differences between supplemented and non-

supplemented groups (44). Thus, the literature has showed that fortified milk may either increase or decrease iron status.

The effect of zinc-fortified milk on nutrient status may be related to extraneous factors or fortified milk program. First, measurements of biomarkers at different seasons may be influenced by inflammation or infections. A study of Kenya showed a lower prevalence of malaria and high CRP levels at the end of the study (44). Since inflammation may increase ferritin and copper concentrations, less inflammation will reduce their concentrations (45). In our Guatemalan study, the serum CRP concentrations and levels were similar in baseline and 6-month follow-up and serum ferritin and copper concentrations were adjusted for inflammation; thus, inflammation is unlikely to have influenced iron and copper status. Second, deteriorating socio-economic conditions or seasonal changes during the period from February to October 2006 may have led to reduced iron and copper dietary intakes (e.g. reduced meat & animal products). This was not likely as the children lived in a peri-urban area and were not subject to any major changes in this region during this study. Third, high calcium content in whole milk may have reduced iron absorption at the intestinal membrane. The calcium content in whole milk is estimated at 119 to 143 mg/100 g (238-286 mg/200 g) following the table of food composition for Central America and Panama (46). Previous studies suggest that giving children 165 mg calcium in the form of milk may reduce iron absorption by 50-60% (47). Together with low-bioavailability of native food iron (only 15%) (48), and common diets with low meat and high non-digestible carbohydrates in Guatemala (49), the calcium in the milk may have compromised iron status at 6-month follow-up. Fourth, 200 ml fortified milk in the morning may suppress the appetite of children and reduce their food consumption at lunchtime. This

resulted in a decrease in serum ferritin, ALP, and albumin concentrations in our study. Finally, the changes in iron and copper biomarkers in both groups after 6-months of intervention may be explained by the Hawthorne effect (50). This effect may occur during any intervention, due to extra attention by researchers, higher levels of clinical surveillance, and influence on both treatment and control groups. Overall, fortified milk program in this study was effective in improving zinc status but possibly harmful for iron and copper status.

Strengths. This study had several strengths. This was a carefully controlled trial of zinc supplementation with good randomization and high rates of retention and follow-up. Therefore, the effective randomization balanced all potential confounding factors between two groups. In addition, the study used standard methods for measuring biomarkers, had duplicate measurements in 20% of blood samples, and the results had high precision (small coefficients of variation 3.43-6.34%). Thus, measurement errors were minimized and estimates had high precision.

Limitations. There were some important limitations. First, the unexpected school fortified milk program concurrent with the zinc supplementation trial did not affect the validity but may have reduced the impact of zinc supplementation on nutrient status. Missing data on copper status (n = 158 or 21.9% missing) may have reduced the precision of estimates and underestimated the effect of zinc in the supplementation or the fortified milk on copper status.

In conclusion, this study showed that zinc supplementation improves serum zinc and serum ALP activity in school-aged children. The study does not provide evidence to support a significant impact of zinc supplementation on biomarkers of iron and copper status. The

concurrence of daily zinc- and iron-fortified milk program improved zinc status but reduced iron and copper status.

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Table 6.1: Selected baseline demographic and nutritional characteristics of school-age children in Guatemala (n = 672)[1]

Variable	Zinc n = 337	Placebo n = 335
Age, year	9.0 (1.2)	9.0 (1.2)
Gender, %		
Male	50.7	48.7
Female	49.3	51.3
Grades, %		
1	20.2	18.8
2	31.8	32.2
3	29.7	30.8
4	18.4	18.2
WAZ	-0.53 (1.11)	-0.53 (1.02)
HAZ	-1.25 (0.89)	-1.17 (0.86)
Serum CRP, mg/L	0.57 (0.26, 1.54)	0.56 (0.27, 1.57)
Serum zinc, μg/dL	75.4 (12.9)	75.1 (13.0)
Zinc <65 μg/dL, %	22.6	20.3
Serum ferritin, μg/L	48.4 (34.2, 64.0)	46.9 (33.3, 62.2)
Ferritin <15 μg/L, %	2.7	1.8
Serum copper, μg/dL	100.8 (12.6)	100.0 (12.9)
Copper <90 μg/dl, %	23.1	23.5
Hemoglobin, g/dL	13.8 (0.8)	13.8 (0.7)
Alkaline phosphatase, IU/L	548.9 (122.6)	561.5 (116.8)
Albumin, g/dL	6.0 (0.5)	6.0 (0.5)

[1] Values are mean (SD) or median (25th, 75th).

Table 6.2: Changes in concentrations of nutritional biochemical variables between treatment groups in school-aged children in Guatemala[1]

Outcomes	Zinc n = 337	Placebo n = 335	MD (95%CI)[2]	p[3]	p[4]
Serum zinc, μg/dL					
Baseline	75.1 (13.0)	75.7 (12.9)			
6-month Fu.	111.2 (31.0)	106.1 (31.3)			
Change	36.1 (32.8)	30.5 (34.0)	5.6 (0.6, 10.7)	0.03	0.03
p[5]	<0.0001	<0.0001			
Alkaline phosphatase, IU/L					
Baseline	548.9 (122.6)	561.5 (116.8)			
6-month Fu.	663.7 (151.7)	655.7 (149.2)			
Change	114.8 (124.2)	94.2 (124.2)	20.6 (1.8, 39.4)	0.03	0.03
p[5]	<0.0001	<0.0001			
Albumin, g/dL					
Baseline	6.0 (0.5)	6.0 (0.6)			
6-month Fu.	5.8 (0.3)	5.8 (0.4)			
Change	-0.2 (0.3)	-0.2 (0.6)	0.04 (-0.04, 0.12)	0.35	0.35
p[5]	<0.0001	<0.0001			
Hemoglobin, g/dL					
Baseline	13.8 (0.8)	13.8 (0.7)			
6-month Fu.	13.7 (0.7)	13.7 (0.7)			
Change	-0.1 (0.7)	-0.1 (0.6)	0.0 (-0.1, 0.1)	1.00	1
p[5]	<0.0001	<0.0001			
Log(ferritin), log(μg/L) [6]					
Baseline	3.8 (0.5)	3.7 (0.5)			
6-month Fu.	3.6 (0.6)	3.6 (0.6)			
Change	-0.2 (0.5)	-0.2 (0.5)	0.00 (-0.08, 0.08)	0.99	0.99
p[5]	<0.0001	<0.0001			
Copper, μg/dL[6, 7]					
Baseline	100.3 (12.4)	99.6 (12.8)			
6-month Fu.	85.4 (13.9)	85.8 (15.2)			
Change	-15.2 (18.8)	-13.7 (20.6)	-1.4(-4.7, 1.8)	0.39	0.47
p[5]	<0.0001	<0.0001			

[1] Values were mean (SD).

[2] Mean difference between the changes in nutrient concentrations between 2 intervened groups without adjustments. Adjustments for time, age, and gender give similar values (GLIMMIX).

[3] P value by t-test comparing the changes in 2 treatment groups.

[3] P value by comparing the changes in 2 treatment groups, adjusted for autocorrelation between time-points, age and gender (GLIMMIX).

[5] P-value by t-test comparing the concentrations at the baseline and the 6-month follow-up in each treatment group. Adjusted for autocorrelation between time-points, age and gender using GLMM give the same p-values (GLIMMIX).

[6] Serum ferritin and copper concentrations are adjusted for inflammation using Thurham method (multiplying with correction factors as ratio of geometric means of the referent group (CRP ≤5 mg/L) by the high CRP group (CRP >5 mg/L). Adjustment using Thurham method with the CRP cutoff of 0.5 mg/L did not change the significances of the associations.

[7] Numbers of data with completed copper measurements at 2 time points were 280 and 282 for zinc and placebo groups, respectively.

Table 6.3: Changes in the prevalence of low serum zinc, ferritin, and copper levels between 2 treatment groups

Outcomes	Zinc n = 337	Placebo n = 335	OR (95% CI)[1]	p[2]	p[3]
Zinc < 65 μg/dL, %					
Baseline	22.6	20.3			
6-month	1.2	4.2	0.28 (0.09, 0.85)	0.02	0.02
Change	-21.4	-16.1			
p[4]	<0.0001	<0.0001			
Ferritin < 15 μg/L, % [5]					
Baseline	2.7	1.8			
6-month	4.5	6.6	0.66 (0.34, 1.30)	0.24	0.20
Change	1.8	4.8			
p[4]	0.3	0.004			
Copper < 90 μg/dL,% [5]					
Baseline	25.6	26.4			
6-month	71.6	71.0	1.03 (0.71, 1.49)	0.93	0.78
Change	0.21	0.21			
p[4]	<0.0001	<0.0001			

[1] Odds ratio for low nutrient level of zinc versus placebo supplementation at 6-month follow-up without adjustments. Adjustments for autocorrelation between time-points, age, and gender give similar estimates (GLIMMIX).

[2] P-values by Fisher-exact tests comparisons of the change in the prevalence between 2 groups.

[3] P-values by comparisons of the change in the prevalence between 2 groups adjusted for time, age, and gender (GLIMMIX).

[4] P-values by Fisher-exact tests comparing the prevalence at 6-month follow-up and the baseline in each treatment group. Adjusted for time-points, age and gender (GLIMMIX) similar results.

[5] Serum ferritin and copper concentrations are adjusted for inflammation using Thurham method (multiplying with correction factors as ratio of geometric means of the referent group (CRP ≤5 mg/L) by the high CRP group (CRP >5 mg/L).

Table 6.4: Associations between the changes of serum zinc concentration and the changes of other biochemical concentrations (N = 672)

Variable changes	MD (95% CI)[1]	p[1]	Adj. MD (95% CI)[2]	p[2]
Serum ALP, IU/L	0.41 (0.13, 0.69)	0.04	-0.29 (-0.91, 0.34)	0.36
Serum albumin, mg/L	0.00 (0.00, 0.00)	0.24	-0.00 (-0.01, 0.00)	0.26
Log(serum ferritin, μg/dL)[3]	0.00 (0.00, 0.00)	0.24	-0.00 (-0.01, 0.00)	0.08
Hemoglobin, g/dL	0.01 (0.00, 0.01)	<0.0001	-0.00 (-0.01, 0.00)	0.13
Serum copper, μg/L[3]	0.11 (0.06, 0.16)	<0.0001	-0.00 (-0.01, 0.00)	0.13

[1] Changes and p-values in serum nutrient concentrations after treatments due to a unit change in serum zinc concentrations using simple linear regression.

[2] Changes and p-values in serum nutrient concentrations after treatments due to a unit change in serum zinc concentrations, adjusted for autocorrelation between time-points, treatment group, age and gender (GLIMMIX).

[3] Values of serum ferritin and copper are adjusted for inflammation using correction factor as ratio of nutrient geometric means of CRP ≤5 mg/L by CRP >5 mg/L.

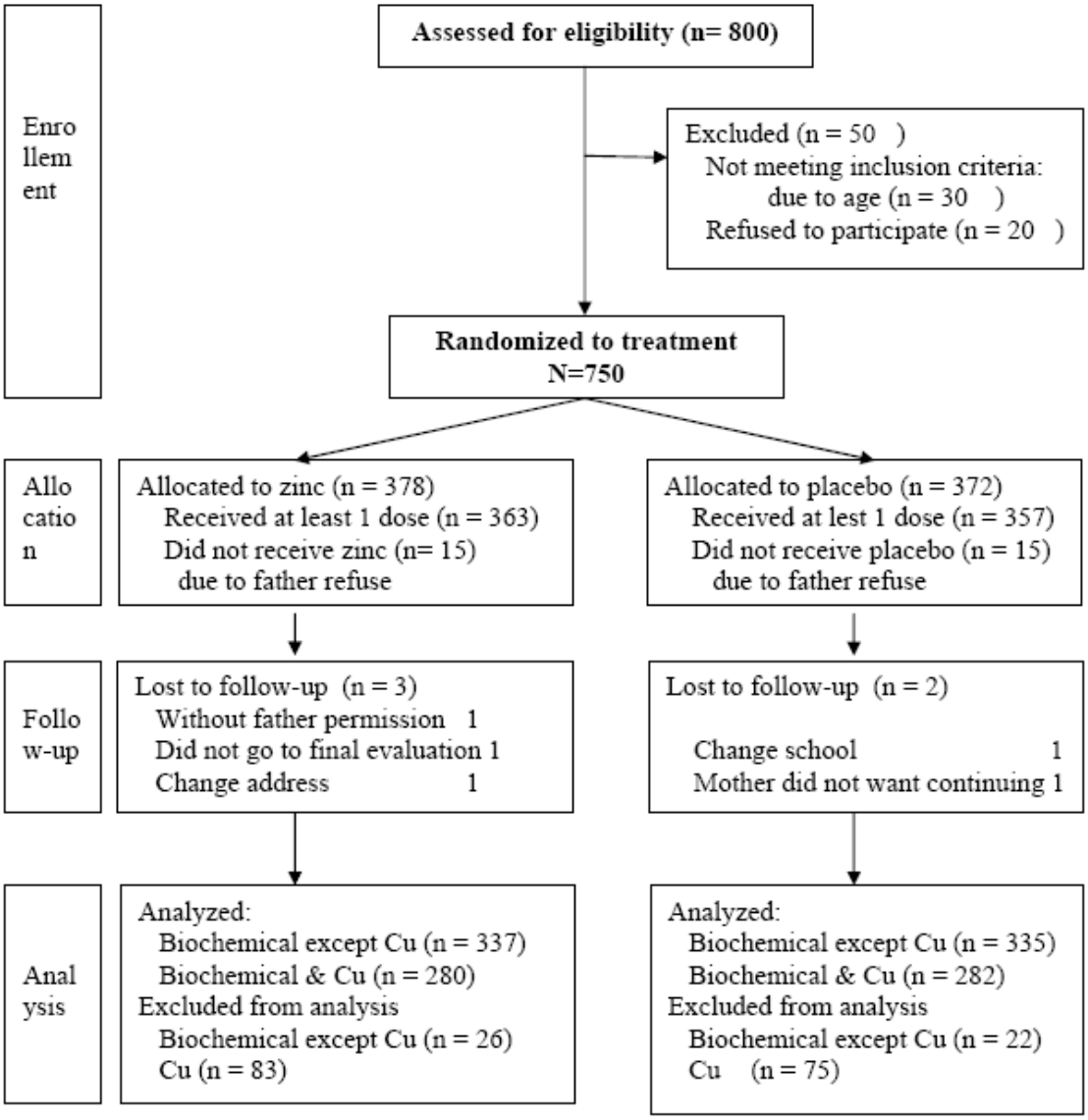

Figure 6.1: Flow chart of participants (n = 672)

CHAPTER VII

SUMMARY AND CONCLUSIONS

This thesis is about results from a double-blind, placebo-controlled randomized trial of zinc supplementation. It was conducted in 2006, with 720 healthy Guatemalan school-aged children (6-11 years) in a low-income urban area of Guatemala City, Guatemala. During the six-month period of this study, each participant was randomly allocated to receive daily chewable tablets of either zinc oxide of 10 mg or placebo. The main purpose of this thesis is to investigate the effect of zinc supplementation on serum biomarkers of zinc, iron, and copper status. However, associations between zinc supplementation and biomarker measurements may be confounded by many factors. Subclinical inflammation in healthy children may have transient influences on circulating plasma concentrations of biomarkers, resulting in misclassifications of zinc, iron, and copper status (186, 360). My first paper assesses associations between serum CRP, a biomarker of inflammation and the biomarkers of nutrient status at baseline, and quantify the magnitude of the effect of inflammation in significant associations. The assessment of zinc status in healthy children is of concern due to the lack of a sensitive and specific marker for zinc status. Serum zinc concentration has shown to be a reliable marker of zinc status (31). Other clinical variables, such as dietary zinc intake, serum alkaline phosphatase, and serum albumin, are related to zinc deficiency and associated with serum zinc (1, 27-29). These may influence the association between serum zinc and zinc supplementation. My second paper studies the associations between serum

zinc and other indicators of zinc status, as either continuous or dichotomized variables. Finally, my third paper, investigates the effect of zinc supplementation on the biomarkers of zinc, iron, and copper status using longitudinal data at baseline and six-month follow-up.

7.1. Key Findings

Associations between serum CRP and serum zinc, ferritin, and copper in school-aged children

The first paper shows that serum CRP concentration was not associated with biomarkers of zinc status, but was positively associated with serum ferritin and serum copper concentrations. The adjustment for inflammation had the greatest influence on recalculated prevalence in the case of the CRP 0.5 mg/L cut off. Given the very low prevalence of iron deficiency, the adjustment had little effect on low ferritin prevalence, but larger impact on low copper prevalence.

The prevalence of low serum zinc < 65 μg/dL, ferritin <15 μg/L, and copper <90 μg/dL were 21%, 2.1%, and 23.8%. Serum CRP median (25^{th}, 75^{th} percentiles) was 0.56 (0.26, 1.54). CRP concentration was positively associated with ferritin and copper concentrations ($r = 0.23$ and 0.29, respectively; $p < 0.0001$); but not with serum zinc and other biomarkers ($p > 0.05$). Regardless of dichotomized CRP cutoffs, high CRP levels had higher ferritin concentrations, higher copper concentrations, and lower prevalence of copper <90 μg/dL than low CRP levels ($p < 0.05$). The adjustment for inflammation using correction factors reduced nutrient concentrations and increased the prevalence of low nutrient levels. Lower CRP cutoffs had stronger influence than higher CRP cutoffs and the greatest influence on recalculated prevalence was observed with the CRP 0.5 mg/L cutoff.

Compared to the prevalence with no adjustment, the adjustment using CRP 0.5 mg/L changed the low ferritin prevalence from 2.1% to 2.5% and the low copper prevalence from 23.8% to 31.2%. Therefore, given a very low prevalence of iron deficiency in our sample, the adjustment for inflammation had little influence on ferritin status but great impact on copper status (from 23.8% to 31.2%).

Previous studies observed null associations between serum CRP and serum zinc (187, 361-363), significant associations between serum zinc and serum ferritin (186, 187, 238, 244, 294), and serum copper in healthy adults and hospitalized children (360, 364-366).

Differences in the influence of inflammation of biomarkers of zinc status, ferritin, and copper status in healthy children can be explained by different mechanisms of the inflammation effect on biochemical concentrations. The change in serum zinc concentrations during inflammation may involve increases in urinary zinc loss or increased metallothionein synthesis in liver (192-196). Inflammation has a strong effect on serum ferritin and copper because they are acute-phase proteins (i.e., ferritin) or measured within a complex with another acute-phase protein (i.e., ceruloplasmin).

In the adjustment of serum ferritin for inflammation using CRP, many authors used various serum CRP cutoffs such as 10, 8, 5, 3 mg/L (186, 187, 189, 240, 241, 244, 245, 285, 286). However, this is the first study to investigate the cutoffs of serum CRP as low as 0.5 mg/L. We demonstrated that the use of the CRP 0.5 mg/L cutoff decreased the prevalence of low ferritin and low copper levels, compared to the traditional CRP cutoffs of 5 mg/L or 10 mg/L. The impact of this low CRP cutoff is low in populations with very low prevalence

of iron or copper deficiencies but will be substantial in those with high prevalence of nutrient deficiencies.

Finally, this paper has two new findings. First, it is the first study to investigate the association between serum CRP and serum copper concentrations in healthy children. Second, it is also the first to use the CRP cutoff point of 0.5 mg/L in the adjustment for inflammation based on the significant differences in ferritin and copper concentrations between high and low CRP levels (>0.5 mg/L vs. ≤0.5 mg/L).

Associations among zinc-related variables in school-aged children

The second paper shows that dietary zinc intake was associated consistently but weakly with serum zinc concentration at baseline and after six-month follow-up ($r = 0.20$, $p < 0.05$ and $r = 0.21$, $p = 0.06$). Serum zinc concentration was associated with serum ALP, but not after, zinc supplementation.

The children ($n = 691$) were 9 ± 1.2 years old at baseline. Low serum zinc (<65 μg/dL) and low dietary zinc intake (estimated average requirements <4 mg/d and <7 mg/d in 4-8 and 9-13 year-olds, respectively) were observed in 21.6% and 39.4% children. Serum zinc concentration was weakly associated with dietary zinc intake, serum ALP, and albumin ($r = 0.20, 0.15, 0.07$; $p < 0.05, < 0.0001$, and $= 0.08$; respectively). At six-month follow-up, 659 children had complete data. Low serum zinc and low dietary zinc intakes were observed in 2.6% and 16.8% children. Serum zinc concentration was associated with only dietary zinc intake ($r = 0.21$, $p = 0.06$).

Weak associations among the zinc-related variables in healthy children at risk of zinc deficiency may be due to strong zinc homeostasis. The concentrations of serum zinc or

other zinc-related biomarkers remain constant for a long period until negative zinc balance decreases by about 30% of the whole body content (98). Therefore, in healthy people with marginal zinc deficiency, dietary zinc intake is not sensitive to other biomarkers. Serum ALP and albumin may be less sensitive to zinc status because their synthesis in bone and liver depends on many other factors besides zinc status. The associations among zinc-related status at marginal stages of deficiency are weaker than in the severe stage of zinc deficiency.

This study confirms that serum zinc concentration is the only biomarker of zinc status in individuals with either low or high dietary zinc intakes. Additional measurements of other biomarkers have no benefit on the assessment of marginal zinc status, but their uses as zinc-related variables may be useful in advanced stages of zinc deficiency for diagnosis and treatment.

The effect of zinc supplementation on biomarkers of zinc, iron, and copper status

The third paper demonstrates that six-month zinc supplementation increased serum ALP activity and zinc status but had no significant effects on iron and copper indicators among school-aged children. In addition, the six-month follow-up observed higher zinc status but lower hemoglobin, albumin, iron and copper status.

A total of 672 children completed the study and had complete data for key variables (zinc 337, placebo 335). At baseline, the mean (SD) age was 9.0 ± 1.0 years, 5.5% were underweight, 14.7% stunted, and 21.5% had serum zinc levels < 65 μg/dL; no differences were observed between groups at baseline. Compared to the placebo, the zinc group had larger 6-month increases in serum zinc concentration and ALP activity (both $p = 0.03$), a lower risk of low serum zinc ($p = 0.02$), but no differences in copper, albumin, hemoglobin, and ferritin concentrations ($p > 0.05$). Changes in serum zinc concentration were not

associated with changes in serum ALP, albumin, hemoglobin, ferritin, and copper ($p > 0.05$). Compared to the baseline, the 6-month follow-up presented higher serum zinc and ALP concentrations, but lower hemoglobin, serum ferritin, copper, and albumin concentrations in the placebo as well as in the zinc group ($p < 0.001$).

The results of this study are consistent with the results of the effects of preventive zinc supplementation on biomarkers in a recent systematic review of Brown *et al.* (14). However, an unexpected school program with iron-and-zinc-enriched fresh milk occurred simultaneously with the zinc versus placebo supplementation. Given randomization in a controlled trial, the school milk program did not bias the estimates, but may have decreased the precision to detect the association between zinc supplementation and iron or copper status. Further analyses demonstrated that the change in serum zinc concentrations was not associated with those in ferritin and copper concentrations. Therefore, zinc supplementation is less likely to induce significant changes in iron and copper status.

Fortified milk improved zinc status but compromised iron and copper status in this study. The reduction of hemoglobin, serum albumin, ferritin, and copper concentrations after the six-month fortified milk program in both treatment groups was unusual according to reviewed literature (367). It may be either related to the inhibitory effect of school milk in the morning, to food consumptions at lunch, or the reduced absorption of iron and copper due to high calcium content in liquid milk. Other factors such as lower inflammation status, deteriorating socio-economic conditions, seasonal changes at the six-month follow-up compared to the baseline were less likely. Given no control group without zinc supplementation or fortified milk, the effect of fortified milk on iron and copper status were inconclusive.

The third paper does not add new information given that zinc supplementation has been proven to be beneficial to zinc status and safe in iron and copper status in children. However, new evidence shows serum ALP activity and zinc status. The fact that serum ALP is sensitive to zinc supplementation suggests that serum ALP could be a reliable marker in monitoring zinc status during a zinc intervention. In addition, fortified milk may present harmful effects on iron and copper status, and more well-design studies are necessary to verify this effect.

7.2. Strengths and Limitations of the Current Study

Strengths

The strengths of the thesis are many. Participants for the study were selected from a community setting with well defined criteria for inclusion and exclusion. The randomization was performed with standard procedures and assured good balance with respect to all potential confounding factors. Information of socio-demographic and dietary food intakes of the child were carefully obtained from the mother at their respective homes using structured questionnaires. The food frequency questionnaires were validated by comparison of data from the food frequency questionnaire to the 24-hour recall method in a subset of children; the correlation coefficients were low (i.e., 0.18, 0.30, 0.27 for protein, fat, zinc intakes, respectively) but similar to many other studies in adults (169). Blood samples were collected using standard procedures to avoid trace element contamination. In measurements of biomarkers, standard methods were used with calibration and replication in a subset. The biochemical measurements had a coefficient of variation ranging from 1.54% to 6.34%;

ideally, the analytical CV for an assay used in epidemiologic study is around 5% (176). Therefore, the study had minimal measurement errors in data collection.

For the intervention, the zinc and placebo tablets were identical in physical appearance and were administered in the presence of investigators. No adverse effects of intervention (average duration of 5.8 months/6 months = 96.7%) were noted. Very few participants were lost to follow-up (n = 5 among 720 participants).

The post-hoc analysis showed that the sample size of participants (n = 720) were sufficient to detect the differences between two groups as small as 1.5 μg zinc/dL, 5.2 μg ferritin/L, and 2.5 μg copper/dL with type-1 error α of 0.05 and power of 0.8. Missing data were only 11.9%, 4.0%, and 6.7% in papers 1, 2, and 3, respectively. The measures of associations between key variables were adjusted for appropriate covariates (e.g., age, gender, serum CRP, intervened groups) by regression models. The covariates were selected based on their possible associations with the independent and dependent variables in the literature (i.e., age, gender, serum CRP). In addition, based on the results in paper 1, serum CRP was included as a covariate for the analysis of ferritin and copper concentrations because serum CRP was associated with ferritin and copper. Based on the results in paper 2, serum ALP and albumin were not included as covariates in the association between zinc supplementation and serum zinc. We categorized biomarkers and dietary intakes based on conventional definitions. For serum CRP concentration, the cutoffs were suggested from clinical values, CRP distributions, and statistical differences in outcomes. Dietary zinc intakes from the FFQ were adjusted for total calorie intake using the residual regression method of Willett, and the estimates in regression models were later adjusted for measurement errors.

In multiple regression models, possible confounding and interaction factors were considered.

Limitations

The study had some limitations. A sample size of 720 participants was initially calculated for cognition and behavior outcomes. This number may be not sufficient to detect associations between biomarkers of inflammation and nutrient status. In the first two papers, data were analyzed using a cross-sectional analysis, but participants were selected for a randomized controlled trial. Therefore, this may affect the ability to generalize to other populations with different characteristics.

In paper 1, the associations between serum CRP concentration and biomarkers were not strictly linear. Therefore, we used non-parametric statistic tests, which have less power to detect associations. In addition, we used serum CRP as the only biomarker of inflammation; a better approach would have been to include at least two measures of inflammation (246). Thus, the adjusted prevalence of low ferritin in this study may be underestimated.

In paper 2, one of four key variables was dietary zinc intake, which may contain considerable measurement error. The FFQ method is less accurate than multiple 24-hour recalls or records, especially in children. Few previous studies have validated FFQ in children. Dietary phytate intake in participants could not be estimated because phytate content is absent in the food composition table of Guatemala. Therefore, the associations between dietary zinc intakes and other biomarkers may be underestimated and the estimates may be biased towards the null.

In paper 3, the striking problem is the unexpected school milk program, which simultaneously occurred in both intervention and control groups during the six-month zinc supplementation. Although it did not affect the validity of the results, it may have undermined the estimates and falsely led to null associations.

7.3. Future Studies

In paper 1, we used only one biomarker of inflammation (i.e., serum CRP). Future studies using two biomarkers (e.g., serum CRP and AGP) will permit us to identify a subgroup of healthy children with low serum CRP and low AGP levels and better assess the impact of high CRP levels > 1 mg/L on serum nutrient concentrations.

In paper 2, the consistent association between dietary zinc intakes and serum zinc concentration before and after zinc interventions was demonstrated in a sample of school children from 6 to 11 years of age. Future studies are recommended to investigate the association among zinc-related variables in children under 5 years.

In paper 3, the zinc supplementation trial was compromised by the zinc-fortified school milk program which had no control group. It is advisable to conduct randomized controlled studies (with a placebo group) of fortified milk to properly assess the potential harmful effects of fortified milk program on iron and copper status.

7.4. Implication of the Study Findings

The results of this study, if verified, have several implications. Paper 1 suggests that serum CRP is not associated with biomarkers of zinc status in healthy school children. Therefore, measuring biomarkers of inflammation may not be necessary in the assessment of

zinc deficiency in healthy children. In addition, the CRP cutoffs of 1 mg/L or lower require the use of high-sensitive CRP methods instead of traditional CRP methods. Although the cost of hs-CRP methods is greater than traditional CRP methods, its use may improve the estimation of iron deficiency prevalence in community-based studies in children.

Paper 2 suggests that serum zinc concentration is a reliable biomarker of zinc status in individuals with either low or high dietary zinc intakes. Serum ALP and albumin are not useful in marginal zinc deficiency.

Paper 3 suggests that zinc supplementation with a daily chewable zinc tablet of 10 mg zinc oxide during 6 months is safe and beneficial in the improvement of zinc status in children. Therefore, zinc oxide tablets are safe at the RDA dosage for at least six months in order to improve zinc status in children in regions with high risk of zinc deficiency.

7.5. Conclusions

This study presents several new facts. First, this is the first study which investigates the association between CRP concentration and copper concentrations in healthy children. Second, this is the first study which detects significant increases in serum ferritin and copper concentration beginning at CRP levels as low as >0.5 mg/L. It suggests that low CRP cutoffs (e.g., 0.5 mg/L, 1 mg/L) can be more effective than commonly used higher cutoff points in adjusting serum ferritin and copper concentrations for inflammation.

In summary, this study showed that serum CRP was associated with serum ferritin and copper but not with zinc status. In a population at very low risk of iron deficiency, inflammation had a small impact on ferritin levels and may not need further adjustment. However, the low risk of inflammation still influenced copper status. In adjusting for

inflammation, low CRP cutoffs led to higher estimated prevalence of iron and copper deficiencies, compared to conventional CRP cutoffs of 5 mg/L or 10 mg/L. Serum zinc concentration was consistently associated with dietary zinc intakes before and after zinc supplementation, and thus, was a reliable biomarker of zinc status in individuals. Finally, zinc supplementation improved zinc status but did not influence iron and copper status. The fortified-milk program benefited zinc status but may have been harmful for iron and copper status.

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APPENDICES

Appendix 1: Form for Summary of Admission and Group Assignment

INSTITUTE OF NUTRITION OF CENTRAL AMERICA AND PANAMA (INCAP)
EMORY UNIVERSITY
"Zinc, Mental Health and School Performance in Guatemala"

EMORY UNIVERSITY

SUMMARY OF ADMISSION & GROUP ASSIGNMENT

1) Form 0 3

2) ID child

3) School

4) Grade and section

5) Date of application (dd) (mm) (aa)

6) Code of the interviewer

7) Child's initial

8) Gender (1=Male 2=Femal)

9) Date of birth of the child (a) (dd) (mm) (yy)

10) Age of last birthday (L.B) (yy) (mm)

(Child must be 7 years old, but no more than 10 years and 6 months to baseline, in grades 1 to 4)

11. Does the child understand and speak Spanish fluently? 0= NO 1= YES

12. Does the mother understand and speak Spanish fluently? 0= NO 1= YES

13. Are eligible for age 0= NO 1= YES

14. Are eligible for language (for child) 0= NO 1= YES

15. Are eligible for health 0= NO 1= YES

16. Final decision: 1= Eligible to participate in the study

2= Is eligible but not applying the questionnaires to the mother

3= Not eligible and will not participate

Appendix 2: Form for Socio-Economic Information

INSTITUTE OF NUTRITION OF CENTRAL AMERICA AND PANAMA (INCAP)
EMORY UNIVERSITY
"Zinc, Mental Health and School Performance in Guatemala"

EMORY UNIVERSITY

DEMOGRAPHIC INFORMATION
At Baseline

Form 0 1

Child ID (a)

Interviewer Code

Date of Interview (dd) (mm) (aa)

Grade and section

VII. Housing characteristics

7.1 What type of housing does the family occupy? 7.1
1) Improvised house
2) Rancho
3) Room in house neighborhood
4) Appartment
5) Formal house
6) Other

7.2 Is
1) Bor
2) Leased
3) Owned
4) Shared
5) Other
9) Do not know 7.2

7.3 What is the main material of the walls of the house? 7.3
1) Print
2) Adobe
3) Lepa, stick or cane
4) brick or block
5) wo
6) oth

7.4 What is the main material of the lafloor of the house? 7.4
1) Soil
2) Cement
3) Wood
4) Granite/ceramic floor
5) Oth

7.6 Where do you get main water used for your household? 7.6
1) Pipe (red), within housing
2) Pipe, outside the house but on the filed
3) Pipe jet public
4) Public or private Pozo
5) River, lake, spring
6) Tank truck
7) Rain
8) Othe

7.7 Is there electricity in the house? 7.7
0= NO 1= YES

7.8 What type of service health is the home? 7.8
1) Network attached toilet drainage
2) Toilet connected to a septic hole
3) Washable
4) Latrine or cesspool
5) No hay

7.9 ¿ Where is the kitchen? 7.9
0= No
1= In the bedroom
2= Separate place

7.11 ,Where do you cook? How many room does the house have? (Not included kitchen, bathrooms, hallways, garages and dedicated to business) TOTAL ROOMS 7.11

7.12 doe (Not included kitchen, bathrooms, hallways, garages and dedicated to business) ROOMS 7.12

7.13
0= 1=
a) radio/record 7.13.a
b) television 7.13.b
c) refrigerador 7.13c
d) closet 7.13d
e) iron 7.13e
f) cell 7.13f
g) car 7.13g
h) mot 7.13h
i) bike 7.13i
j) dogs/ cats 7.13j
k) birds 7.13k
l) Cable TV 7.13l
m) VHS/DVD 7.13m
n) microwave 7.13n
o) cow 7.13o

7.5 What is the main material of the roof of the house 1) Palma or similar 2) Metal sheet 3) Teja 4) Concrete 5) Abestos sheet cement 6) Other (specified) _____	7.5 ☐	7.10 **What Where do you use** most of the time for cooking 1) Gas cooktop 2) Electric cooktop 3) Firewood and charcoal cooker 4) Other (specified) _____ _____	7.10 ☐	7.14 Do you own this house? hogar? **0= NO 1= YES** a) Father salary b) Mother salary or sales c) Work from other members in household d) Support of children work e) Remittance of a relative in the foreign f) Other _____	a ☐ b ☐ c ☐ d ☐ e ☐ f ☐
				7.15 This interview is conducted in: 1= Home 2= Clinic	☐

Appendix 3: Food Frequency Questionnaire Form

INSTITUTE OF NUTRITION OF CENTRAL AMERICA AND PANAMA (INCAP) FREQUENCY OF FOOD CONSUMPTION OF CHILD

Form | 1 | 9 |

Stage of study [ ]

1=pilot, 2= basal, 3= final

Child ID [ ][ ][ ][ ]

Date of interview [ ][ ][ ][ ][ ][ ]

dd mm yy

Initial of child [ ][ ][ ][ ]

Interviewer code [ ][ ]

Code of person interviewed [ ]

1=mother; 2=caretaker if mother absent; 3=other

I. KNOWLEDGE OF MOTHER/GUARDIAN ABOUT CHILD'S FEEDING MAIN FOOD

(Complete this section only if the respondent is the mother or, in her absence, the primary caretaker)

At this point begin to ask about food consumed by (Child's name)

1. Do you know what (child's name) in the last 7 days, not counting what as given in school?

0=NO ↴ 1=YES (Go to section II)

___

2.1.a. Do you know what (child's name) ate at breakfast in the last 7 days? ***0=NO, none of the days*** ***1=Yes, a few days*** ***2=Yes, every day*** ***8=Do not eat at that time***	⇨	___	2.1.b How many days can you tell me what (child's name) ate at breakfast?	___
2.2.a Do you know what (child's name) ate brought for snack at chool in the last 7 days ***0=NO, none of the days*** ***1=Yes, a few days*** ***2=Yes, every day*** ***8=Do not eat at that time***	⇨	___	2.2.b How many days can you tell me what (child's name) brought for snack at school?	___
2.3.a Do you know what (child's name) ate at lunch in the last 7 days? ***0=NO, none of the days*** ***1=Yes, a few days*** ***2=Yes, every day*** ***8=Do not eat at that time***	⇨	___	2.3.b How many days can you tell me what (child's name) ate at lunch?	___
2.4.a Do you know what (child's name) ate at dinner in the last 7 days? ***0=NO, none of the days*** ***1=Yes, a few days*** ***2=Yes, every day*** ***8=Do not eat at that time***	⇨	___	2.4.b How many days can you tell me what (child's name) ate at dinner?	___
2.5.a Do you know what (child's name) ate between meals in the last 7 days? ***0=NO, none of the days***	⇨	___	2.5.b How many day can you tell me what (child's name) ate at meals?	___

1=Yes, a few days ***2=Yes, every day*** ***8=Do not eat at that time***			

3. Who knows most of the items that you were not able to report on for what (child's name) ate in the last 7 days?

1. **Name**

_____

II. FREQUENCY OF FOOD CONSUMPTION

The following questions are about what (child's name) eats in the last 7 days. I'll ask how many days a week and how many time a day (child's name) eats each food mentioned.

	FOOD	**FREQUENCY**		
		Do you eat (food) in the last 7 days 0=NO, 1=YES	How many day is (food) given in the last 7 days **(1-7)**	How many times a day is (food) given?
4.	**BEANS**			
4.1	Beans without processed, drained, or liquefied	___	___	___
4.2	Fried or refried beans	___	___	___
5.	**TORTILLAS**	___	___	___
6.	**BREAD**			
6.1	French bread	___	___	___
6.2	Sweet bread	___	___	___
6.3	Pancakes	___	___	___
7.	**ATOL**			
7.1	Incaparina	___	___	___

7.2	Quaker Oats	___	___	___
7.3	Mosh (oats per pound)	___	___	___
8.	**INSTANT DRY CEREAL**			
8.1	Cereal type or other non-sweet cornflakes	___	___	___
8.2	Cereal type, frest flakes or other sweet Chococrispis	___	___	___
9.	**MILK**			
9.1	Fluid milk	___	___	___
9.2	Powder milk	___	___	___
9.3	Sliced cheddar cheese	___	___	___
9.4	Fresh or hard cheese	___	___	___
9.5	Cream cheese	___	___	___

	FOOD	**FREQUENCY**		
		Do you eat (food) in the last 7 days 0=NO, 1=YES	How many day is (food) given in the last 7 days **(1 - 7)**	How many times a day is (food) given?
9.6	Cream	___	___	___
10.	**EGG**			
10.1	Hard-boiled or cooked egg	___	___	___
10.2	Fried or scrambled cake	___	___	___
11.	**WINES AND SOUPS**			
11.1	Chicken or chicken broth	___	___	___
11.2	Broth beef or rib	___	___	___
11.3	Maggi Soup on or Mahler type	___	___	___
11.4	Pepian of chicken, ribs, or beef	___	___	___
12.	**PASTA AND RICE**			
12.1	Cooked rice	___	___	___
12.2	Fried rice or rice soup	___	___	___

12.3	Noodle Sauce	___	___	___
12.4	Noodles with cream or margarine	___	___	___
12.5	Chaomein	___	___	___
13.	**POTATO/BANANA**			
13.1	Beaked, steamed or pureed potato	___	___	___
13.2	French fries or golden cake	___	___	___
13.3	Baked banana	___	___	___
13.4	Fried banana	___	___	___
14.	GREEN VEGETABLES			
14.1	Chayote	___	___	___
14.2	Herbs (watercress, or quilete macuy)	___	___	___
14.3	Lettuce	___	___	___

	FOOD	**FREQUENCY**		
		Do you eat (food) in the last 7 days 0=NO, 1=YES	How many day is (food) given in the last 7 days **(1 - 7)**	How many times a day is (food) given?
14.4	Carrot	___	___	___
14.5	Avocado	___	___	___
14.6	Cucumber	___	___	___
14.7	Skin of green beans or cauliflower	___	___	___
14.8	Green beans	___	___	___
14.9	Coauliflower	___	___	___
14.10	Corn	___	___	___
14.11	Tomato or chirmol	___	___	___
14.12	Radish	___	___	___
14.14	Would you eat (name) any other vegetable in the last 7 days? 0=NO (go to question 15.1) 1=YES (What? Record in 13.15 to 14.17)	___		
14.15	_____		___	___
14.16	_____		___	___

14.17	_____		___	___
15.	**MEAT**			
15.1	Roast, stewed or baked chicken	___	___	___
15.2	Yellow, fried, breaded or medallone chicken	___	___	___
15.3	Roast or cooked beef	___	___	___
15.4	Steak, fried or bread beef	___	___	___
15.5	Cooled pork	___	___	___
15,6	Fried pork	___	___	___
15.7	Pork tortitas	___	___	___
15.8	Sausages (salchicha, longaniza, chorizo)	___	___	___

	FOOD	**FREQUENCY**		
		Do you eat (food) in the last 7 days 0=NO, 1=YES	How many day is (food) given in the last 7 days **(1 - 7)**	How many times a day is (food) given?
15.9	Ham	___	___	___
15.10	Fried fish	___	___	___
15.11	Viscerales (kidney, liver of chicken, beef)	___	___	___
16.	**FASTFOOD**			
16.1	Burger	___	___	___
16.2	Hot-dog	___	___	___
16.3	Pizza	___	___	___
17.	**TYPICAL FOOD**			
17.1	Tostadas	___	___	___
17.2	Tamal	___	___	___
18.	**BEVERAGES**			
18.1	Coffee, with or without milk	___	___	___
18.2	Natural fresh (lemons,	___	___	___

	oranges, jamaica)			
18.3	Fresh/artificial juice (toki, tang, Tampico)	___	___	___
18.4	Soda	___	___	___
18.5	Fruit liquide	___	___	___
19.	**GOODIES**			
19.1	Bag of goodies (curls, tortrix, Sabritas poporopos, pork)	___	___	___
20.	**COMPLEMENT OR ADD**			
20.1	Margarine added to bread or other food preparation	___	___	___
20.2	Mayonnaise added to bread or other food preparation	___	___	___
20.3	Sugar added to coffee or other food preparation	___	___	___

	FOOD	**FREQUENCY**		
		Do you eat (food) in the last 7 days 0=NO, 1=YES	How many day is (food) given in the last 7 days **(1 - 7)**	How many times a day is (food) given?
21.	**FRUIT**			
21.1	Banana	___	___	___
21.2	Orange	___	___	___
21.3	Watermelon	___	___	___
21.4	Melon	___	___	___
21.5	Green mango	___	___	___
21.6	Fresh mango	___	___	___
21.7	Would you eat (name) any other fruit in the last 7 days? 0=NO (go to question ***22.1***) 1=YES (What? Record of ***21.8 a 21.9***)	___		
21.8	_____		___	___
21.9	_____		___	___
22.	**TREATS**			

22.1	Cookies	___	___	___
22.2	Gelatin	___	___	___
22.3	Dulcitos, gum, candy or any kind of candy	___	___	___
22.4	Ice cream	___	___	___
22.5	Water ice or snow	___	___	___
23.	**OILS AND FATS**			
23.1	What brand of oil used for cooking in the last 7 days?	___ ___	___	___
23.2	Did you use margarine for cooking in the last 7 days? 0=NO (go to question ***23.4***) 1=YES	___		
23.3	Which brand of margarine do you use?	___ ___	___	___

	FOOD	**FREQUENCY**		
		Do you eat (food) in the last 7 days 0=NO, 1=YES	How many day is (food) given in the last 7 days **(1 - 7)**	How many times a day is (food) given?
23.4	Did you use butter for cooking in the last 7 days? 0=NO (go to question ***24***) 1=YES	___		
23.5	Which kind of butter do you use? 1=vegetable 2=animal	___	___	___
24.	Did you eat (name) any other food in the last 7 days? 0=NO (go to question ***25***) 1=YES (What? Anotate ***24.1 a 24.5***)	___		
24.1	_____		___	___
24.2	_____		___	___
24.3	_____		___	___
24.4	_____		___	___
24.5	_____		___	___

25.	**MICRONUTRIENT SUPPLEMENTATION**			
25.1	Vitamin tablets or syrup Brand: _____ Iron (mg) ___ ___ . ___ Zinc (mg) ___ ___ . ___ Folic acid or folate (mcg) ___ ___ ___	___	___	___

Comments:

_____

_____

_____

_____