ZINC SUPPLEMENTATION AND INSULIN SECRETION IN CHILDREN

by

ANDREA J. LOBENE

(Under the Direction of Richard Lewis)

ABSTRACT

Many children in the US are not meeting the Recommended Dietary Allowance (RDA) for zinc. Altered zinc status has been observed in prediabetic and diabetic individuals, and the incidence of type-2 diabetes is growing among children. Zinc is required for the maturation of insulin in beta cells, and its effect on insulin in children is uncertain. The objective of this study was to determine the effect of supplementation with 9 mg/d zinc over 4 weeks on insulin in healthy children (N = 147), and to determine if the effect differs by race. Serum insulin, glucose, and C-peptide were assessed, and homeostatic model assessment (HOMA2) was calculated. An increase in C-peptide was observed in the PL group, but not the ZN group, in blacks, but not whites (p=0.06). No changes were observed for HOMA2. Our results suggest 9 mg/d zinc may attenuate an increase in insulin secretion in black children, suggesting a potential role for zinc in the treatment of type-2 diabetes.

INDEX WORDS: Zinc; insulin; insulin secretion; children; beta cell function; C-peptide; HOMA; pubertal growth
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ANDREA J. LOBENE

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ANDREA J. LOBENE

Major Professor: Richard Lewis
Committee: Arthur Grider
           Nathan Jenkins

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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CHAPTER 1
INTRODUCTION

Though zinc deficiency in developed countries is rare, evidence suggests that many children in the U.S. aren’t meeting the recommendations for zinc intake (1, 2). Indeed, adolescents may be susceptible to marginal zinc deficiency as a result of poor eating habits combined with increased requirements for growth (3). Zinc is necessary for maturation, storage and secretion of insulin in pancreatic β-cells (4). Cell culture studies have suggested that zinc ions co-secreted from the β-cells with insulin may have an inhibitory effect on glucose-stimulated insulin secretion (5). However, in human studies the effects of zinc supplementation on insulin levels and glycemic control are unclear. Some studies in adults report no difference in insulin concentration or insulin resistance after supplementation (6), while others report slight increases in insulin and C-peptide (9) or decreases in insulin and insulin resistance (8). The equivocal findings are likely due to differences in the age and health status of participants, zinc doses, duration of supplementation, and outcome variables. Cross-sectional studies in adults have shown that serum zinc is positively associated with insulin sensitivity (9) and negatively associated with insulin resistance (10).

Diabetes is a growing problem in the United States, and altered zinc status has been observed in prediabetic and diabetic individuals (11, 12). Diabetics often exhibit increased urinary zinc excretion and decreased plasma zinc status compared to healthy subjects (13, 14). Type-2 diabetes is an increasing concern for children as well as adults. Nearly one in four U.S. children are prediabetic (15), and the incidence of type-2 diabetes is growing among children
Developing type-2 diabetes during childhood increases the risk of diabetes complications early in adulthood (17). Zinc supplementation has been explored as a possible adjunct therapy for management of type-2 diabetes, but the outcomes have been mixed (14).

It is known that children experience variations in insulin secretion and insulin sensitivity during adolescence (18-20). Even in healthy children, normal adolescent growth is characterized by variability in insulin sensitivity and insulin secretion (18, 19). During the early stages of sexual maturation, a dip in insulin sensitivity is observed in both boys and girls (18, 21). These changes have been observed in lean and obese children alike, though the insulin resistance may worsen with longer duration of obesity (21). Children seem to experience a progressive decrease in insulin sensitivity from Tanner stage I to Tanner stage III and IV (19). Insulin secretion increases in response to the decreased insulin sensitivity, but whether or not insulin secretion fully compensates for this normal progression in insulin insensitivity is still unclear (18-20). Moreover, it has been shown that normal insulin secretion and sensitivity are different in black vs. white adolescents, with blacks having greater insulin secretion and lower insulin sensitivity than whites (22-24).

The relationship between zinc and insulin has not been well researched in healthy children. Cross-sectional analyses in both normal weight and overweight and obese children and adolescents have shown that those with lower serum zinc concentrations have significantly higher insulin concentrations and insulin resistance indices (25-28). Significantly higher homeostatic model assessment (HOMA) values have also been observed in children with low dietary density of zinc, defined as <0.87 mg/megajoule (MJ) compared to those with higher dietary density (26). To date, few intervention studies have been conducted in children, and no similar studies have been conducted in children of different races. One study by Hashemipour et
al found that fasting insulin and HOMA-IR decreased significantly after four weeks of zinc supplementation in obese children (29). Taken together, these studies suggest that children with poor zinc status, assessed by either zinc intake or serum zinc, may exhibit altered insulin secretion and sensitivity, and that zinc supplementation may help correct these alterations. The current study will add to the limited literature on zinc supplementation and insulin in children, and will be the first study to address racial differences in the effects of zinc supplementation on insulin outcomes. Unlike most previous studies, the current study will use C-peptide to assess insulin secretion, which is a more reliable indicator than serum insulin (30, 31).

The purpose of the study presented in this thesis was to examine the influence of zinc supplementation on beta cell function, insulin secretion and insulin resistance in healthy females in the early stages of puberty. The specific aims were to: 1) determine if zinc supplementation has an effect on beta cell function and insulin secretion in healthy female adolescents and 2) determine if there is a difference in the effect of zinc supplementation on beta cell function and insulin secretion in blacks vs. whites. This project will fill in the knowledge gaps about the relationship between zinc and insulin secretion in black and white adolescents through the measurement of C-peptide and calculation of percent beta cell function (HOMA-%β). The original focus of this project was on strictly insulin secretion. While insulin secretion still remains the primary outcome, after further review of the literature, the manuscript (chapter 3) will reflect a more clinical approach to prevention and management of diabetes.
References


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CHAPTER 2
REVIEW OF LITERATURE

Introduction

Though zinc deficiency in developed countries is rare, evidence suggests that many children in the U.S. aren’t meeting the recommendations for zinc intake (1). Zinc is necessary for maturation, storage and secretion of insulin in pancreatic β-cells (2), and altered zinc status has been observed in prediabetic and diabetic individuals (3, 4). Diabetes is a growing problem in the United States for adults, but also for children. Nearly one in four U.S. children are prediabetic (5), and the incidence of type-2 diabetes is growing among children (6). From 2008-2009, in the U.S. 5,089 people younger than 20 years old were newly diagnosed with type-2 diabetes annually (6). Developing type-2 diabetes during childhood increases the risk of diabetes complications early in adulthood (7). Zinc supplementation has been explored as a possible adjunct therapy for management of type-2 diabetes, but the outcomes have been mixed (8). To date, few zinc intervention studies have been conducted in children, and no similar studies have been conducted in children of different races. To our knowledge, only one zinc intervention trial has been conducted in children, and they found that fasting insulin and HOMA-IR decreased significantly after four weeks of zinc supplementation in obese children (9). In the following literature review, I will present background information on diabetes, the mechanism of insulin synthesis, racial differences in insulin secretion and sensitivity, insulin secretion and sensitivity during childhood, zinc recommendations and biomarkers of zinc status, preliminary studies on zinc and insulin, and a review of methods that provide the basis for my thesis research project.
Diabetes

Type-2 diabetes is characterized by an alteration in the balance between insulin sensitivity and insulin secretion (10). Specifically, type-2 diabetes is characterized by insulin resistance and beta cell dysfunction (11). Additionally, a deficit of beta cell mass is shown to be a pathophysiological feature of type-2 diabetes (11). A deficit of beta cell functional mass is present in type-2 diabetics, and progressively declines with disease duration (11). Development of early onset type-2 diabetes (before the age of 45) results from a combination of genetic and environmental factors, including obesity, family history of type-2 diabetes, being of minority race or ethnicity, and physical inactivity (10).

Diabetes is a growing problem in the U.S. As of 2012, nearly 10% of the U.S. population has diabetes, and it is estimated that nearly 28% of those with diabetes have not been diagnosed (12). Over 12% of the adult population has diabetes, and the prevalence is higher in minorities than in non-Hispanic whites (12). Among those with diabetes, nearly 90% have type-2 diabetes (11). The incidence of type-2 diabetes is growing among children as well, with more than 5,000 estimated new cases diagnosed among youth younger than age 20 each year (12). Nearly one in four U.S. children are prediabetic or diabetic, and a greater prevalence of prediabetes/diabetes is observed in children who are overweight or obese (13). Though diabetes is associated with serious complications such as kidney disease, blindness and heart disease, some of these can be reduced with early detection and good blood glucose control (12). Current treatment strategies for diabetes include healthful eating and medical nutrition therapy to improve management, staying physically active, and medications to help lower blood glucose (12, 14). Because beta-cell dysfunction is associated with poor glycemic control in type-2 diabetics, preservation and recovery of beta cell functional mass is an important therapeutic strategy, with lifestyle
modification and weight reduction being the most important therapies to do so (11). Metformin is also considered a first-line therapy in most guidelines for the treatment of type-2 diabetes (11).

**Insulin synthesis, storage and secretion**

Zinc is necessary for maturation, storage and secretion of insulin in β-cells (2). Insulin is first synthesized at the ribosome of the β-cell as preproinsulin (2, 15) (Figure 1). Preproinsulin is translated in the endoplasmic reticulum (ER) (Figure 1, step 1) and secreted into the lumen of the rough ER, where it is cleaved to form proinsulin, the precursor to the active form of insulin (2) (Figure 1, step 2). Proinsulin is then transported to the Golgi apparatus, where it is packaged into secretory granules (Figure 1, step 3) and cleaved to form insulin and C-peptide (2, 15) (Figure 1, step 4). Meanwhile, zinc ions are transported into the secretory granules (Figure 1, step 5), and in the presence of these zinc ions insulin undergoes a maturation process, forming 2-Zn-hexameric complexes (2) (Figure 1, step 6). This process increases the insulin storage capacity (2). Upon receiving a stimulatory signal, such as a change in plasma glucose, the mature insulin and C-peptide are secreted via exocytosis into the circulation (2, 15, 16) (Figure 1, step 7). When the insulin hexamers are secreted into the blood stream, they dissociate into monomers, the active form of insulin (15).

**FIGURE 1.** Mechanism of insulin synthesis, storage, and secretion in the β-cell.
Racial differences in insulin secretion and sensitivity

Differences in insulin sensitivity and insulin secretion have been observed in subjects of different races. African American adults demonstrate decreased insulin sensitivity and increased insulin secretion compared to Caucasians (17, 18). While researchers have hypothesized that these differences could be due to the greater prevalence of obesity among African Americans, inherent ethnic differences in insulin sensitivity and beta cell function may exist. A study conducted in healthy girls and women found African Americans had lower insulin sensitivity and greater beta cell responsivity to a glucose tolerance test relative to their European American counterparts despite adjusting for percent body fat and intra-abdominal adipose tissue (19). The same study also found greater beta cell responsivity was independent of insulin sensitivity in African American women, providing further support for the existence of inherent racial differences (19). Similar results have been shown in adolescents, demonstrating that healthy black versus white adolescents had lower insulin sensitivity (20, 21), higher fasting insulin levels (22), and higher insulin concentrations in response to a glucose load (20, 21, 23). Additionally, a study conducted in black and white children found that black children had an upregulated beta cell function, as assessed by plasma insulin concentrations in response to a glucose infusion, relative to insulin sensitivity (20). A similar relationship has been observed in obese type-2 diabetic adolescents as well (24).

Insulin secretion and sensitivity in childhood/adolescence

Even in healthy children, adolescence is characterized by variability in insulin sensitivity and insulin secretion (25, 26). During the early stages of sexual maturation, a dip in insulin sensitivity is observed in both boys and girls (25, 27). These changes have been observed in lean
and obese children alike, though the insulin resistance may worsen with longer duration of obesity (27). Children seem to experience a progressive decrease in insulin sensitivity from Tanner stage I to Tanner stage III and IV (26), but the exact mechanism has yet to be determined (27). In later stages of sexual maturation, the level of insulin sensitivity seems to recover in girls, but remains low in boys (25). Insulin secretion increases in response to the decreased insulin sensitivity, but whether or not it is able to fully compensate is still unclear (25, 26, 28). Aside from this normal physiological change with maturation, children and adolescents are also increasingly being diagnosed with type-2 diabetes, a disease characterized by increased insulin resistance and compensatory increased insulin secretion (7).

**Zinc deficiency, RDA, dietary intakes and markers of zinc status**

Dietary sources of zinc include red meat, shellfish, legumes, nuts, seeds, fortified cereals, and whole grains (29, 30). Dietary zinc derived from plants is less bioavailable than that from animal products due to the presence of phytic acid that binds to zinc, which forms insoluble complexes and inhibits zinc absorption (29, 30). Overall, zinc deficiency in developed countries is uncommon. However, evidence suggests that many children in the U.S. aren’t meeting the recommendations for zinc intake (1, 31). The primary clinical feature of zinc deficiency is impaired growth velocity, which may be corrected with zinc supplementation (32). Indeed, adolescents may be susceptible to marginal zinc deficiency as a result of poor eating habits combined with increased requirements for growth (33). In addition, individuals with impaired gastrointestinal function may be at risk of zinc deficiency related to decreased nutrient absorption and increased excretion (32).

The RDA for zinc for children ages 4-8 years and 9-13 years is 5 mg/day and 8 mg/day,
respectively (34). The RDA for adolescents between the ages of 14-18 years differs based on gender (34), as the RDA for boys is 11 mg/day while the RDA for girls is 9 mg/day. The RDA is further broken down by life stage group for adults ages 19 and older. Adult men and adult women are recommended to consume 11 mg/day and 8 mg/day, respectively. The RDA is greater in pregnant and lactating females (34).

A reliable, sensitive, and specific index of zinc is still under investigation (35). Although a few functional indicators may be associated with zinc status, they are not specific to zinc and may be associated with other nutrient deficiencies or infection (36). Plasma/serum zinc concentrations are the most commonly used indices for evaluating zinc deficiency; however plasma zinc is under tight homeostatic control, and therefore may not reflect cellular zinc status (30). While assessment of plasma (or serum) zinc concentration does not reflect subtle changes in zinc status (35) it is the best available biomarker for determining zinc deficiency in populations (36), and there is evidence to suggest that plasma/serum zinc increases in response to zinc supplementation (37). The lower limit of normal fasting plasma zinc has been set at 10.7 μmol/L (70 μg/dL) (30). Suggested lower cutoffs of serum zinc concentration for assessment of risk of zinc deficiency have been developed from NHANES data, and are set at 70 μg/dL (10.7 μmol/L) in females ≥10 years old, and 74 μg/dL in males ≥10 years old (38). It is important to note that serum zinc does not necessarily reflect individual zinc status, and the use of serum zinc for diagnosis and treatment of individuals is not recommended at this time (36). Height- or length-for-age is the best functional outcome associated with the risk of zinc deficiency because low height- or length-for-age is often responsive to zinc supplementation (36). However, this outcome is not specific to zinc status and is only appropriate for use in children (36). In individuals, urinary excretion does not accurately reflect changes in zinc intake (35). There is
little evidence that hair zinc concentration is an effective marker of zinc status, and data suggests that platelet, mononuclear cell, and erythrocyte zinc concentration and alkaline phosphatase activity are also ineffective biomarkers (29). Other biomarkers for assessing zinc status have been explored. In particular, certain microRNAs (miRNAs) are shown to be responsive biomarkers to acute zinc depletion and repletion, and thus may be useful biomarkers of zinc status (39).

**Zinc and insulin and beta cell function**

**Animal/cell studies**

Cell culture studies have explored the potential mechanistic link between zinc and insulin secretion. Two studies by Slepchenko et al sought to determine how zinc regulates insulin availability and secretion (40, 41). The results of these studies in both β-cells and pancreatic islets suggest that insulin secretion is regulated by co-secreted zinc (40, 41). Specifically, the researchers showed that zinc ions co-secreted from the β-cells with insulin may inhibit glucose-stimulated insulin secretion through a negative feedback mechanism (40, 41).

Animal studies have been used to study the mechanisms of zinc transport and insulin maturation in beta cells. The ZIP transporters in the pancreatic beta cells take zinc from the extracellular space or from intracellular organelles and transfers them into the cytosol (42). From there, zinc transporter-8 (ZnT8) delivers zinc ion from the cytoplasm of pancreatic beta cells into intracellular vesicles (43). In tissue harvested from rats with diabetogenic conditions induced by an intermittent hypoxic challenge, a lower ZIP8 expression was shown. Additionally, as the level of ZIP8 transporters decreased, the level of insulin also decreased, and as the expression of ZIP8 in the plasma membrane decreased, the level of zinc imported by ZIP8 decreased (44). The
decrease in secreted insulin and C-peptide were likely not due to lack of production, but rather an inability of insulin molecules to precipitate and crystalize due to a lack of zinc (44). Importantly, no studies to date have determined whether zinc administration increases ZIP8 concentration in beta cells. ZIP6 and ZIP7 have also been shown to play a role in regulating cytosolic zinc concentrations and insulin secretion in beta cells (45). In a separate study conducted in mice, a deficiency in ZnT8 expression was associated with low zinc content in beta cells, insulin crystallization failure, and presence of atypical insulin granules lacking a detectable dense core (46).

The relationship between zinc and diabetes has been studied in the animal model. Zinc concentrations in the serum and femur were shown to be lower in genetically diabetic mice, which are representative of type II diabetes, compared to nondiabetic littermates (47). Urinary zinc concentrations were also shown to be higher in genetically diabetic mice compared to control animals (47). In genetically diabetic mice, dietary zinc supplementation was shown to attenuate fasting hyperglycemia, whereas a marginally zinc-deficient diet exacerbated fasting hyperglycemia (48). In the same study, diabetic zinc-supplemented mice also had higher pancreatic zinc concentrations than diabetic control mice (48).

Cross-sectional studies

Cross-sectional analysis in humans has demonstrated that diabetic patients have lower serum zinc concentrations than nondiabetics (3). Serum zinc concentration has also been shown to be significantly lower in prediabetics compared to normal groups, suggesting low zinc precedes the development of diabetes (4). Additionally, serum zinc has been shown in adults to be positively associated with insulin sensitivity (49) and negatively associated with insulin
resistance (50). Though one cross-sectional study found that in normal adults higher zinc was associated with a lower beta cell function, this was likely due to the fact that higher zinc was also associated with lower insulin resistance (4). Cross-sectional analyses in both normal weight and overweight and obese children and adolescents have shown that those with lower serum zinc concentrations (near or below the 10.7 μmol/L cutoff) have significantly higher insulin concentrations and insulin resistance indices (51-53). Significantly higher HOMA values have also been observed in children with low dietary density of zinc, defined as <0.87 mg/MJ, compared to those with higher dietary density (52).

**Preliminary zinc supplementation studies**

Zinc supplementation trials have been performed in individuals who have an increased risk of type-2 diabetes due to a polymorphism in the gene that codes the ZnT8 transporter in the beta cell. In one such study researchers hypothesized that zinc supplementation would improve insulin response to glucose stimulation in those with the susceptible genotypes, however they found no significant changes (54). Studies in obese adults have produced mixed findings. One study conducted in obese adults found no significant differences in fasting insulin, HOMA-IR, or insulin sensitivity following zinc supplementation (55), while another found significant decreases in fasting insulin and HOMA-IR following zinc supplementation (56). Studies of individuals diagnosed with diabetes have found more promising results. One study found significant increases in fasting insulin and C-peptide concentrations in subjects with more than 4 years’ duration of diabetes (57). Another study found zinc+multivitamin/mineral supplementation significantly decreased fasting blood glucose, postprandial blood glucose, and hemoglobin A1c values (58).
Few similar studies have been conducted in children. One study of obese children reported a decrease in insulin concentration, insulin resistance, and fasting plasma glucose with zinc supplementation (59). Another study by Kelishadi et al found that markers of insulin resistance decreased significantly after eight weeks of zinc supplementation in obese children (60). However, another study of type-1 diabetic children found no differences in fasting blood sugar or insulin concentrations after zinc and vitamin A supplementation (61). One meta-analysis of studies in both adults and children reported a significant, modest reduction in blood glucose concentrations with zinc supplementation, particularly in obese and diabetic patients (62). Overall, results from zinc supplementation studies have been inconsistent, likely due to differences in age and health status of the study sample, differences in amount of zinc given, and an inconsistency in outcome measures used. Moreover, no study has used C-peptide to assess insulin secretion.

**Serum glucose and insulin—methods**

**Homeostatic model assessment**

Homeostasis model assessment of percent beta cell function (HOMA-%β) is a model used to predict beta cell function based on fasting plasma insulin and glucose concentrations developed by Matthews et al (63). The equation for calculating HOMA-%β is as follows:

\[
OMA - %\beta = \frac{(20 \times FPI)}{(FPG - 3.5)}
\]

where FIP is fasting plasma insulin concentration (mU/l) and FPG is fasting plasma glucose (mmol/l) (64). This equation provides an estimate of beta cell function, and is correlated with measures of beta cell function from the hyperglycemic clamp, the intravenous glucose tolerance test, and the continuous infusion of glucose with model assessment in both normal and diabetic subjects (63, 64). HOMA-%β can be used in studies to
measure beta cell function and allows comparisons of beta cell function between normal subjects and those with abnormal glucose tolerance (64). When reporting HOMA-%β, the researcher should also report insulin sensitivity, as level of beta cell function is closely linked to level of insulin sensitivity (64). The use of HOMA-%β to estimate pancreatic beta cell function has been validated in nondiabetic children (65). HOMA2 is an updated computer model of the original HOMA that is useful for assessing absolute insulin resistance or β-cell function and has been recalibrated in line with current insulin assays (64).

**C-peptide**

C-peptide provides a reliable estimate of insulin secretion. C-peptide is a byproduct of insulin synthesis and is produced in equimolar amounts to insulin by the pancreatic beta cells (66-68). This makes C-peptide a good marker of the amount of endogenous insulin production (68). In addition, it has negligible extraction by the liver, constant peripheral clearance, and its half-life is much longer than that of insulin (66-68). C-peptide is the preferred method to assess endogenous insulin secretion in patients on insulin because an insulin assay will detect exogenous insulin (66, 68). C-peptide can be assessed by radioimmunoassay (average intra-assay CV 4%) (69). In the current study, C-peptide was analyzed on TOSOH 600 II analyzer in triplicate using immunofluorescence technology. The minimum sensitivity is 0.2 ng/mL, and the mean intra- and interassay CVs were 1.67% and 1.20%, respectively.

**Summary**

In summary, diabetes is a growing problem in the U.S, and the incidence of type-2 diabetes is rising among children (6). Zinc is necessary for maturation, storage and secretion of
insulin in β-cells (2). African American adults demonstrate decreased insulin sensitivity and increased insulin secretion compared to Caucasians (17, 18), and similar results have been shown in adolescents (20, 21, 23). Even in healthy children, adolescence is a period of time characterized by variability in insulin sensitivity and insulin secretion (25, 26).

While zinc deficiency in developed countries is rare, evidence suggests that many children in the U.S. aren’t meeting the recommendations for zinc intake (1, 31). Plasma or serum zinc is the most commonly used index for evaluating zinc deficiency. While assessment of plasma (or serum) zinc concentration does not reflect subtle changes in zinc status (35), it is the best available biomarker for determining zinc deficiency in populations (36), and there is evidence to suggest that plasma/serum zinc increases in response to zinc supplementation (31, 37). Animal studies have been used to study the mechanisms of zinc transport and insulin maturation in beta cells as well as the relationship between zinc and diabetes. Cross-sectional studies in humans have demonstrated that prediabetic and diabetic patients have lower serum zinc concentrations than nondiabetics (3, 4). Additionally, serum zinc has been shown in adults to be positively associated with insulin sensitivity (49) and negatively associated with insulin resistance (50).

Overall results from zinc supplementation studies have been mixed. Some studies have found no significant differences in fasting insulin, HOMA-IR, or insulin sensitivity following zinc supplementation (55). On the other hand, some have found significant decreases in fasting insulin and HOMA-IR following zinc supplementation (56, 59). The inconsistency in results may be due to differences in age and health status of the study sample, differences in amount of zinc given, and an inconsistency in outcome measures used. Few zinc supplementation studies of this sort have been conducted in children, and no zinc supplementation studies have been conducted
in blacks or children of different races. The purpose of the current project was to determine the influence of zinc supplementation on beta cell function, insulin secretion (C-peptide), and insulin resistance in healthy female children. A secondary purpose was to determine if the effect of zinc supplementation on these outcomes differs by race.
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CHAPTER 3

ZINC SUPPLEMENTATION DOES NOT ALTER INDICATORS OF INSULIN SECRETION AND SENSITIVITY IN BLACK AND WHITE FEMALE ADOLESCENTS

Zinc supplementation does not alter insulin secretion and sensitivity in black and white female adolescents¹-³

Authors: Andrea J. Lobene⁴, Joseph M. Kindler⁴, Nathan T. Jenkins⁵, Norman K. Pollock⁶, Emma M. Laing⁴, Arthur Grider⁴, Richard D. Lewis⁴∗

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³Abbreviations: BMI, body mass index; HOMA2, homeostatic model assessment; ZN, zinc group; PL, placebo group; %β, beta cell function; IR, insulin resistance; %S, insulin sensitivity; SD, standard deviation

⁴Department of Foods and Nutrition, University of Georgia, Athens, GA, USA; ⁵Department of Kinesiology, University of Georgia, Athens, GA, USA; ⁶Department of Pediatrics, Georgia Regents University, Augusta, GA, USA

*Corresponding Author: Richard D. Lewis, The University of Georgia, 279 Dawson Hall, Athens, GA 30602. Phone: 706-542-4901. Fax: 706-542-5059. Email: rlewis@fcs.uga.edu

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Running Title: Zinc supplementation and insulin in children
ABSTRACT

Background: Zinc is a micronutrient involved in various biological processes including the production of, and peripheral sensitivity to, the pancreatic B-cell-derived insulin. Despite being an essential nutrient, less than 70% of US youth meet the Recommended Dietary Allowance for zinc. The effect of zinc supplementation on insulin outcomes during youth has not been thoroughly investigated.

Objective: The objective of this study was to determine the influence of zinc supplementation on insulin outcomes in early adolescent girls. A secondary objective was to determine whether the effect of zinc supplementation on these outcomes differed by race (i.e., blacks versus whites).

Methods: Healthy non-Hispanic black and white girls ages 9-11 years were randomly assigned to receive either a daily zinc supplement (ZN; 9 mg elemental zinc per day; n = 75) or an identical placebo (PL; n = 72) for 4 weeks. Fasting insulin, glucose, and C-peptide were assessed at baseline and 4 weeks. C-peptide and glucose values were used to calculate homeostatic model assessment (HOMA2) of beta cell function (%β), insulin resistance (IR), and insulin sensitivity (%S). Baseline differences among all four race by treatment groups were determined by one-way ANOVA. Changes in outcome measures were compared using mixed design ANOVA.

Results: At baseline, blacks had higher plasma zinc concentration (P < 0.001) and HOMA-%β than whites. No significant interactions were observed for HOMA2-%β, HOMA2-IR and HOMA2-%S. However, treatment × race × time interactions for C-peptide and HOMA2-IR approached significance (P = 0.08) such that changes in C-peptide and HOMA2-IR was partially attenuated in the black children who were in the ZN group (P = 0.06).
**Conclusions:** Low dose supplementation (i.e., 9 mg/day) of elemental zinc did not affect any insulin outcomes in the subjects in the current study. Considering our marginally significant finding involving zinc supplementation and plasma c-peptide concentration, a measure of insulin secretion, additional trials that are appropriately powered should further explore this relationship.

6**Clinical Trial Registry Identifier:** ClinicalTrials.gov, NCT01892098

**Keywords:** Zinc; insulin; insulin secretion; children; beta cell function; C-peptide; HOMA; pubertal growth
INTRODUCTION

Though the occurrence of zinc deficiency in developed countries is uncommon, almost 25% of U.S. children do not meet the Recommended Dietary Allowance (RDA) for zinc (1). This is of particular concern considering the requirement for zinc increases during growth (2). Zinc is necessary for the maturation, storage and secretion of insulin from the pancreatic β-cells (3), perhaps contributing to the altered zinc status of prediabetic and diabetic individuals (4, 5). Results from cell culture studies have suggested that zinc ions co-secreted from the β-cells with insulin may have an inhibitory effect on glucose-stimulated insulin secretion, thus acting through a negative feedback loop (6). However, in human studies the effects of zinc supplementation on insulin levels and glycemic control are unclear. For instance, some studies in adults have reported a null effect of zinc supplementation on insulin concentration and insulin resistance after zinc supplementation (7), while others report slight increases in insulin and C-peptide (8) or decreases in insulin and insulin resistance (9). The equivocal findings are likely due to differences in the age and health status of participants, zinc doses, duration of supplementation, and outcome variables. Cross-sectional studies in adults have shown that serum zinc is positively associated with insulin sensitivity (10) and negatively associated with insulin resistance (11).

Diabetes is a growing problem in the United States, and disturbances in zinc homeostasis have been associated with diabetes. Diabetics often exhibit increased urinary zinc excretion and decreased plasma zinc status compared to healthy subjects (12, 13). Type-2 diabetes is an increasing concern for children as well as adults. Nearly one in four U.S. children are prediabetic (14), and the incidence of type-2 diabetes is growing among children (15). Developing type-2
diabetes during childhood increases the risk of diabetes complications early in adulthood (16). Zinc supplementation has been explored as a possible adjunct therapy for management of type-2 diabetes, but the outcomes have been mixed (13).

Fluctuations in insulin secretion and insulin sensitivity are common throughout pubertal development (17-19). During the early stages of sexual maturation, a decline in insulin sensitivity is observed in both boys and girls (17, 20). These changes have been observed in lean and obese children alike, though the insulin resistance may worsen with longer duration of obesity (20). Children seem to experience a progressive decrease in insulin sensitivity from Tanner stage I to Tanner stages III and IV (18). Insulin secretion increases in response to the decreased insulin sensitivity, but whether or not it is able to fully compensate is still unclear (17-19). Moreover, it has been shown that insulin secretion and sensitivity are different in black vs. white adolescents, with blacks having greater insulin secretion and lower insulin sensitivity than whites (21-23).

The relationship between zinc and insulin has not been well researched in healthy children. Cross-sectional analyses in both normal weight and overweight and obese children and adolescents have shown that those with lower serum zinc concentrations have significantly higher insulin concentrations and insulin resistance indices (24-27). Significantly higher HOMA values have also been observed in children with low dietary density of zinc compared to those with higher dietary density (25). To date, few intervention studies have been conducted in children, and no similar studies have been conducted in children of different races. One study by Hashemipour et al found that fasting insulin and HOMA-IR decreased significantly after four
weeks of zinc supplementation in obese children (28). Taken together, these studies indicate that zinc supplementation may decrease insulin secretion and insulin resistance, which in turn suggests zinc could have potential clinical applications for prevention or management of type-2 diabetes. The current literature is lacking in zinc intervention trials in children, and no studies have addressed racial differences in the response to zinc supplementation on insulin outcomes. In addition, no previous zinc trial in children has used C-peptide to assess insulin secretion, which is a more reliable indicator than serum insulin (29, 30).

The primary objective of this study was to conduct a secondary analysis of a previously conducted randomized placebo controlled trial to determine the influence of low-dose elemental zinc supplementation on insulin resistance and insulin secretion (C-peptide) in healthy early adolescent girls. A secondary objective was to determine if the effect of zinc supplementation on these outcomes differs by race.

METHODS

Study design and participants

This study is an ancillary analysis to a previously completed double blind, randomized, placebo-controlled zinc supplementation trial. Details on recruitment, randomization, enrollment and compliance following the CONSORT guidelines have been published previously (31). Participants were healthy non-Hispanic white and black girls ages 9-11 years. Exclusion criteria included onset of menses, as measured by self-report, and unwillingness to provide a blood
sample. All participants were required to have been at the early stages of breast development (32). All participants and legal guardians provided written consent and permission, respectively, and the Institutional Review Board on Human Subjects at the University of Georgia approved all study protocols and procedures.

**Zinc supplementation**

Tablets containing 9 mg of elemental zinc (zinc sulfate; 23 mg) and identical placebo pills (ie, in color, size and odor) were provided by Vesta Pharmaceuticals, Inc. (Indianapolis, IN). The pills were analyzed by an independent laboratory. Enrolled participants were randomly assigned to either the supplemental zinc \( n = 75 \) or placebo \( n = 72 \) group. All investigators, research personnel, and participants were blinded to the treatment conditions. Participants were made aware of the potential adverse events with consuming 9 mg Zn/d, though this level is considered safe. At the baseline visit participants were provided a 4-week supply of zinc or placebo tablets and were instructed to take one tablet per day. Empty pill bottles and unused tablets were returned to the laboratory staff at the completion of the 4-week trial. Compliance was measured by pill count and was defined as consuming greater than 80% of the supplement regimen over 4 weeks.

**Biochemical analyses**

Fasting blood samples were collected by a trained phlebotomist following an overnight fast at the baseline and 4 wk timepoints. Samples were stored at -80°C until analyses were
performed. Plasma zinc was determined by atomic absorption spectrophotometry using a Perkin
Elmer Analyst 400 (Shelton, CT) and accuracy was verified based on standards from the United
States Institute of Standards and Technology (Gaithersberg, MD). Serum glucose concentrations
were determined in triplicate using a microtiter modification of the enzymatic Autokit Glucose
method (Wako Chemicals USA). The detection limit for this assay is 0-500 mg/dL, and the mean
intra- and interassay coefficient of variations (CVs) were 1.8% and 2.2%, respectively. Serum
insulin was analyzed in duplicate by Human Insulin Specific RIA (HI-14K; Millipore). The
detection limit is 3.125-100 uU/mL. The mean intra- and interassay CVs were 3.5% and 5.3%,
respectively. C-peptide was analyzed on TOSOH 600 II analyzer in triplicate using
immunofluorescence technology. The minimum sensitivity is 0.2 ng/mL, and the mean intra- and
interassay CVs were 1.67% and 1.20%, respectively. As a function of fasting C-peptide and
glucose concentrations, HOMA2-%B, HOMA2-IR, and HOMA2-%S were calculated using the
HOMA2 calculator v2.2.3 (33).

Dietary assessment

All participants were instructed not to alter their dietary intakes during the study period.
Energy, protein and zinc intakes were assessed at baseline and at 4 weeks using 3-d diet records,
and each subject and their parent received instructions on how to complete these records at
home. Diet records were analyzed using the Food Processor SQL version 9.7.3 (ESHA Research,
Salem, OR) and the average over three days reported. Average measure (3-day) ICCs were
calculated in girls aged 6-10 years (n = 10), whose 3-day diet records were completed twice over
a 2-week period and calculated for vitamin D, calcium, and energy (all ≥ 0.86).
Anthropometry and sexual maturation

Height and body weight were measured at baseline and at 4 weeks. Weight was measured to the nearest 0.1 kg using an electric scale (Seca Bella 840, Columbia, MD). Height was measured using a wall-mounted stadiometer to the nearest 0.10 cm (Novel Products Inc., Rockton, IL). Height and weight were used to calculate BMI-for-age percentiles (34). Sexual maturation stage was determined by self-assessment using the stages of breast development method described by Tanner (32).

Statistical analyses

Power analyses were computed based on the primary outcomes of C-peptide and HOMA-%β using the Simple Interactive Statistical Analysis Program (http://www.quantitativeskills.com/sisa/index.htm). Alpha was set at 0.05 using 80% power and a two-tailed approach for both outcomes. Using a zinc supplementation study in diabetic and nondiabetic adults by Oh et al (8) a sample size of 127 was estimated for observing significant changes in C-peptide with zinc supplementation. It is hypothesized that HOMA-%β will be modestly correlated with plasma zinc values; for correlations of \( r = 0.2, 0.3, \) or \( 0.4 \), sample sizes of approximately \( n = 194, 85, \) or \( 47 \), would be needed for these correlations to be considered statistically significant (http://www.cct.cuhk.edu.hk/stat/other/correlation.htm). Thus, a sample size of 130 would allow detection of a correlation as low as \( r = 0.3 \). Therefore, a sample size of 130 would be sufficient to see significant results for both C-peptide and HOMA-%β.
Data were examined for normality and logarithmic transformations were applied if necessary for analyses, but backtransformed for presentation of results. Baseline differences among all four race-treatment groups were determined by one-way ANOVA. Changes in outcome measures were compared using mixed ANOVA design in an intention-to-treat analysis strategy using all available data. Time was used as the within-subjects factor and treatment and race as between-subjects factors. Further analyses used Pearson bivariate correlations to quantify relationships between plasma zinc status and the insulin outcomes of interest, and partial correlations to quantify associations between baseline plasma zinc status and biochemical measures of insulin secretion controlling for insulin resistance. All statistical analyses were performed using SPSS software (version 22, IBM SPSS Statistics, Chicago, IL) and statistical significance was set at $P < 0.05$.

RESULTS

Of the 147 total participants, four did not complete the study (2 from ZN, 2 from PL). Baseline descriptive participant characteristics for the total cohort as well as our race-treatment groups are provided in Table 1. At baseline, blacks had significantly higher plasma zinc than whites ($P < 0.001$). There were no other group differences in baseline characteristics. Forty-five participants (30.6%) had a BMI-for-age percentile at or above the 95th percentile for age. The mean zinc intake for both treatment groups was less than the RDA of 8 mg/d for children in this age group (35), and 53.7% of the subjects ($n = 79$) consumed less than 2/3 the RDA. In addition, mean fasting blood glucose levels were normal in both groups, although four subjects had fasting glucose levels within the prediabetic range (100-125 mg/dL).
Baseline plasma zinc was positively associated with C-peptide after controlling for HOMA2-IR ($r = 0.205$, $P < 0.05$). No other significant associations were found between baseline plasma zinc status and cardiometabolic outcomes. No significant interactions or changes were observed for HOMA2-%$\beta$, HOMA2-IR and HOMA2-%$S. However, the treatment $\times$ race $\times$ time interaction for C-peptide approached significance ($P = 0.08$) (Table 2), such that a change in C-peptide was partially attenuated in the black children who were in the ZN group ($P = 0.06$) (Figure 1). Similarly, the treatment x race x time interaction for HOMA2-IR approached significance ($P = 0.08$) (Table 2), such that the change in HOMA2-IR was partially attenuated in the black children in the ZN group ($P = 0.06$).

**DISCUSSION**

Much of the previous work related to zinc and insulin outcomes has focused on its potential clinical benefits for type-2 diabetes (36-39). To our knowledge this is the first study to examine the effects of zinc supplementation on insulin secretion and sensitivity in otherwise healthy early adolescent girls through a randomized, double-blind, placebo-controlled study design. Four-week supplementation of low-dose (i.e., 9 mg/day) elemental zinc did not have an effect on any indices of insulin secretion (i.e., fasting insulin and c-peptide) or sensitivity (i.e., HOMA-IR). However, in the black but not white children, zinc supplementation had a marginal attenuating effect on changes in fasting c-peptide over the 4-week supplementation period.

Our findings are more consistent with those who found reductions in insulin outcomes with zinc supplementation (9, 28). A previous study found that 20 mg of elemental zinc given
over eight weeks to non-diabetic obese children significantly decreased fasting insulin and HOMA-IR (28). A similar study in adults found that fasting insulin decreased significantly after supplementing with 30 mg zinc daily for 4 weeks (9). The current study gave a lower amount of zinc, only 9 mg/d elemental zinc, compared to these two previous studies, which could explain why the changes in the outcomes measured in the current study did not reach statistical significance. However, it was also previously demonstrated by our lab that 9 mg/d of zinc given to children over 4 weeks was sufficient to significantly increase plasma zinc levels, demonstrating that 9 mg/d is sufficient to produce changes in markers of zinc status, and thus should be sufficient to elicit changes in C-peptide and HOMA2-IR (31).

While most previous studies have used insulin resistance and fasting insulin as the primary outcome measures (7, 9, 28), a strength of the current study is the additional use of C-peptide, which is a more reliable indicator of insulin secretion (29, 30). One other zinc supplementation study that used C-peptide as an outcome measure found that C-peptide was significantly increased from baseline after zinc supplementation in diabetic adults with more than four years’ duration of diabetes and in those with marginal zinc status as determined by plasma zinc (8). The differences between these results and those of the current study are likely due to the differences in age and health status of the populations studied.

A secondary aim of this study was to determine whether the effect of zinc supplementation on C-peptide and insulin resistance differed by race. Black versus white children are often more insulin resistant and present with greater values of fasting insulin and c-peptide (21-23, 40). Therefore, the marginal increase in c-peptide in the black, but not white,
children may be attributed to the characteristically normal fluctuations in insulin secretion that are evident throughout the early stages of adolescent growth (17, 18). A previous study from our lab, which considered children of the same age as those in the current study, found a significant increase in fasting insulin and HOMA-IR over 12 weeks (41). In addition, it has been shown that African-American adolescents secrete more insulin than their white peers with comparable insulin sensitivity and body composition (22). These racial differences in insulin secretion may explain why the increase in C-peptide after 4 weeks was greater in the black children given placebo than it was in the white children given placebo. While the population in the current study was more heterogeneous than those in other studies, our findings may only be applicable to black and white children during the early stages of maturation.

Strengths of the current study include the use of C-peptide as an outcome measure, which is a more reliable indicator of insulin secretion than fasting insulin (29, 30), and the inclusion of both black and white participants. Previous studies have assessed outcomes in a single-race population, and no previous study has looked at outcomes in blacks. We also acknowledge potential limitations with the current study. As discussed earlier, we gave a lower amount of zinc, 9 mg/d compared to previous studies (7-9). However, it was previously shown by our lab that 9 mg/d of zinc given over 4 weeks was sufficient to significantly increase plasma zinc levels (31), suggesting that 9 mg/d should also be sufficient to elicit changes in the intended biochemical outcomes in the current study. We were also limited by a 4-week study duration, which may have been insufficient to detect changes in our outcomes of interest. However, other zinc supplementation studies have found significant changes in fasting insulin and HOMA-IR.
after 4 weeks (8, 9, 28), suggesting 4 weeks should be sufficient to produce significant changes in C-peptide and HOMA2-IR.

In conclusion, low-dose zinc supplementation over 4 weeks did not alter insulin secretion or insulin resistance in healthy female adolescents. Supplementation with 9 mg/d elemental zinc/day may attenuate an increase in C-peptide and HOMA2-IR in black children, but not necessarily their white counterparts. The use of zinc to modify metabolic risk factors in adolescents in the early stages of maturation, especially racial differences, should be explored further.

ACKNOWLEDGEMENTS

RDL, EML, AJL, JMK were responsible for the study concept and design. AJL and NKP conducted the statistical analyses. AG was responsible for the plasma Zn assays; Dr. Dorothy Hausman responsible for insulin and glucose assays; NKP responsible for calculating HOMA2 model. AJL was responsible for writing the manuscript. All authors contributed to the revision of the manuscript. RDL, AJL, and JMK were responsible for the interpretation of the data and drafting of the manuscript. All authors read and approved the final manuscript.
REFERENCES


<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall ((n = 147))</th>
<th>ZN-White ((n = 40))</th>
<th>ZN-Black ((n = 35))</th>
<th>PL-White ((n = 40))</th>
<th>PL-Black ((n = 32))</th>
<th>(p^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>10.5 ± 0.7</td>
<td>10.6 ± 0.7</td>
<td>10.5 ± 0.1</td>
<td>10.7 ± 0.7</td>
<td>10.3 ± 0.7</td>
<td>0.08</td>
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<tr>
<td>Tanner stage (1-3)</td>
<td>2.3 ± 0.5</td>
<td>2.4 ± 0.5</td>
<td>2.3 ± 0.5</td>
<td>2.3 ± 0.44</td>
<td>2.3 ± 0.5</td>
<td>0.77</td>
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<tr>
<td>Weight (kg)</td>
<td>47.0 ± 11.3</td>
<td>45.6 ± 10.6</td>
<td>48.2 ± 12.7</td>
<td>46.8 ± 9.5</td>
<td>48.0 ± 12.6</td>
<td>0.74</td>
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<tr>
<td>Height (cm)</td>
<td>148 ± 6.7</td>
<td>147 ± 6.2</td>
<td>149 ± 7.5</td>
<td>148 ± 5.5</td>
<td>149 ± 7.7</td>
<td>0.50</td>
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<tr>
<td>BMI-for-age-percentile (%)</td>
<td>75.1 ± 26.1</td>
<td>75.7 ± 21.4</td>
<td>73.0 ± 31.3</td>
<td>77.4 ± 23.5</td>
<td>73.7 ± 29.0</td>
<td>0.88</td>
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<td>Percent body fat (%)</td>
<td>30.7 ± 8.1</td>
<td>31.2 ± 7.7</td>
<td>30.6 ± 8.7</td>
<td>31.5 ± 7.6</td>
<td>9.3 ± 8.7</td>
<td>0.71</td>
</tr>
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<td>Energy intake (kcal/d)</td>
<td>1902 ± 553</td>
<td>1996 ± 522</td>
<td>1901 ± 652</td>
<td>1894 ± 513</td>
<td>1786 ± 532</td>
<td>0.49</td>
</tr>
<tr>
<td>Zinc intake (mg/d)</td>
<td>4.6 ± 1.7</td>
<td>4.6 ± 1.7</td>
<td>4.8 ± 1.7</td>
<td>4.8 ± 1.7</td>
<td>4.0 ± 1.8</td>
<td>0.44</td>
</tr>
<tr>
<td>Plazma zinc (μmol/L)</td>
<td>11.7 ± 1.3</td>
<td>10.0 ± 1.3</td>
<td>13.9 ± 1.2</td>
<td>10.0 ± 1.3</td>
<td>14.4 ± 1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (μU/mL)</td>
<td>24.0 ± 1.6</td>
<td>21.2 ± 1.5</td>
<td>27.2 ± 1.6</td>
<td>22.9 ± 1.4</td>
<td>26.0 ± 1.6</td>
<td>0.06</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>86.1 ± 7.6</td>
<td>86.6 ± 7.3</td>
<td>84.9 ± 8.8</td>
<td>87.1 ± 7.2</td>
<td>85.3 ± 7.1</td>
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<tr>
<td>C-peptide (ng/mL)</td>
<td>1.9 ± 1.5</td>
<td>1.8 ± 1.5</td>
<td>2.0 ± 1.6</td>
<td>2.0 ± 1.6</td>
<td>1.7 ± 1.6</td>
<td>0.39</td>
</tr>
<tr>
<td>HOMA2-%β (%)</td>
<td>131 ± 1.4</td>
<td>125 ± 1.3</td>
<td>139 ± 1.4</td>
<td>134 ± 1.4</td>
<td>126 ± 1.4</td>
<td>0.41</td>
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<td>HOMA2-%S (%)</td>
<td>80.4 ± 33.6</td>
<td>81.9 ± 32.7</td>
<td>78.0 ± 33.1</td>
<td>75.8 ± 37.3</td>
<td>86.8 ± 30.4</td>
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<tr>
<td>HOMA2-IR (mol × μU/L^2)</td>
<td>1.4 ± 1.6</td>
<td>1.3 ± 1.5</td>
<td>1.4 ± 1.6</td>
<td>2.0 ± 1.6</td>
<td>1.5 ± 1.6</td>
<td>0.40</td>
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^1Values are means ± SD

^2Tests of significance between groups were based one-way ANOVA

HOMA2-%β, homeostatic model assessment of percent beta cell function; HOMA2-%S, homeostatic model assessment of insulin sensitivity; HOMA2-IR, homeostatic model assessment of insulin resistance; PL, placebo; ZN, zinc; B, blacks; W, white
TABLE 2. C-peptide and HOMA2 outcomes at baseline and 4 weeks in black and white girls

<table>
<thead>
<tr>
<th></th>
<th>ZN (n = 75)</th>
<th>PL (n = 72)</th>
<th>Treatment x race x time (P)</th>
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<tr>
<td></td>
<td>White (n = 40)</td>
<td>Black (n = 35)</td>
<td>White (n = 40)</td>
</tr>
<tr>
<td>C-peptide (ng/mL)</td>
<td>1.8 ± 1.5</td>
<td>1.9 ± 1.6</td>
<td>2.0 ± 1.6</td>
</tr>
<tr>
<td>HOMA2-%β (%)</td>
<td>125 ± 1.3</td>
<td>130 ± 1.4</td>
<td>139 ± 1.4</td>
</tr>
<tr>
<td>HOMA2-%S (%)</td>
<td>81.9 ± 32.7</td>
<td>80.6 ± 37.2</td>
<td>78.0 ± 33.1</td>
</tr>
<tr>
<td>HOMA2-IR (mol × μU/L²)</td>
<td>1.3 ± 1.5</td>
<td>1.4 ± 1.6</td>
<td>1.4 ± 1.6</td>
</tr>
</tbody>
</table>

1Values are means ± SD

HOMA, homeostatic model assessment; PL, placebo; ZN, zinc.
FIGURE 1. Mean ± SD for C-peptide and HOMA2 outcomes at baseline and 4 weeks
CHAPTER 4
SUMMARY AND CONCLUSIONS

Though zinc deficiency in developed countries is rare, evidence suggests that many children in the U.S. aren’t meeting the recommendations for zinc intake (1). Zinc is necessary for maturation, storage and secretion of insulin in pancreatic β-cells (2), and altered zinc status has been observed in prediabetic and diabetic individuals (3, 4). Type-2 diabetes is an increasing concern for children as well as adults; nearly one in four U.S. children are prediabetic (5), and the incidence of type-2 diabetes is growing among children (6). Even in the absence of diabetes, healthy children experience variations in insulin secretion and insulin sensitivity during adolescence (7-9). Insulin secretion increases in response to the decreased insulin sensitivity, but whether or not it is able to fully compensate is still unclear (7-9). Moreover, it has been shown that insulin secretion and sensitivity are different in black vs. white adolescents, with blacks having greater insulin secretion and lower insulin sensitivity than whites (10-12).

Cell culture studies have suggested that zinc ions co-secreted from the β-cells with insulin may have an inhibitory effect on glucose-stimulated insulin secretion (13). However, in human studies the effects of zinc supplementation on insulin levels and glycemic control are unclear. Some studies in adults report no difference in insulin concentration or insulin resistance after supplementation (14), while others report slight increases in insulin and C-peptide (15) or decreases in insulin and insulin resistance (16). The relationship between zinc and insulin has not been well researched in healthy children. Cross-sectional analyses in both normal weight and overweight and obese children and adolescents have shown that those with lower serum zinc
concentrations have significantly higher insulin concentrations and insulin resistance indices (17-20). Significantly higher HOMA values have also been observed in children with low dietary density of zinc compared to those with higher dietary density (18). To date, few intervention studies have been conducted in children, and no similar studies have been conducted in children of different races. One study by Hashemipour et al found that fasting insulin and HOMA-IR decreased significantly after four weeks of zinc supplementation in obese children (21). Taken together, these studies suggest that children with poor zinc status, assessed by either zinc intake or serum zinc, may exhibit altered insulin secretion and sensitivity, and that zinc supplementation may help correct these alterations.

To determine the influence of zinc supplementation on insulin secretion (C-peptide) and insulin resistance in healthy female children, and the racial differences in these effects, we conducted an ancillary study to a completed randomized, double blind, placebo-controlled zinc supplementation trial (22). Data and samples for the project were collected on healthy white and black females (N = 147) ages 9-11 y and in Tanner stages 2/3 for breast development (23). The mean zinc intake in the current population was below the RDA of 8 mg/d, and more than 50% of participants consumed less than 2/3 of the RDA. We found an increase in C-peptide after 4 weeks that approached significance in the PL group, but not the ZN group, in blacks, but not in whites (p = .06). These results are similar to previous studies in adults and children that have found significant decreases in insulin outcomes after zinc supplementation (16, 21). The current study gave a lower amount of zinc, only 9 mg/d elemental zinc, compared to these two previous studies, which could explain why the changes in the outcomes measured in the current study did not reach statistical significance. However, it was previously demonstrated by our lab that 9 mg of zinc given over 4 weeks was sufficient to significantly increase plasma zinc levels.
demonstrating that 9 mg/d is sufficient to produce changes in markers of zinc status, and thus should be sufficient to elicit changes in the intended outcomes (22). It is also possible that a 4-week study duration was insufficient to detect significant changes in the biochemical markers of interest. However, other intervention studies in obese and diabetic adults have found significant changes in fasting insulin and HOMA-IR after 4 weeks, suggesting 4 weeks should be sufficient to produce significant changes in insulin outcomes (15, 16). The trend ($p = 0.06$) of increased insulin secretion observed in the blacks given placebo may be due to the normal physiological increase in insulin secretion observed in the early stages of puberty (7, 8). Even in healthy children, adolescence is a period of time characterized by variability in insulin sensitivity and insulin secretion (7, 8). In addition, it has been shown that African-American adolescents secrete more insulin than their white peers with comparable insulin sensitivity and body composition (11). These racial differences in insulin secretion may explain why the increase in C-peptide after 4 weeks was greater in black children given placebo than it was in the white children given placebo.

In conclusion, low-dose zinc supplementation over 4 weeks did not increase insulin secretion in healthy female adolescents. Supplementation with 9 mg/d elemental zinc may attenuate an increase in insulin secretion experienced during the early stages of puberty, especially in black children who are slightly insulin resistant. The use of zinc to modify metabolic risk factors in adolescents in the early stages of insulin resistance, especially race differences, should be explored further.
REFERENCES


APPENDIX A

ZINC FLYER
Nutrition Study for Females 9 to 11 years old

Those who qualify will receive:

• Up to $75
• FREE bone test
• FREE body composition test
• FREE dietary & growth info

Contact: (706)542-4918 or bone@uga.edu
APPENDIX B

VERBAL CONSENT SCRIPT
Verbal consent script (pilot):

I am ______________ from the University of Georgia-Athens, from the Department of Foods and Nutrition. I am conducting research entitled the EFFICACY OF SUPPLEMENTAL ZINC FOR IMPROVING BONE STRENGTH IN EARLY PUBERTAL CHILDREN and would like to know more about whether zinc supplements improve bone health in young females. This interview should only take 15 minutes.

The purpose of the research is to see whether zinc supplements taken each day for one month will improve blood and urine markers of bone health. To determine if your child qualifies, I will conduct a telephone screening questionnaire with you. If your child meets our criteria, you will first be asked to complete a sexual maturation questionnaire that I will mail to you right after our phone conversation. The sexual maturation questionnaire will have several drawings of sexual maturation stages. Your child will simply circle the picture that best represents her level of maturation. If your child is in the stage of sexual maturation that we are looking for, you and your child will come to our laboratory on the University of Georgia campus so that we may measure your child’s height, sitting height and leg length. This will allow us to calculate how far away your child is from her growth spurt. If your child does not qualify for the study, she will receive $10 for coming to our laboratory for these measurements. If your child is within the range we are looking for, your child will be invited to participate in this study, which lasts one month. Your child will be placed in either the zinc group or the placebo group. Participants in the zinc group will take 24mg of zinc sulfate per day for one month. This level is a safe level of zinc. Participants in the placebo group will take a pill identical in appearance to the zinc pill each day for one month.

If your child decides to participate in our study, the following procedures will take place. Your child will come to our laboratory for testing twice: once at the beginning and once at the end (at one month). For the first and last testing sessions, the following procedures will be done: your child will fill out consent forms and questionnaires about nutrition, health, bone, physical activity, sun exposure, and demographics; give a fasting blood sample and a urine sample; be measured for height and weight; be given two different types of bone scans; and be given a take-home 3-day diet record. You will be asked to mail the diet record back to our laboratory in an envelope we will provide. These testing sessions will last about 1½ hours. For the 1-month testing session, your child will complete all of the things I listed except the bone scans.

We will count the pills to document how many pills your child has taken. We will also give you a calendar to help keep track of each day. Your child will receive $25 for the baseline testing session, and $50 at the end of the study for a total of $75 for the entire study.

Do you have any questions?

Let me assure you that any information you and your child provide will be kept strictly confidential. In final research products I will disguise your identity by only presenting aggregate (group) data. Your child’s participation in taking zinc supplements and providing me with information on your health is completely voluntary and you may discontinue our interaction at any time or skip any question you don’t want to answer. If your child does not qualify or decides to not participate after responding to some or all of the questions, her information will be destroyed.

Do you agree to complete the screening questionnaire for your child?  
[If yes, continue with telephone screening questionnaire]  
[If not, say “Thank you for your time, goodbye.”]

The contact information for the researcher conducting this project is:

Emma Laing  
279 Dawson Hall  
University of Georgia  
706-542-4918
APPENDIX C

TELEPHONE SCREENING QUESTIONNAIRE
### UGA ZINC STUDY
#### Telephone Screening Questionnaire

This interview should take approximately ten minutes:

- **Date:** ____________
- **Time:** ____________
- **Screen completed by:** ____________

1. **How would you describe your child’s ethnicity/race?**

   **Ethnicity:**
   - Hispanic or Latino
   - Non-Hispanic or Latino

   **Race:**
   - American Indian or Alaska Native
   - Asian
   - Black or African American
   - Native Hawaiian or other Pacific Islander
   - White
   - any combination of the above

   *Parents of participants may indicate one or more of the following (mixed racial heritage should be indicated by selecting more than one category):*

2. **We would also like to know the ethnicity/race of the child’s biological parents. How do you describe your child’s mother and father?**

   **Child’s Mother**
   - **Ethnicity:**
     - Hispanic or Latino
     - Non-Hispanic or Latino
   
   **Race:**
   - American Indian or Alaska Native
   - Asian
   - Black or African American
   - Native Hawaiian/ Pacific Islander
   - White
   - any combination of the above

   **Child’s Father**
   - **Ethnicity:**
     - Hispanic or Latino
     - Non-Hispanic or Latino
   
   **Race:**
   - American Indian or Alaska Native
   - Asian
   - Black or African American
   - Native Hawaiian/other Islander
   - White
   - any combination of the above

3. **How old is your child? ____________ Years; **

   **DOB:** ____________
   - mm
   - dd
   - yy

4. **What grade does she attend in school? __________**

5. **How tall is your child? ____ft____ in**

   **How much does she weigh? __________ lbs.**

6. **Has your child lost or gained weight in the past 3 months? YES _____ NO _____**

   If yes, how much? ___________ lbs
7. Has your child ever been diagnosed with any of the following diseases/conditions?

- Bone Disease
- Diabetes
- High Blood Pressure
- High Cholesterol
- Renal Disease or Kidney Stones
- Cerebral Palsy
- Intestinal Malabsorption
- Juvenile Rheumatoid Arthritis
- Growth Disorders
- Thyroid Disease
- Zinc Malabsorption (e.g. acrodermatitis enteropathica)
- Psychological Illness

8. Is your child currently taking any medications? YES ________  NO ________

If yes, what medication(s)? ______________________________________________

(check specifically for Adderall, Ritalin, and steroid medications)

9. Is your child taking an herbal, vitamin or mineral supplement? YES ____ NO _____

If yes, how much and how often? __________________________________________

If yes, would your child be willing to stop taking the supplement? YES ____ NO _____

(child would be eligible to enroll in the study after a 4-week washout period)

10. Has your child started her menstrual cycles? YES ____ NO _____

11. In this study, all participants must provide blood and urine samples 2 times (at the start and after 3-4 weeks). Is your child willing to do this? YES ____ NO _____

12. Before initiation of this study, we will ask your child to give a self-assessment of sexual maturation. We will send you the form for your child to complete. Would your child be willing to fill out a self-assessment of sexual maturation form and mail it back in a self-addressed envelope we will provide? YES ______ NO ______

13. If your child meets our criteria for sexual maturation, then he/she will come to our laboratory to have his/her height, weight, sitting height, and leg length measured to calculate how far away he/she is from her peak time of growth. Your child will receive $10 for her time. Would your child be willing to come to our laboratory for these measurements? YES_______NO______
If the caller is still interested, explain more about the study and why we are doing it and collect the following information:

Parent’s name: _______________________________________________

Child’s name: ________________________________________________

Address: ____________________________________________________

Zip Code: ____________________________________________________

Daytime Phone Number: _________________________________ (home or work?)

Email Address: _________________________________

Is it okay to call in the evening? If yes, evening phone: ________________

How did you hear about the study? ________________________________

If selected to participate, what mornings during the week would you be available to come to the UGA Bone and Body Composition Lab, located in Dawson Hall, for testing? M____ T____ W_____ Th_____ F_____ S _____

“This is the end of our telephone screening. We will review this and determine your child’s eligibility for the study. We will get back to you within one week to let you know the status of her eligibility. Do you have any additional questions for me?”

Make sure the potential volunteer has contact numbers for future questions.
APPENDIX D

PARENTAL PERMISSION FORM
I, _______________________, agree to give permission for my child, _________________, to participate in the research titled “EFFICACY OF SUPPLEMENTAL ZINC FOR IMPROVING BONE STRENGTH IN EARLY PUBERTAL CHILDREN,” which is being conducted by Drs. Richard Lewis and Emma Laing of the Department of Foods and Nutrition at the University of Georgia. Dr. Lewis may be reached in room 279 Dawson Hall at 542-4901. I understand that the participation of my child is completely voluntary. I can refuse to grant permission or withdraw permission at any time without penalty or loss of benefits to which my child is otherwise entitled. I can have the results of the participation, to the extent that which it can be identified as my child’s, returned to me, removed from the research records, or destroyed.

The following points have been explained to me:
1) The reason for the research is to study the impact of zinc supplementation on growth factors and markers of bone turnover in children. The benefits that my child and I can expect from participation are the assessment of diet, maturation, growth, bone health (bone mineral density), and body composition (percentage of body fat and nonfat tissue). Additionally, if my child does not qualify for the study but completes a screening session, she will receive a monetary payment of $10. Once enrolled in the study, my child will receive $25 for completion of baseline testing and another $50 at the completion of the 1-month study. Payments will be distributed only if all testing sessions are completed for a given time point and supplements are taken as directed. In order to process the payment for my child’s participation, the researchers need to collect my child’s name and mailing address on a separate payment form. This completed form will be sent to the Department of Foods and Nutrition business office and then to the UGA Business Office. The researchers have been informed that these offices will keep my child’s information private, but may have to release her name and the payment amount to the IRS, if ever asked. The researchers connected with this study have gone to great lengths to protect my child’s private information and will keep this confidential in their locked files. However, they are not responsible once her name and mailing address leave their office/laboratory for processing of payment. Refusal to participate in this study will involve no penalty. All measurements are being used for research purposes only, not medical purposes. However, if abnormalities are found in any measure, I and/or my child will be notified and referred to an appropriate health care professional.

2) The procedures are as follows:
   a) Prior to enrolling in the study, my child will be mailed a sexual maturation self-assessment form for her to complete at home and mail back to the Bone and Body Composition Laboratory (BBCL). My child will compare her own appearance to drawings representative of each sexual maturation stage and circle the drawing she most closely resembles. If my child meets the criteria for inclusion for sexual maturation, she will be scheduled to attend a screening session during which her height, sitting height and leg length will be recorded. If my child meets the criteria for inclusion for these growth measures, she will be enrolled in the study and scheduled to attend Session 1 (described below).
   b) Session 1 of testing will be conducted at two different time points [at the beginning of the study and after one month].
   c) My child will fast the night before Session 1. On the day of testing, my child and I will arrive in the BBCL in Dawson Hall or a determined safe location in the community (community center, health department, school, clinic, etc) at the scheduled time. Prior to any testing or participation, a permission form will be read to me and an assent form will be read to my child. After which, the researcher and I will sign the permission form and my child will sign the assent form. During the reading of the permission and assent forms, my child and I will be briefed and familiarized with the testing procedures that will be used during the study (15 minutes). My child and I will be given the opportunity to reread the permission and assent forms and ask any questions that we may have about the study. Prior to any testing, my child and I will be walked through all procedures and reminded that we are free to withdraw without penalty at any time.
My child will provide her second morning urine sample in a private restroom. A trained phlebotomist will then draw approximately 20 mL of blood from my child’s arm, after which she will be given a snack (15-20 minutes). My child’s blood and urine will be analyzed for compounds that reflect how her bones are responding to the supplements, and if she has certain genetic factors that may affect this response. Any unused portions of the blood that is collected will be discarded after 10 years. If a blood sample cannot be obtained after two attempts, no further attempts will be made. My child and I will complete a general information/health questionnaire, sun exposure questionnaire, and a seven-day recall physical activity recall (approximately 15 minutes). We will also be given a three-day diet record to be mailed back to the BBCL in a stamped, self-addressed envelope provided by the researcher. Session 1 will require approximately 45 minutes.

Session 2 of testing will be conducted at one time point [at the beginning of the study only]. After completion of the questionnaires, my child’s height, sitting height, leg length, and weight, as well as my height will be measured. She will also have her bone mineral density and body composition measured using a non-invasive bone scanning machine. If testing occurs at locations other than the BBCL, the bone and body composition scans are optional, and my child will be offered the opportunity to travel to the BBCL at a time during the study for the bone scan measures, if desired. These measurements will require approximately 30 minutes, which includes a small break in between each scan (four scans total). My child will also have her bone structure analyzed using a different kind of bone scanner (a pQCT machine). This measurement will require about 20 minutes. I understand that a trained laboratory technician under the supervision of Dr. Richard D. Lewis will conduct all measurements. Because our current knowledge of the risk of DXA to the unborn child is limited, prior to conducting the bone scans, my daughter will sign a consent form developed for use with the DXA and pQCT that asks if she is currently pregnant or believes she may be pregnant. If my daughter is pregnant, she will be told that she cannot participate because the X-rays from the DXA pose a risk to the fetus. If my child expresses any doubts regarding pregnancy, a pregnancy test will be provided to complete in the privacy of her own home prior to DXA testing. If pregnancy test is refused or if determined to be pregnant, my daughter may maintain confidentiality by electing not to disclose the pregnancy test results to the research group, but must voluntarily withdraw from the study. Refusal will be documented. If my daughter and I elect to notify the research group of the pregnancy, she/we will receive a referral to her pediatrician or primary care physician.

Information from all analyses will be stored in locked filing cabinets. My child and I will be instructed on the proper use of the provided supplements. We agree to follow the instructions on the label of the supplements. I understand that the supplement is either 24 mg of zinc or a placebo (a substance of no medical value), neither of which is expected to cause harm to my child if taken properly. If supplementation should cause any noticeable, negative side effects (i.e., Nausea, stomachache, or dizziness), my child may opt to either continue the study without taking supplements, or may discontinue the study completely. Session 2 will require approximately 1½ hours.

3) The discomforts or stresses that may be faced during this research are minor physical discomfort from blood draws and minor psychological discomfort from the questions about my child’s diet or medical history. To minimize this stress, participants will be interviewed in private rooms. If undue discomfort occurs, my child has the right to discontinue the testing at any time.

4) I understand that one of the foreseen risks to my child is discomfort during the blood draw. I understand that if a blood sample cannot be obtained after two attempts, no further attempts will be made. I understand that another foreseen risk to my child is exposure to a small amount of radiation when assessing body composition and bone status with the bone scanning machines. The scans for the entire study will give a total radiation dose of 165.75 microsieverts (µSv). This dose is very small, as radiation doses from an adult chest X-ray ranges from 500 to 800 µSv and environmental background radiation per week totals 35 µSv. Thus the total radiation exposure for the study is 21-33% of standard chest X-rays. In the event that information from any scan is lost or unusable, no additional scans will be performed.
 Radiation Doses Per Testing Session:

<table>
<thead>
<tr>
<th>Testing Session</th>
<th>DXA</th>
<th>pQCT</th>
<th>Times Session Conducted</th>
<th>Total Radiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening</td>
<td>40 µSv</td>
<td></td>
<td>1</td>
<td>40 µSv</td>
</tr>
<tr>
<td>Baseline</td>
<td>165 µSv</td>
<td>4 µSv</td>
<td>1</td>
<td>169 µSv</td>
</tr>
</tbody>
</table>

**TOTAL:** 209 µSv

5) The results of my participation and that of my child will be confidential and will not be released in any identifiable form without my child’s prior permission and mine unless required by law. My signature on this form authorizes that use of my data and my child’s data in group analyses which may be prepared for public dissemination, without breaching my own or my child’s confidentiality. To accomplish this, my child will be assigned a four digit subject participation code, which will be used on all data collected during my child’s participation in this research. A master list with my child’s name and corresponding code number will be kept separate from testing data and locked at all times.

6) The investigator will answer any further questions that my child or I may have about this research, either now or during the course of the project.

My child was given the opportunity to complete a simple urine test for pregnancy:

(Check one): **YES**  ____  **NO**  ____

__________________________  _______________
Signature        Date

I refuse for my child to take the pregnancy test:

(Check one): **YES**  ____  **NO**  ____

__________________________  _______________
Signature        Date

I understand the procedures described above. My questions have been answered to my satisfaction, and I agree to give permission for my child to participate in this study. I have been given a copy of this form.

Richard Lewis/Emma Laing  ____________________________  _______________
Name of Researcher        Signature        Date
Telephone: 542-4901
Email: rlewis@fcs.uga.edu

__________________________  ____________________________  _______________
Name of Parent or Guardian Signature        Date

Please sign both copies, keep one and return one to the researcher.

Additional questions or problems regarding your child’s rights as a research participant should be addressed to The Chairperson, Institutional Review Board, University of Georgia, 612 Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411; Telephone (706) 542-3199; E-Mail Address IRB@uga.edu.
APPENDIX E

ASSENT FORM (CHILD)
I, _____________________________, agree to take part in a research study about bone health and growth.

I do not have to be in the study if I do not want to be. I have the right to leave the study at any time without giving any reason, and without penalty.

I will take my zinc or placebo supplement every day according to the directions. I will bring my unused supplements to the researcher after 1 month so that she may count how many I missed.

Too much zinc in the diet can cause stomach aches, dizziness and/or nausea. If I feel any of these symptoms, I will report them to my parent and the researcher, and I may either choose to continue to take the supplements, or to discontinue the study. I will also be asked to answer questions about how the supplements are affecting me.

Before entering the study:
- I will receive a sexual maturation self-assessment form in the mail that I will complete in private at home. I will compare my own appearance to drawings of growth stages and circle the drawing that looks most like me.
- I will have my height measured against a wall and my weight measured on a scale.
- If I complete these measures listed above but do not qualify for the study, I will receive $10.
- If any of these procedures cause me to be uncomfortable, I may skip those procedures and any information about me will not be shared with anyone else.

At the beginning of the study and 1 month later:
- A trained nurse will take a blood sample from my arm.
- I will provide a urine sample in a private bathroom.
- I will have my height measured against a wall and my weight measured on a scale.
- My parent and I will write down what I eat during two weekdays and one weekend day.
- I will answer questions about my physical activity.
- If I complete these measures listed above, I will receive $25 at the beginning of the study and $50 after 1 month.
- I may experience hunger before the blood and urine collection, but I will receive a snack once the tests have been completed.
- I may experience a bruise under my skin after the blood draw, which should disappear within a few days.
- If any of these procedures or questions asked of me cause me to be uncomfortable, I may skip those procedures/ questions and any information about me will not be shared with anyone else.

If the equipment is available, at the beginning of the study, I will have pictures taken of my bones. During one set of pictures I will lie on a table for approximately 30 minutes. I will take short breaks between the different pictures that are taken. During another set of pictures, I will place my leg in the circular part of a machine for about 10 minutes. During the last set of pictures, I will place my arm on a box for about 5 minutes. These pictures provide a small amount of radiation, similar to the X-ray pictures taken at the dentist’s office. If any of these procedures or questions
cause me to be uncomfortable, I may skip those procedures/questions and any information about me will not be shared with anyone else. If the equipment is unavailable, I have the option to travel to the Bone & Body Composition Lab in Athens, Georgia to have a picture of my bones at another time during the study. Before I have a picture of my bones taken, I will be asked if I am pregnant. If I am not sure, I will be given a pregnancy test. If I am pregnant or refuse the pregnancy test, I will not participate in the study.

If I have any questions or concerns, I can always call the researcher, Dr. Richard Lewis at the following number: 542-4901.

Sincerely,

Emma Laing, PhD, RD, LD
Department of Foods and Nutrition
University of Georgia
279 Dawson Hall

I was given the opportunity to complete a simple urine test for pregnancy:
(Check one): YES ____  NO ____

________________________________________  ______________
Signature                                     Date

I refuse to take the pregnancy test: (Check one): YES ____  NO ____

________________________________________  ______________
Signature                                     Date

I understand the project described above. My questions have been answered and I agree to participate in this project. I have received a copy of this form.

________________________________________
Signature of the Participant/Date

Please sign both copies, keep one and return one to the researcher.

Additional questions or problems regarding your rights as a research participant should be addressed to The Chairperson, Institutional Review Board, University of Georgia, 612 Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411; Telephone (706) 542-3199; E-Mail Address IRB@uga.edu.
SEXUAL MATURATION QUESTIONNAIRE (GIRLS)

We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.

Stage 1: Elevation of papilla only.
Stage 2: Elevation of breast and papilla as small mound, areola diameter enlarged.
Stage 3: Further enlargement without separation of breast and areola.
Stage 4: Secondary mound of areola and papilla above the breast.
Stage 5: Recession of areola to contour of breast.

Thank you for answering this question. Please send this questionnaire back to the researcher in the stamped envelope provided.

APPENDIX G

ANTHROPOMETRIC DATA SHEET
UGA BONE STUDY

ANTHROPOMETRIC DATA SHEET

ID NUMBER: ____________

HEIGHT  _______________ (TO NEAREST 1/4 INCH)
WEIGHT  _______________ (TO NEAREST 1/4 POUND)
LEG LENGTH ______________ (TO NEAREST 1/4 INCH)
SITTING HEIGHT ______________ (TO NEAREST 1/4 INCH)
MOM’S HEIGHT ______________ (TO NEAREST 1/4 INCH)
   SELF-REPORT?   YES   NO
DAD’S HEIGHT ______________ (TO NEAREST 1/4 INCH)
   SELF-REPORT?    YES    NO
LENGTH OF RADIUS IN CENTIMETERS _______________  R or L?___
NUMBER OF BLOCKS USED FOR SPINE SCAN ________

Hip Circumference:           Belly Circumference:      Waist Circumference:

BIRTHDATE ___________________________

____________________________________

TO BE COMPLETED BY INVESTIGATOR:

PREDICTED HEIGHT _______________
% PREDICTED HEIGHT ______________

GROWTH VELOCITY
   HEIGHT _______________________
   LEG LENGTH __________________
   SITTING HEIGHT ______________
% HEIGHT FOR AGE  ______________
% WEIGHT FOR AGE    ______________
% WEIGHT FOR HEIGHT _____________
BMI___________________________________
APPENDIX H

GENERAL INFORMATION QUESTIONNAIRE
GENERAL INFORMATION QUESTIONNAIRE

Demographic Data:

I am going to ask you some questions about your age, family and education. Your mother or father can help you answer.

1. What is your date of birth? Month_________ Day ___________ Year_______
2. What is your age? Years _________________ Months ________________
3. Gender: (Circle One) Female Male
4. What is your grade in school? ________________
5. How do you describe yourself? (Circle One or More: Mixed racial heritage should be indicated by checking more than one category)
   American Indian or Alaska Native
   Asian
   Black or African American
   Hispanic or Latino
   Native Hawaiian or Other Pacific Islander
   White
6. Do you live with your parents? (Circle One) YES NO
   6a. If no, with whom do you live? ________________________________
7. Do you have any brothers or sisters? (Circle One) YES NO
   7a. If yes, list ages of: _____Years (Brother) _____Years (Sister)
      _____Years (Brother) _____Years (Sister)
      _____Years (Brother) _____Years (Sister)
   7b. If yes, do they participate in sports? (Circle One) YES NO
   7c. If yes, list the sport and gender of sibling. Sport___________(Brother or Sister)
      Sport___________(Brother or Sister)
      Sport___________(Brother or Sister)
      Sport___________(Brother or Sister)
8. Do you have a twin brother or sister? (Circle One) YES NO
9. What is your parents’ income? (Circle One)

<table>
<thead>
<tr>
<th>Income Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than $9,999</td>
</tr>
<tr>
<td>$10,000 - $19,999</td>
</tr>
<tr>
<td>$20,000 - $29,999</td>
</tr>
<tr>
<td>$30,000 - $39,999</td>
</tr>
<tr>
<td>$40,000 - $49,999</td>
</tr>
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<tr>
<td>$60,000 - $69,999</td>
</tr>
<tr>
<td>$70,000 - $79,999</td>
</tr>
<tr>
<td>$80,000 - $89,999</td>
</tr>
<tr>
<td>$90,000 - $99,999</td>
</tr>
<tr>
<td>Over $100,000</td>
</tr>
</tbody>
</table>

10. What is your mother’s occupation? __________________________________________

11. What is your father’s occupation? __________________________________________
GENERAL INFORMATION QUESTIONNAIRE

Health Data

Now, I am going to ask you to respond to a few questions about your health. I am the only one that will know how you answer these questions, so please be honest with your answers.

1. Have you gained or lost any weight (≥ 10 pounds) in the last 3 months? (Circle One) YES NO
   1a. If yes, how much? +__________ pounds OR -__________ pounds

2. Have you had any height changes in the past 3 months? (Circle One) YES NO
   2a. If yes, how much? __________ feet __________ inches

3. How would you rate your present health? (Circle One)
   Poor Fair Good Excellent

4. Have you started your menstrual cycles? (Circle One) YES NO If so, what date?

5. Do you have any diseases or illnesses? (Circle One) YES NO
   10a. If yes, what diseases? ___________________________
       ___________________________

6. Are you taking any medications either prescribed by a doctor or over-the-counter (self-prescribed)? (Circle One) YES NO
   11a. If yes, what medications? __________________________ Amount per day ________
       __________________________ Amount per day ________
       __________________________ Amount per day ________

Those were some difficult questions to answer because the questions were so private. I want to assure you again that I am the only person who knows how you answered these questions. Thank you for being so honest with your answers.
GENERAL INFORMATION QUESTIONNAIRE

Nutrition Data:

These next questions are about your diet and eating habits. Try to think about how you eat.

1. Do you eat three meals per day? (Circle One) YES NO
   1a. If no, why not? ________________________________

2. Do you eat snacks during the day? (Circle One) YES NO
   2a. If yes, how many snacks per day do you eat? ___________ snacks per day

3. Are you following a special kind of diet? (Circle One) YES NO
   3a. If yes, what kind of diet? ________________________________

4. Do you take any vitamin or mineral supplements or any “nutrition pills”? (Circle One) YES NO
   4a. If yes, what kind? ________________________________ Amount per day ___________
       ________________________________ Amount per day ___________
       ________________________________ Amount per day ___________

5. Have you ever been on a diet to lose weight? (Circle One) YES NO
   5a. If yes, what kind of a diet was it? ________________________________
   5b. How old were you when you were on this diet? _____ years _____ months
       _____ years _____ months

6. Have you ever eaten a large amount of food and then vomited to get rid of the food? (Circle One) YES NO
   6a. If yes, how old were you? ___________ years _____ months
       ___________ years _____ months

7. Were you breastfed as an infant? (Circle One) YES NO
   7a. If yes, for how many months? ____________

Thank you for answering all of those questions. You did really well, and I appreciate your being so truthful with your answers.
GENERAL INFORMATION QUESTIONNAIRE

Physical Activity

The next questions that I will ask you are about your physical activity such as P.E. and exercise. There are no right or wrong answers, so please answer these questions the best that you can.

1. How would you rate your physical activity level? (Circle One)
   - Inactive
   - Below average
   - Average
   - Above average
   - Very high

2. Do you have any health problems that limit your activity? (Circle One)
   - YES
   - NO
   2a. If yes, what health problem?

3. Do you exercise or do physical activity regularly (not including P.E. class)?
   (Circle One)
   - YES
   - NO
   3a. If yes, how often? ______ hours per day/week/month (Circle One)

4. Do you participate in P.E. at school? (Circle One)
   (Circle One)
   - YES
   - NO
   4a. If yes, how often? ______ hours per day/week/month (Circle One)
GENERAL INFORMATION QUESTIONNAIRE

**Bone Health Data:**
The next questions have to do with your bones and your family’s bones.

1. Does anyone in your family (including your parent’s, grandparents, aunts, uncles, cousins) have osteoporosis or “humpback”? (Circle One) **YES NO**
   1a. If yes, who? __________________________

2. Has anyone in your family (including your parents, grandparents, aunts, uncles, cousins) had a hip or wrist fracture? (Circle One) **YES NO**
   2a. If yes, who? __________________________

3. Have you ever had a bone fracture or broken bone? (Circle One) **YES NO**
   3a. If yes, which bone(s)? __________________________
   3b. If yes, how old were you? _________ years _________ months

4. Have you ever been told by a doctor that you have bone disease? (Circle One) **YES NO**
   4a. If yes, what disease? __________________________
   4b. If yes, how old were you? _________ years _________ months
APPENDIX I

SUN EXPOSURE QUESTIONNAIRE
UGA ZINC STUDY

Your Name: ________________________________

(Once your vitamin D results and this questionnaire have been matched, your name will be removed from the records)

Date: ___ / ___ / 2008

SUN EXPOSURE QUESTIONNAIRE

Thank you for agreeing to take part in this study. It is very important that you answer all the questions. If you have any difficulties, feel free to discuss them with the Researcher(s).

If you have any problems or queries please do not hesitate to contact:
  ▪ Dr. Richard Lewis at 706-542-4901 or rlewis@fcs.uga.edu
  ▪ Dr. Emma Laing at 706-583-0040 or emonk@uga.edu

All of the information that you provide will remain strictly confidential

This project is funded through the National Institutes of Health, Grant Number: xxxxxx
Firstly we would like to ask you some questions about your background:

1. What gender are you? *(Please tick the appropriate box)*
   - Male
   - Female

2. What is your date of birth? / / (b) current age: ___ years

3. In what country were you born?

4. IF YOU WERE NOT BORN IN THE USA
   In what year did you come to live in the USA?

5. We are interested in your parents’ **ETHNIC ORIGIN** (that is, the place where most of their ancestors came from) and the **COUNTRY THEY WERE BORN IN**.

<table>
<thead>
<tr>
<th>Country of Birth</th>
<th>Ethnic Origin <em>(Please tick the appropriate box)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td>□ North European  □ Asian  □ African  □ Aboriginal/TSI</td>
</tr>
<tr>
<td></td>
<td>□ South European  □ Other  □ Don’t know</td>
</tr>
<tr>
<td>Father</td>
<td>□ North European  □ Asian  □ African  □ Aboriginal/TSI</td>
</tr>
<tr>
<td></td>
<td>□ South European  □ Other  □ Don’t know</td>
</tr>
</tbody>
</table>

6. What is the **highest** technical, professional or academic **qualification** you have **completed**? *(Please tick one of the boxes below)*
   - Bachelor’s degree or higher
   - Some High School (Year 11 or under)
   - Certificate or Diploma
   - Primary school
   - Trade/Apprenticeship
   - Did not complete primary school
   - Year 12 Senior Certificate (or HSC)

6a. What is your main occupation?

6b. Please tick the box below which best describes your main occupation:
   - Mainly indoors
   - Half indoors and half outdoors
   - Mainly outdoors
The questions in this section are about your skin and how it reacts to the sun.

7. **We are interested in whether your skin BURNS.**
   
   SUPPOSE your skin is exposed to strong sunlight for the first time in summer, WITH NO PROTECTION such as sunscreen or shade. If you stayed in the sun for 30 minutes would your skin…
   
<table>
<thead>
<tr>
<th>Option</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Never</td>
<td>Burn</td>
</tr>
<tr>
<td>Rarely</td>
<td>Burn</td>
</tr>
<tr>
<td>Sometimes</td>
<td>Burn</td>
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<tr>
<td>Mostly</td>
<td>Burn</td>
</tr>
<tr>
<td>Always</td>
<td>Burn</td>
</tr>
</tbody>
</table>

8. **We are also interested in whether your skin TANS.**
   
   IMAGINE you spend several weeks at the beach in strong sunlight, without any protection such as sunscreen or clothing. What would your skin be like? *(Please tick the appropriate box)*
   
<table>
<thead>
<tr>
<th>Option</th>
<th>Tanning Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very brown &amp; Deeply tanned</td>
<td>Moderately tanned</td>
</tr>
<tr>
<td>Moderately tanned</td>
<td>Slightly tanned</td>
</tr>
<tr>
<td>Slightly tanned</td>
<td>Not tanned at all</td>
</tr>
</tbody>
</table>

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**SECTION 3: TIME SPENT IN THE SUN & SUN-PROTECTION**

10. **During the PAST MONTH, how much time did you usually spend outside each day** between sunrise & sunset on:

<table>
<thead>
<tr>
<th>Time</th>
<th>Hours</th>
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</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td>typical WEEKDAYS (Monday - Friday)?</td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td></td>
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<tr>
<td>typical WEEKENDS (Saturday and Sunday)?</td>
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</tr>
</tbody>
</table>

11. **Is the PATTERN of sun exposure described above in questions 10 fairly TYPICAL for you at this time of year?**

<table>
<thead>
<tr>
<th>Option</th>
<th>Reason</th>
</tr>
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<tbody>
<tr>
<td>Yes</td>
<td>(IF YES, <em>Please go to Question 14</em>)</td>
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<tr>
<td>No</td>
<td>IF NO, do you usually spend MORE or LESS time in the sun?</td>
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</tbody>
</table>

12. **If you answered NO to question 11, please tell us WHY your pattern of sun exposure has been DIFFERENT over the PAST MONTH?** (e.g. indoors more because you were sick, or outdoors more because you were on holidays, or working outdoors in a new job etc).

   ____________________________________________________________
   ____________________________________________________________

13. **This question asks about the SUN PROTECTION you may have used in the PAST MONTH.**

<table>
<thead>
<tr>
<th>Action</th>
<th>Frequency</th>
<th>Never</th>
<th>Rarely</th>
<th>Sometimes</th>
<th>Usually</th>
<th>Almost always</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wear a hat?</td>
<td></td>
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<tr>
<td>Wear sunscreen?</td>
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<tr>
<td>Wear a shirt with long sleeves?</td>
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<tr>
<td>Wear long trousers</td>
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</tbody>
</table>
or clothing that covers all or most of your legs?

14. If you have used sunscreen in the PAST MONTH, what is the sun-protection factor (SPF) of the sunscreen that you have been using? (e.g. SPF 30+)

   SPF? __________

15. Over the PAST MONTH, when you have used sunscreen, HOW OFTEN have you applied it?

   □ once /day   □ 2 times /day   □ 3 or more times/day   □ Other: ____________________

16. Over the PAST MONTH, when you used sunscreen, WHERE did you usually apply it?

   *(Please shade the parts of the body where you usually applied sunscreen).*

*THANK YOU VERY MUCH FOR TAKING THE TIME TO PARTICIPATE IN THE STUDY*
APPENDIX J

HIGH ZINC FOODS TO AVOID
High Zinc Foods to Avoid:

- Mollusks
- Oysters
- Kellogg’s Product 19
- Kellogg’s Complete Wheat Bran Flakes
- General Mills Total Raisin Bran
- General Mills Total Corn Flakes
- General Mills Whole Grain Total
- Baked Beans
- General Mills Frosted Wheaties
- General Mills Wheaties
- Crustaceans
- Crab

Look for zinc listed here. Avoid food items with zinc levels above 8 milligrams (mg) per serving. Avoid foods that have more than 50% of the Daily Value.

<table>
<thead>
<tr>
<th>Nutrition Facts</th>
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<tbody>
<tr>
<td>Serving Size 2 crackers (14 g)</td>
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<td>Servings Per Container About 21</td>
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</table>

<table>
<thead>
<tr>
<th>Amount Per Serving</th>
<th>% Daily Value*</th>
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<tr>
<td>Calories 60</td>
<td>2%</td>
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<td>Calories from Fat 15</td>
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<tr>
<td>Total Fat 1.5g</td>
<td>2%</td>
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<tr>
<td>Saturated Fat 0g</td>
<td>0%</td>
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<td>Trans Fat 0g</td>
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<tr>
<td>Cholesterol 0mg</td>
<td>0%</td>
</tr>
<tr>
<td>Sodium 70mg</td>
<td>3%</td>
</tr>
<tr>
<td>Total Carbohydrate 10g</td>
<td>3%</td>
</tr>
<tr>
<td>Dietary Fiber Less than 1g</td>
<td>3%</td>
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<tr>
<td>Sugars 0g</td>
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<tr>
<td>Protein 2g</td>
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</table>

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs.

Look for zinc listed here. Avoid food items with zinc levels above 8 milligrams (mg) per serving. Avoid foods that have more than 50% of the Daily Value.
APPENDIX K

3-DAY DIET RECORD
DIRECTIONS FOR KEEPING A 3-DAY DIET DIARY

Please write down everything you eat (meals, snacks, beverages) for three days on these forms. Please select TWO WEEKDAYS AND ONE WEEKEND DAY. Use as much space as you need.

1. Write down the date and day at the top of the form.

2. Write down the first foods you ate for that day. Write down:
   - The time of day you ate the food(s).
   - Each food that you ate.
   - How the food was prepared (baked, boiled, fried, microwaved).
   - How much you ate (cup, 1/2 cup, pieces, tablespoons, teaspoons).

3. It is important to describe each food you eat in detail.
   For example:
   - Write down brand names for each food you ate if you know them.
   - Write down the type of milk (whole, 2%, or skim) and bread (white, wheat, etc).
   - Write down if the food was fresh, frozen, or canned.
   - If you ate a casserole or a salad, write down the foods there were in it and amounts.
   - If you add things like butter, jelly, sugar, honey, or cream to foods or beverages, please write them down with the amounts used.

4. Do you drink whole ______, 2% ________, 1% ______, or skim ____ milk?

5. Do you use white _______ or whole-wheat ______ bread?

6. What is the complete name and brand name of bread that you eat most often?

7. About how many glasses of water do you drink each day? ______________
DAY 1 OF THE DIET DIARY

ID: ___________________________ CHECKED BY: ____________________

DATE: _________________________ DAY OF THE WEEK: ______________

Did you drink a calcium-fortified beverage today (e.g. Calcium-fortified orange juice) or eat a calcium-fortified food (e.g. Total breakfast cereal)?  Yes  No

If yes, list all the calcium-fortified beverages/foods, with the BRAND name, and how much:

_________________________________________________________________

_________________________________________________________________

Write down everything you eat, beginning with the first thing you have for breakfast. Be sure to include very detailed information such as how the food was prepared, how much you ate, and the brand names.

<table>
<thead>
<tr>
<th>Time Eaten</th>
<th>Foods Eaten</th>
<th>Preparation Methods</th>
<th>Amount (cup, 1/2 cup, piece, etc)</th>
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DAY 2 OF THE DIET DIARY

ID: ___________________________ CHECKED BY: ___________________________

DATE: ___________________________ DAY OF THE WEEK: __________

Did you drink a calcium-fortified beverage today (e.g. Calcium-fortified orange juice) or eat a calcium-
fortified food (e.g. Total breakfast cereal)? Yes No

If yes, list all the calcium-fortified beverages/foods, with the BRAND name, and how much:

______________________________________________________________________________

______________________________________________________________________________

______________________________________________________________________________

Write down everything you eat, beginning with the first thing you have for breakfast. Be sure to include very
detailed information such as how the food was prepared, how much you ate, and the brand names.

<table>
<thead>
<tr>
<th>Time Eaten</th>
<th>Foods Eaten</th>
<th>Preparation Methods</th>
<th>Amount (cup, 1/2 cup, piece, etc)</th>
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If yes, list all the calcium-fortified beverages/foods, with the BRAND name, and how much:

Write down everything you eat, beginning with the first thing you have for breakfast. Be sure to include very detailed information such as how the food was prepared, how much you ate, and the brand names.

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APPENDIX L

7-DAY PHYSICAL ACTIVITY RECALL QUESTIONNAIRE
7-DAY PHYSICAL ACTIVITY RECALL QUESTIONNAIRE

1. On the average, how many hours did you sleep each night during the last 5 weekday nights (Sunday-Thursday)? Record to nearest quarter-hour.
   
   Hours: _______   Minutes: _______

2. On the average, how many hours did you sleep each night last Friday and Saturday nights?
   
   Hours: _______   Minutes: _______

3. First let’s consider moderate activities. What activities did you do and how many total hours did you spend during the last 5 weekdays doing these moderate activities or others like them? Please tell me to the nearest half-hour.
   
   Hours: _______   Minutes: _______

4. Last Saturday and Sunday, how many hours did you spend on moderate activities and what did you do? (Can you think of any other sport, job, or household activities that would fit in this category?)
   
   Hours: _______   Minutes: _______

5. Now let’s look at hard activities. What activities did you do and how many total hours did you spend during the last 5 weekdays doing these hard activities or others like them? Please tell me to the nearest half-hour.
   
   Hours: _______   Minutes: _______

6. Last Saturday and Sunday, how many hours did you spend on hard activities and what did you do? (Can you think of any other sport, job, or household activities that would fit in this category?)
   
   Hours: _______   Minutes: _______

7. Now let’s look at very hard activities. What activities did you do and how many total hours did you spend during the last 5 weekdays doing these very hard activities or others like them? Please tell me to the nearest half-hour.
   
   Hours: _______   Minutes: _______

8. Last Saturday and Sunday, how many hours did you spend on very hard activities and what did you do? (Can you think of other sport, job, or household activities that would fit in this category?)
   
   Hours: _______   Minutes: _______
Physical Activity List

Moderate Activities

*Occupational Tasks:*
9. Delivering mail or patrolling on foot
10. House painting
11. Truck driving (making deliveries – lifting and carrying light objects)

*Household activities:*
1. Raking the lawn
2. Sweeping and mopping
3. Mowing the lawn with a power mower
4. Cleaning windows

*Sports Activities (Actual playing time)*
1. Volleyball
2. Ping pong
3. Brisk walking for pleasure or to work (3 mph or 20 min/mile)
4. Golf-walking and pulling or carrying clubs
5. Calisthenic exercises

Hard Activities

*Occupational Tasks:*
1. Heavy carpentry
2. Construction work – doing physical labor

*Household Tasks:*
1. Scrubbing floors

*Sports Activities (Actual playing time):*
1. Doubles tennis
2. Disco, Square, or Folk dancing

Very Hard Activity

*Occupational Tasks:*
1. Very Hard physical labor – digging or chopping with heavy tools
2. Carrying heavy loads, such as bricks or lumber

*Sports Activities (Actual playing time):*
1. Jogging or swimming
2. Singles tennis
3. Racquetball
4. Soccer
5. Aerobics
6. Stair climbing
7. Weight training
8. Gymnastics
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<tr>
<th>Activity</th>
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Worksheet for Calculating Daily Energy Expenditure

1. Add up all the hours of sleep and naps you had. 
2. Multiply the total number of hours of sleep and naps (line 1) by 1. 
   \[ X \times 1 = \]
3. Add up the total number of hours spent in moderate activity. 
4. Multiply the hours spent in moderate activity (line 3) by 4. 
   \[ X \times 4 = \]
5. Add up the total number of hours spent in hard activity. 
6. Multiply the hours spent in hard activity (line 5) by 6. 
   \[ X \times 6 = \]
7. Add up the total number of hours spent in very hard activity. 
8. Multiply the hours spent in very hard activity (line 7) by 10. 
   \[ X \times 10 = \]
9. Add up the figures in lines 1, 3, 5, and 7. 
   \[ (1 + 3 + 5 + 7) = \]
10. Hours spent in light activity is equal to 24 hours minus the hours in 
    lines 1, 3, 5, and 7. 
    \[ 24 - (1 + 3 + 5 + 7) = \]
11. Multiply the figure in line 10 by 1.5. 
    \[ X \times 1.5 = \]
12. Add up the figures in lines 2, 4, 6, 8, and 11. 
    \[ (2 + 4 + 6 + 8 + 11) = \]
13. The figure you arrived at in line 12 is the total kilocalories per 
    kilogram of body weight expended per day. 
    \[ (\text{kcal} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}) = \]
14. To calculate the total number of calories you expended in one day, 
    multiply your total body weight in kilograms \( \text{body weight in pounds} \div 2.2046 = \text{kilograms} \) by the figure in line 13. 
    \[ \text{Body weight (kg)} \times \text{kcal} \cdot \text{kg}^{-1} \cdot \text{day}^{-1} = \text{total calories expended} = \]

The following are some average kcal \( \cdot \text{kg}^{-1} \cdot \text{day}^{-1} \) for individuals of different ages:

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<th>Age Group</th>
<th>Male</th>
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APPENDIX M

COMPLIANCE CALENDAR
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APPENDIX N

PILL COUNT FORM
Pill Count Form

Subject ID#: _____________
Interviewer: _____________
Date of Interview: ________

Date_______________?

Number of study pills (zinc or placebo) left in the bottle: _____________

Any complaint of side effects?  Yes  No

If yes, please report:

______________________________________________________________________
______________________________________________________________________
______________________________________________________________________

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APPENDIX O

RESPONSE TO SUPPLEMENTATION QUESTIONNAIRE
Response to Supplementation Questionnaire

Subject ID#: _____________
Interviewer: _____________
Date of Interview: _____________

How is your overall health?

How do you feel about the supplements you have been taking?
APPENDIX P

MATURITY OFFSET PREDICTION EQUATIONS
Maturity Offset Prediction Equations

Variables:
- Age (yrs)
- Height (cm)
- Weight (kg)
- Leg Length (cm)
- Sitting Height (cm)

Maturity Offset (Females) = 
\[-9.376 + [0.0001882 \times (\text{Leg Length} \times \text{Sitting Height})] + [0.0022 \times (\text{Age} \times \text{Leg Length})] + [0.005841 \times (\text{Age} \times \text{Sitting Height})] + [-0.002658 \times (\text{Age} \times \text{Weight})] + [0.07693 \times (\text{Weight}/\text{Height} \times 100)]\]