EFFECT OF SHORT-TERM ORAL ZINC SUPPLEMENTATION ON SERUM PROTEIN CONTENT IN EARLY PUBERTAL, CAUCASIAN FEMALES: A SEARCH FOR CANDIDATE BIOMARKERS

by

LAUREN LEE ATWELL

(Under the Direction of Arthur Grider)

ABSTRACT

This study tested if zinc supplementation can induce changes in serum protein levels. ***Hypothesis*:** Early pubertal, Caucasian girls taking 24 mg oral zinc sulfate for 30 days have different contents of serum proteins than matched controls taking a placebo. Twelve, healthy, well-nourished girls, ages 9-11, were selected from an ongoing, randomized, placebo-controlled trial and matched based on maturity offset (years to peak height velocity), body weight (kg), and Tanner stage of maturation. High abundance proteins were removed using Seppro® IgY 14 Spin Columns. Two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) were used to identify changes in serum proteins among baseline and four-week samples. Ten proteins were differentially expressed in the zinc supplementation group. These proteins may serve as biomarkers for zinc status in humans. Future research is necessary to validate these candidate biomarkers and to characterize their roles in zinc metabolism and responses to zinc supplementation.

INDEX WORDS: Zinc, Zinc supplementation, Biomarker, Protein analysis, Prefractionation, Zinc sulfate, Healthy, Early pubertal, Caucasian, Serum proteins, SDS-PAGE, Maturity offset, 2DE, Proteomics, 2D, Girls, Females

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CHAPTER 1

LITERATURE REVIEW

HISTORY OF ZINC DEFICIENCY

The importance of zinc in biological systems has long been recognized.(1-2) In 1963, zinc deficiency was first characterized in humans, further highlighting its physiological significance. Prasad et al. (1963) attribute observed growth stunting in an original study population to zinc deficiency instead of iron deficiency, which was supported by their later studies that found decreased concentrations of zinc in plasma, red blood cells, hair, urine and feces of subjects with evident growth stunting when compared to controls with normal growth. Greater improvements in growth were reported for those supplemented with zinc than for those either supplemented with iron or fed a diet with adequate protein.(3) Many researchers have since tried to uncover the mechanisms by which zinc affects organ function and, particularly, linear growth.

Symptoms characteristic of zinc deficiency are widespread, implying that multiple organ systems are affected by zinc nutriture. Clinical deficiency symptoms include dermatitis, impaired wound healing, diarrhea, hair loss, growth stunting, delayed sexual maturation and abnormal skeletal development.(3) Observable symptoms typically manifest in more severe states of deficiency when homeostatic control mechanisms can no longer keep serum, or plasma, zinc concentrations within a normal range. However, this does not mean that milder states of deficiency have no clinical significance or are not accompanied by symptom presentation. Suboptimal zinc nutriture could relate to altered biochemical measures that influence an

individual's long-term health. For example, if mild zinc deficiency results in a slight decrease in bone growth and mineralization, this may result in future skeletal health problems, such as increased risk of fractures or osteoporosis.(4)

Since the majority of adult bone mass is acquired during early puberty, and a large proportion of total body zinc resides in the skeleton, the effects of zinc on bone growth and development are of great interest. Zinc deficiency, even when mild, is well known for causing abnormal bone development and stunted growth.(4-6) As stunted growth in childhood negatively impacts the adult skeleton, zinc nutriture is a cause for concern.(4) A large number of studies testing the effects of zinc supplementation in infant, children and adolescent populations exhibiting growth stunting report improvements in linear growth.(7-8) In addition, increases in serum levels of insulin-like growth factor I (IGF-I), osteocalcin and alkaline phosphatase (ALP), all of which are involved with growth and development of bone tissue, are reported to increase with zinc supplementation.(9-10) Furthermore, a controlled animal study reports that zinc-deficient rats had narrower epiphyseal plates, implying a decrease in activity of growth hormone (GH).(11) GH is a potent stimulator of chondrocyte enlargement at the epiphyseal plate. GH induces hepatic production and secretion of IGF-I, a hormone also involved with the expansion of chondrocytes.(10) Also, zinc has been associated with increased expression of GH receptors, GH binding proteins, and IGF-I as well as circulating levels of the two hormones.(12-13)

Since the discovery of zinc deficiency in human populations, over 300 zinc-dependent proteins have been identified, many of which are related to growth and development.(14) Zinc plays multiple roles with body proteins, including catalytic, structural, and regulatory roles. Metalloenzymes, such as DNA and RNA polymerases, require zinc to catalyze reactions that support new tissue growth. Zinc-finger proteins require zinc to maintain proper structure and,

thus, function.(15) In addition, the expression of several proteins, including GH receptors, GH binding proteins, and IGF-I, is regulated by zinc at the transcriptional level.(12) Considering the large number of proteins dependent on zinc for adequate synthesis and function, it is no surprise that the symptoms of zinc deficiency are so widespread.

Multiple cellular proteins exist that transport zinc in and out of cellular compartments, shuttling zinc molecules where needed.(16) These transporters are largely being studied to understand the etiology of symptoms accompanying aberrant zinc metabolism. One transporter in particular, ZIP13, allows for zinc to move from the Golgi apparatus to the cytoplasm. It is coded by the gene known as SLC39A13. With decreased levels of ZIP13, zinc accumulates in the Golgi apparatus and is unavailable to various metalloproteins within the cytosol as well as other areas within the cell, such as the nucleus. The extent of ZIP13 functions is not entirely clear, yet animal studies have shown that ZIP13$^{-/-}$ mice have reduced osteoblast activity, altered collagen proliferation, and abnormal bone development.(17) Much of these effects are due to ZIP13's involvement with the functioning of genes involved with bone metabolism, such as BMP and TGF-β, and binding of hydroxylases, such as prolyl 3- and 4-hydroxylase, which are necessary to ensure proper hydroxylation for the synthesis of collagen.(16, 18-19) Amino acids present in procollagen molecules, often proline and lysine, are hydroxylated by such enzymes, which allows for cross-linking with other procollagens to form strong collagen fibers.(20) Bone collagen is reported to increase following increases in zinc intake.(21)

Several populations have an increased risk of developing zinc deficiency, such as pregnant women, low-income populations, and infants and pre-pubertal children, which are populations experiencing rapid growth. Early pubertal children experience peak growth rates and

increased zinc needs, which should be evident with response to supplementation.(22-23) The present study targeted this population.

SOURCES AND BIOAVAILABILITY OF ZINC

Zinc is widely available in the food supply. Some of the richest, most bioavailable sources include oysters, red meat and pork, seafood, fortified cereals, and nuts. Zinc is also present in grains and legumes, but high phytic acid contents reduce zinc bioavailability.(10, 22, 24-25) Diets high in phytates center mostly around cereal grains such as wheat and barley.(26) Diets low in protein also decrease zinc bioavailability.(22, 25)

Developed countries, such as the United States, have an ample supply of zinc-rich foods as well as documented cases of deficiency.(27-28) Prevalence estimates for a given country are indirect and offer limited information, because they are based on the known zinc content and bioavailability of zinc in foods instead of actual intakes or measures of zinc nutriture.(29) Mild or marginal zinc deficiencies can evade diagnosis when a region's typical diet is considered highly bioavailable, such as in the United States, simply because exposure to diets with higher zinc bioavailability does not necessarily translate to higher intakes of zinc. In populations vulnerable to zinc deficiency, undiagnosed deficiencies can have dangerous, long-term health implications. Zinc supplementation may be needed in such populations to ensure optimal zinc nutriture and growth.

ZINC METABOLISM

Several pools of zinc exist within body tissues, with the extracellular plasma compartment playing a central role. Tissue pools are grouped by how rapidly they exchange zinc with the plasma compartment when zinc nutriture is affected. Although some pools have much longer turnover rates, others, such as those found in the liver, turnover within 72 hours.(30) Zinc-

dependent changes in protein synthesis within and secretion from tissues containing more rapidly exchanging zinc pools are expected to be evident by increases in circulating serum proteins well within the one-month period of the present study.

Little is known about the homeostatic regulation of zinc metabolism. Zinc homeostasis occurs by altering rates of intestinal absorption, endogenous secretion, and fecal excretion. When intake is increased, urine zinc output increases until changes in intestinal absorption can restore homeostasis. At the cellular level, cells respond to excess zinc by regulating proteins involved with cellular zinc efflux, intracellular sequestration, and cellular zinc uptake, likely by inducing changes in gene expression rather than translocation.(31-32) The transcription factor, metal-response binding-element transcription factor-1 (MTF-1), is a major intracellular zinc sensor that binds zinc and subsequently alters the transcription of several genes known to regulate zinc homeostasis.(33)

Levels of zinc intake or tissue zinc concentrations indicative of varying degrees of zinc nutriture are not well-defined and often vary substantially in the literature. Metabolic changes are currently the best indicators of zinc nutriture, valued over intake estimates and symptom presentation. A study with experimental rats reports that growth stunting resulting from inadequate levels of zinc intake did not occur when energy intake was normal, indicating that zinc deficiency estimates based on intake data or symptom presentation are likely underestimated, as a positive energy balance can mask a deficiency.(34) This evidence supports the need of a reliable biomarker or biochemical pattern in order to accurately assess zinc nutriture and risk of deficiency. Figure 1.1 represents an adapted model based on the metabolic and homeostatic disturbances occurring with varying stages of zinc nutriture.(35)

ZINC REQUIREMENTS AND RECOMMENDATIONS

Ideally, recommendations should indicate levels of intake needed to optimize zinc nutriture. Determining physiological zinc needs is complicated by variability of food security, zinc bioavailability, food choices, and zinc metabolism. Current recommendations based on assumed bioavailability of diets typically consumed by a population may be inadequate for many population subsets, especially for many at-risk subsets, because they do not account for the variation in actual food items consumed. Also, there is insufficient evidence describing long-term homeostatic adaptations that could occur in those consuming diets of low zinc bioavailability.(36)

A factorial approach considering kinetics of zinc metabolism and estimations for new tissue growth is used to set dietary reference intakes (DRIs).(22) To yield accurate recommendations, extensive knowledge of physiological needs in all populations is a must. At the time current DRIs were being determined, this metabolic information did not exist for early pubertal children. Instead, DRIs for children and adolescents, 9-13 years, which encompasses much of the early and pre-pubertal population, were derived using weight-based multipliers based on data obtained from metabolic studies in adults.(22) Recently, two studies suggest factors other than body weight, such as intestinal length, account for differences in zinc metabolism between children and adults.(37-38) Accuracy of future recommendations relies on identification of biomarkers of zinc nutriture that uncover the physiological manifestations of deficiency, adequacy, and excess, aiding in the interpretation of accompanying metabolic changes in children.

BIOMARKER IDENTIFICATION

There is a dire need among all areas of research relating to physiological needs of zinc to identify a biomarker reflective of zinc nutriture. Specifically, such biomarkers can enhance interpretations of data from metabolic studies by indicating causes for zinc redistribution among tissues, identifying mechanisms of homeostasis, and determining functional and metabolic differences among various biological zinc compounds. Biomarker identification for disease risk, diagnosis, and pathology is a common goal of current proteomic studies.(39) Proteomic techniques are continually being improved to propel the search for biomarkers of many disease states.

A reliable biomarker is both sensitive and specific to variations of zinc nutriture. Sensitive indicators will respond to fluctuations in dietary intake or metabolic use and will reveal acute changes in intake or needs. Specific indicators will only change in response to changes in zinc nutriture. An acceptable biomarker must also be obtained from a tissue within which its metabolism is well-characterized. The tissue must also be appropriate for routine clinical use and unaffected by disease processes.(35) Serum is an ideal tissue, because it is easy to obtain from patients and provides a whole-body physiological view.

Many candidate biomarkers identified thus far lack the combination of sensitivity and specificity.(22) Therefore, we are currently unable to definitively relate zinc nutriture with functional or biochemical indices.

Analysis of hair zinc content is complicated by age, disease processes, environmental contaminants and multiple stresses, but little is known about how or to what extent these factors affect hair zinc levels.(10, 20) In addition, the amount of zinc retained in hair may be depended

on the rate of hair growth.(40) With further understanding of these contributing factors, hair zinc content may prove useful as a long-term measure of zinc status in the future.

Measuring serum and erythrocyte metallothionein is only useful to assess severe deficiency or toxicity of zinc. Yet, these measures are also affected by metabolic fluxes of iron, copper and cadmium, so they are not specific or sensitive to zinc nutriture.(20, 40-42) Further lack of specificity is evidenced by the influence of cytokines, stress hormones and time of day on metallothionein concentrations.(40, 42-43)

Plasma and serum zinc levels have also been considered as biomarkers of zinc status, yet these measures cannot distinguish between zinc deficiency and adequacy.(44) Plasma and serum zinc are often synonymous within the literature, as will be the case in this review, despite differences incurred from different methods of collection and separation.(45) Deregulation of homeostasis may result in decreased plasma zinc concentrations below the steady-state range of around 12-15 µmol/L. However, this does not occur in all cases, which could be due to adaptive compensatory processes, which are currently uncharacterized.(46) Only a small amount of total body zinc resides in the serum.(44) Changes in energy and tissue metabolism can contribute to the deregulation of homeostasis by increasing zinc fluxes from tissues to the plasma.(35, 47-48) Inflammatory and diurnal responses in plasma zinc concentration also occur.(20, 35, 45, 49) Despite its apparent lack of specificity and sensitivity, this measure has proven useful to indicate zinc nutriture of a population and predict a response to supplementation.(50) Population intakes as low as 2-3 mg/day correspond to significant decreases in plasma concentrations. At higher intakes, plasma concentrations increase until hitting a plateau at intakes of 25-30 mg/day.(47, 51) Serum zinc is commonly measured by atomic absorption spectrophotometry.(44) At best, these

measures may possibly be used in conjuncture with other measures to verify supplementation if serum levels were tested soon after administering a zinc dose.

Reports on the use of erythrocyte (RBC) zinc concentrations for identifying degrees of zinc nutriture are inconsistent.(52-56) Although RBC zinc levels are seemingly unaffected by inflammatory processes, convincing evidence for potential use of this measure as a biomarker is lacking.(57) Much of RBC zinc is bound to carbonic anhydrase, which prohibits RBC zinc levels from responding sensitively to changes in zinc intake.(40) Also, this measure is complicated by the fact that two kinetically distinct compartments have been identified within RBCs, suggesting that the regulation of RBC zinc levels is intricate.(58)

Analyses of leukocyte (WBC) zinc concentrations typically do not distinguish between cell subclasses, such as neutrophils, monocytes, or T-lymphocytes. Because different half-lives exist among these subclasses, interpretations of collective analyses are inaccurate. By separately analyzing subclass zinc concentrations, such as lymphocyte zinc, one may be able to distinguish among degrees of intake.(35) However, isolation of specific subclasses is too difficult for routine analysis, rendering this measure unsuitable as a biomarker.(20) Additionally, conditions have been reported to cause decreases in WBC zinc, but much more study is needed to characterize these effects and identify other causes for alterations in zinc levels.(40)

Urine zinc content does not correlate with dietary zinc intake and may be subject to long-term adaptation with chronic marginal intakes.(20, 59) At most, acute increases could suggest turnover of zinc pools, because urine zinc levels often increase along with positive markers of growth.(60) As levels respond acutely, often before that of serum, decreases could indicate early zinc deficiency.(61-62) Changes in urine zinc content could also be used to assess acute changes in intake.(35) The amount of zinc excreted can be affected by certain metabolic conditions such

as liver dysfunction, wasting, or refeeding.(40) Perhaps, urine zinc would be a good measure to verify supplementation with zinc, but timing of the measurement would need to be considered.

Analyzing global changes in the content of serum proteins in response to zinc supplementation provides the potential for identifying whole-body states of tissue metabolism. Certain proteins, such as alkaline phosphatase and 5'-nucleotidase, may not be useful as biomarkers of zinc nutriture alone but may serve in conjunction with others to signify whole-body zinc nutriture. Proteomic studies, such as the one described here, are using new and promising techniques that will enhance the ability to identify new candidate biomarkers. Depletion strategies are often used to remove higher abundance proteins (mg/mL) prior to performing two-dimension electrophoresis (2-DE), which increases visualization of lower abundance serum proteins (ng/mL).(63-64) Removing high abundance serum proteins from crude samples was a vital step in the present study for two reasons. Certain zinc-responsive proteins, such as GH, and other identified biomarkers of disease are often present in low abundance.(63-64) Also, our goal was to identify proteins previously not recognized to respond to zinc supplementation that could have been masked by higher abundance proteins in previous studies. Multiple protein removal kits are available for use, differing in terms of which proteins are removed and to what degree.(64) We used the Seppro® IgY 14 Spin Columns (Sigma-Aldrich, #SEP010). This kit removes 14 high abundance proteins: albumin, IgG, IgA, transferrin, α2-macroglobulin, complement C3, α1-acid glycoprotein (Orosomucoid), HDL (apolipoproteins A-I and A-II), LDL (mainly Apolipoprotein B), α1-antitrypsin, IgM, haptoglobin, and fibrinogen. Albumin and IgG alone constitute 60-80% of the total protein content in serum.(64) In addition to being abundant, immunoglobins complicate protein analyses and subsequent identification efforts using mass spectrometry due to their multiple, random protein sequences

that normally function to detect foreign substances in the body. With these 14 proteins removed, various medium and low abundance proteins were revealed on two-dimensional (2D) gels.

HYPOTHESIS AND SPECIFIC AIM

Hypothesis: Early and pre-pubertal, Caucasian girls taking 24 mg oral zinc sulfate for 30 days have different contents of serum proteins following supplementation than matched controls taking a placebo.

Specific Aim: Do significant changes occur in the content of serum proteins in response to four weeks of zinc supplementation?

PRELIMINARY DATA

No previous studies have been conducted to determine changes in serum protein contents following zinc supplementation in early pubertal females. Therefore, methods used in the present study were performed as strategically as possible to ensure maximum reproducibility among samples. Two-dimensional electrophoresis (2-DE) is capable of high reproducibility, with a strong correlation between corresponding protein spot volumes on replicate electrophoretograms ($r = 0.85$). However, as variability existing among subjects can greatly decrease the consistency of differences seen using 2-DE, our study design included measures to eliminate as much of this variability as possible.(65)

The depletion strategy employed to reduce the content of high abundance serum proteins is necessary to narrow the magnitude of concentration differences among serum proteins, because current separation techniques are not able to accurately detect proteins within such a wide range.(66) Figure 1.2 illustrates the results obtained before (A) and after (B) the removal of multiple high abundance proteins from serum samples using the selected method. As many largely abundant serum proteins function as carrier proteins, their removal is sometimes

accompanied by the removal of a certain low abundance proteins. The Seppro® columns contain IgY antibodies, which are considered the most specific in terms of binding substrates. Similar products tested for efficacy are reported to retain very few non-targeted proteins when removing high abundance proteins.(67-68) Figure 1.3 shows the location of several of the proteins that were removed from the serum samples prior to measuring protein content. Many of the proteins that showed differential trends between groups are located near these high abundance proteins, so several of the changes observed may have gone unnoticed if the samples had not first been prefractionated.

The current study included only early and pre-pubertal females to encompass peak growth rates.(23) Serum protein changes in this population are reported in only three zinc supplementation studies, all of which offered limited information as to expectations for this study. After six weeks of zinc supplementation [50 mg/day; > 0.60 mg/kg bw], Imamoglu et al. (2005) report increases in serum levels of bone ALP, osteocalcin, IGF-I, and insulin-like growth factor binding protein-3 (IGFBP-3) in children of idiopathic short stature but not zinc deficient. However, the study design lacks a control group. Thus, normal growth could be partly responsible for the observed increases in serum protein content, which may not actually be significant.(69) Another group following healthy adolescents with no apparent growth stunting for six weeks used much lower supplementation doses [15 mg/day; 0.30 mg/kg bw] and did not find any differences in content of serum bone biomarkers between the supplement and placebo groups.(70) A collaborator of the present study conducted a three-week trial that suggests the inconsistent reports from the two aforementioned studies are a result of the doses used. The trial shows that zinc supplementation [30 mg/day; 0.60 mg/kg bw] in healthy adolescent girls, 11-14 years, increases in IGF-I (7.6%) and IGFBP-3 (16.7%) (unpublished data).

A cross-sectional study(71) reports that serum IGF-I concentrations are influenced by an interaction between age and pubertal stage, not considering any effects of zinc supplementation (Figure 1.4).(71) Older early pubertal females have higher serum IGF-I concentrations compared to younger early pubertal females. This report raises the issue of whether or not baseline content of proteins varying with age will influence changes in protein content occurring in response to zinc supplementation. Our study does not thoroughly address this relationship, but it discussed in more detail in later sections.

Based on our review of the existing literature, changes in serum protein concentrations may depend on the following factors, not all of which are measureable: stage of subject maturation; baseline adequacy of zinc pools; baseline content of serum proteins, genetic differences affecting zinc metabolism; individual dietary zinc content; and methodology.

FIGURES

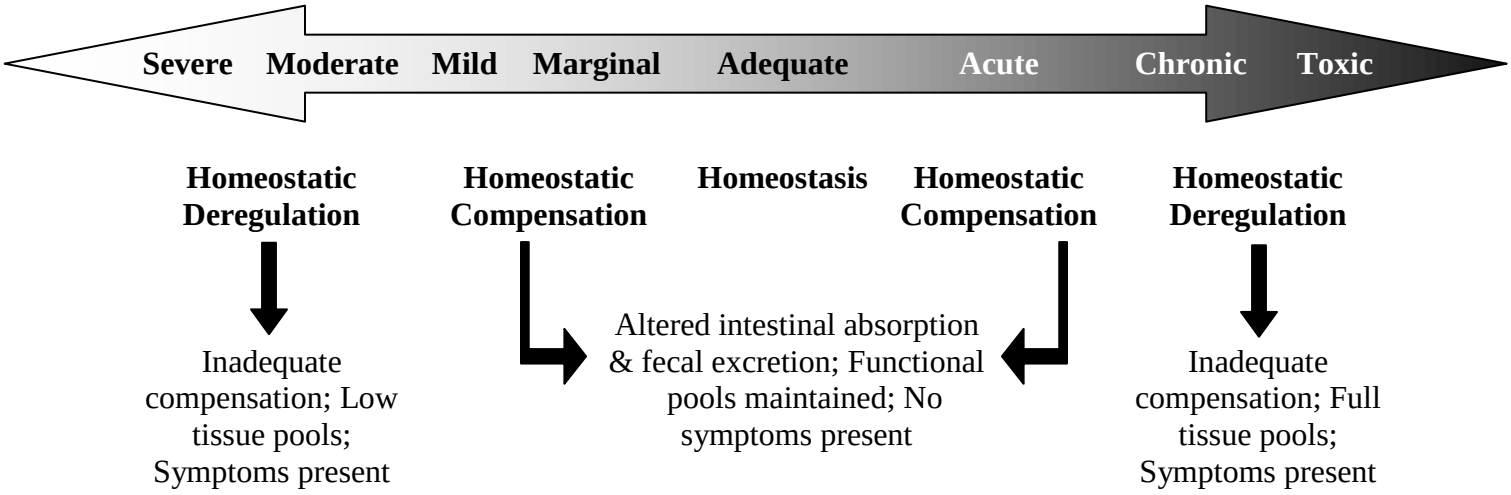

Figure 1.1. **Spectrum of zinc nutriture.** This figure represents a model based on the metabolic and homeostatic disturbances occurring with varying stages of zinc nutriture.

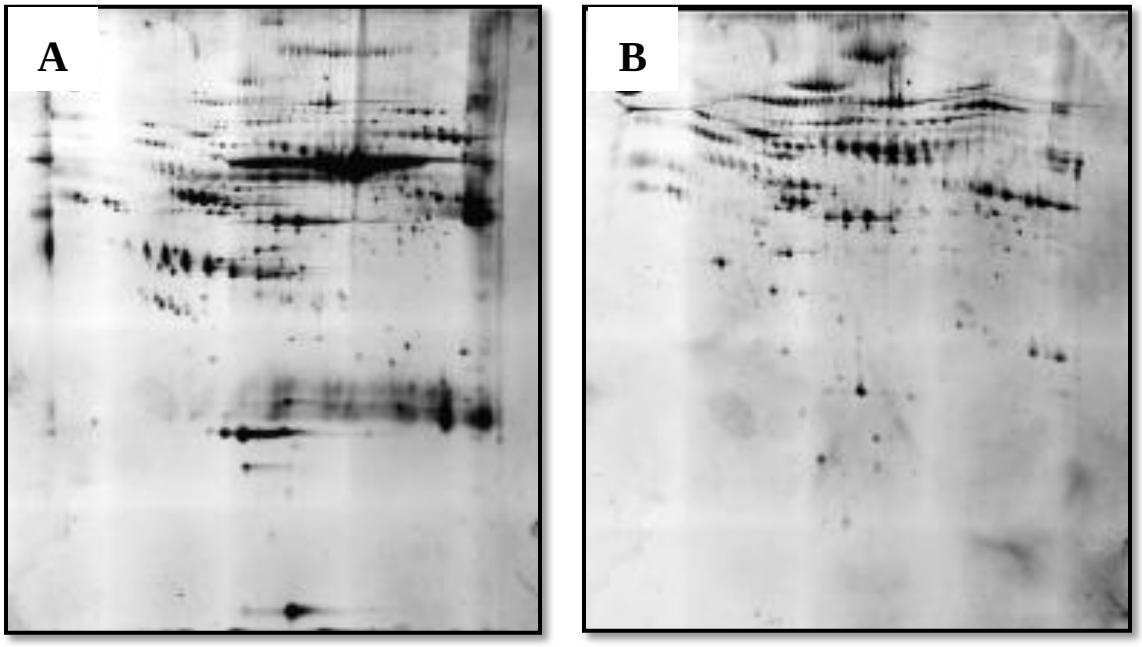

Figure 1.2. **Effect of protein removal from serum.** Image A represents a 2D gel prior to removal of the 14 abundant proteins. Image B represents a 2D gel after removal. These images are results of running samples on 10% SDS-PAGE gels.(72)

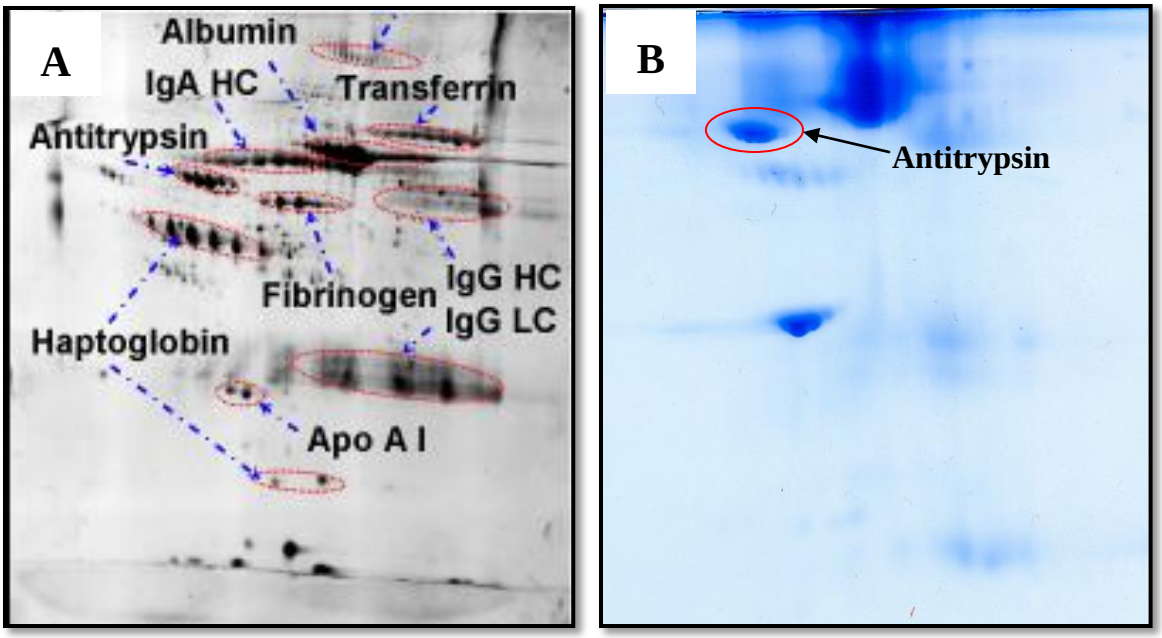

Figure 1.3. Location of high abundance proteins removed from serum samples. Image A shows the relative locations of certain proteins that were removed from serum samples.(72) The protein labeled 'antitrypsin' was also identified in our prefractionated samples. Image B is a 2D gel of the proteins we eluted from immunoaffinity columns. Antitrypsin is also present in our eluted / bound fraction.

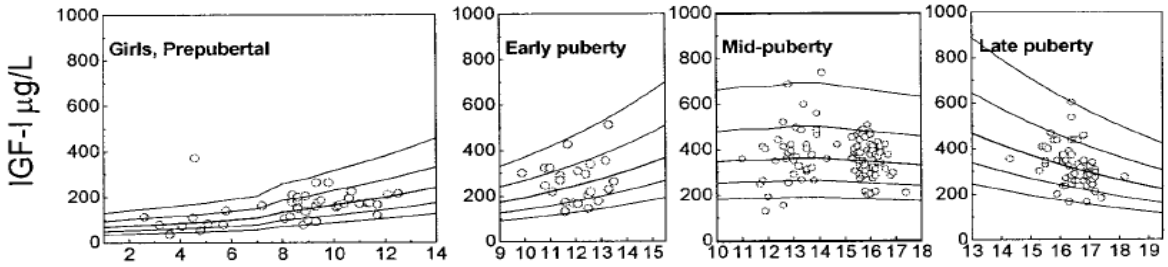

Figure 1.4. Female serum IGF-I concentrations by age and pubertal stage. Levels of IGF-1 appear to depend on age and pubertal stage. The *p*-value of the interaction was < 0.001.(71)

CHAPTER 2

MATERIALS AND METHODS

STUDY POPULATION

Participants were recruited voluntarily from local elementary schools and pediatrician's offices as well as through distribution of flyers and postcards and announcements in newspapers, television and radio. All recruitment tools were approved by the National Institutes of Health Review Board prior to use. Recruitment efforts were conducted throughout Athens-Clarke County, Georgia and surrounding counties.

Participants are 12 healthy, well-nourished, early pubertal females of Non-Hispanic white ethnicity, ages 9-11 years, selected from an ongoing, randomized, placebo controlled trial testing the effects of zinc supplementation on bone health in this population. Healthy is defined as the absence of any disease or illness requiring use of medications. Well-nourished is defined using gender-specific CDC and National Center for Health Statistics body mass index (BMI) growth charts.(73) Exclusion criteria includes having an existing condition requiring medications that could result in multiple treatment interactions or discontinuation of a previously administered treatment regimen, or use of medications or conditions known to influence zinc absorption or nutriture (e.g., Type I Diabetes, Acrodermatitis Enteropathica, Sickle Cell Anemia, cirrhosis, liver disease, parasitic infections) or bone metabolism.(74) Subjects taking any supplements were instructed to undergo a four-week washout period prior to participation.

Subjects were matched based on the following: maturity offset (MO) using a validated equation;(75) Tanner stage of maturation (stage II) determined by self-assessment;(76) body

weight (kg); and compliance measured using a questionnaire and pill count. MO is defined as years preceding onset of peak height velocity (PHV), where a value of "0" indicates onset of PHV.(75)

The present study only included Non-Hispanic white females. Non-Hispanic black females are excluded due to known differences in diet patterns, metabolism, growth patterns and timing of growth, body composition, physical activity and compliance patterns.(77) Also, the MO equation is not validated for use with Non-Hispanic black populations.(75) Only one gender was included due to multiple differences in zinc metabolism and timing of events related to hormonal control and pubertal development.(10, 37) Inclusion of more than one ethnicity or gender would greatly increase variability among subjects, which could mask significant relationships existing in subsets of the study population. Additionally, males were not included in the initial study due to a lack of experience with male participants.

SAMPLE COLLECTION

Blood samples were taken from subjects after an overnight fast. Within an hour of collection, samples were centrifuged to separate serum and plasma fractions. Serum samples were then pipetted into 1.5-mL microtubes in 0.5 mL aliquots and stored immediately at -80°C.

IMMUNOAFFINITY PREFRACTIONATION

Several high abundance proteins were removed using the Seppro® IgY 14 Spin Columns (immobilized cibacron blue/Protein A gel suspended by beaded agarose; 50% slurry, 0.02% sodium azide). The columns arrived pre-assembled in spin columns and packed in storage buffer (25 mM Tris, 25 mM NaCl, 0.01% sodium azide; pH 7.5). Columns were labeled "A" and "B" so that runs using a particular column could be analyzed specifically. Before prefractionating each sample, storage buffer was removed through centrifugation, and the columns were washed

twice using 1X dilution buffer (100 mM Tris-Buffered Saline, 1 M Tris-HCl with 1.5 M NaCl, pH 7.4). After each wash, columns were centrifuged 30 seconds at 2 xg.

Each sample was prepared in duplicate, and both fractions were prefractionated simultaneously. Column A always prefractionated the first sample fraction, and column B, the second. The specified maximum sample volume (20 µL) was combined with 480 µL 1X dilution buffer for a total volume of 500 µL sample fraction dilution, the minimum volume recommended for loading columns. Each fraction was transferred to a 0.45 µm spin filter (one per sample) and centrifuged 1 minute at 10 xg to remove particulates, such as lipids, that could impede analysis. This step was staggered with washing of the columns so that upon final centrifugation of the columns, the filtered samples could be added to the columns immediately, preventing any damage to the beads from excessive drying. Samples were then added to columns and processed according to kit instructions.

PROTEIN EXTRACTION AND MEASUREMENT

Protein content of samples and a standard, 1.46 mg/mL bovine serum albumin (Protein Standard II, Bio-Rad Laboratories, #500-0007) were measured using the Reducing Agent and Detergent Compatible (RC/DC) Protein Assay Kit II (Bio-Rad Laboratories, #500-0122). Five dilutions, totaling 25 µL in volume, of the standard were prepared, while the samples were not diluted. To precipitate the protein, 125 µL of both RC Reagent I (#500-0117) and RC Reagent II (#500-0118) were added to each tube. After centrifuging 5 minutes at 14,000 xg in a non-refrigerated centrifuge, supernatants were removed and discarded, taking care not to disturb protein pellets. All tubes were inverted on a clean paper towel to air-dry. To resolubilize the protein, 125 µL Reagent A' (5 µL DC Reagent S (#500-0115) per 250 µL DC Reagent A (#500-0113) was added to each tube. Finally, 1 mL of DC Reagent B (Folin reagent containing 0.5%

$CuSO_4 \cdot 5H_2O$ in 1% Na^+ of K^+ tartrate; #500-0114) was added. After a 15-minute incubation period, absorbances of standard dilutions and samples were read with the Beckman Coulter DU 800 spectrophotometer at an absorbance of 750 nm. A standard curve was created based on absorbance readings of the bovine standard. Sample protein concentrations were determined by comparing absorbances to the standard curve. Sample concentrations determined via spectrophotometry were then used to determine sample volumes needed for the downstream application. Samples remained on ice throughout the experiment to await acetone precipitation, while the standard was returned to -80°C storage.

ACETONE PRECIPITATION

Volumes remaining in the original sample fractions were estimated and multiplied by the calculated protein concentrations to derive the protein content in each sample. As each sample was run in duplicate, 75 µg protein were needed from each fraction in order to load 150 µg protein downstream. After calculating the volume of each sample fraction containing 75 µg, volumes from each set of fractions were combined and vortexed. To end with volumes easily divisible by 200- or 350-µL, ddH_2O was added to the combined fractions. Samples were then divided into 200- or 350-µL aliquots, where there were several sample fractions, or microtubes, per subject. Four times the volume of acetone was then added to each microtube (i.e., 800 µL added to 200 µL, and 1400 µL added to 350 µL). Samples were frozen at -80°C for at least 30 minutes.

RESOLUBILIZATION

After precipitation, samples were thawed, supernatants were discarded, and tubes were inverted to air-dry. To resolubilize proteins, 125 µL protein solubilization buffer (PSB) was added to the first sample fraction, or microtube, for each subject, and tubes underwent a 10-

second vortex, 2-minute incubation and 5-minute centrifugation at 14,000 xg. After the pellet was well-dissolved, contents of the first microtube were transferred to the second microtube containing the second sample fraction for a particular subject to resolubilize the subsequent pellet. This procedure was repeated until all protein pellets from all microtubes for a given subject were combined into a single microtube for each subject. Residuals were collected from emptied microtubes and added to the final, combined sample. Sample volumes were then estimated and brought to 125 µL using PSB.

REHYDRATION

A rehydration solution, or protein solubilizing buffer, (2 mM TBP, 0.2% ampholyte, 0.0002% bromophenol blue), obtained from Bio-Rad was added to the samples. Samples were then pipetted into a clean, IEF tray, and IPG strips (Bio-Rad ReadyStrips™, 7 cm, #163-2000) containing an immobilized pH gradient (pH 3-10) were laid on top. Immobilized pH gradients are shown to decrease variability among sample analyses and prevent gradient shifts.(78) Mineral oil was applied atop the strips, 1.5 mL per lane, to prevent evaporation of the buffer and drying of strips. The solubilized sample mixture was adsorbed to the strips via active rehydration for approximately 15.5 hours in a Bio-Rad Protean IEF cell set at 20°C.

ISOELECTRIC FOCUSING: 1^{ST} DIMENSION

After rehydration, two wicks, wetted with 10 µL ddH_2O, were placed carefully under each IPG strip to protect the electrodes in the IEF tray. The tray was placed back in the Bio-Rad Protean IEF cell. IEF was run on a rapid preset method with the following settings: 20,000 VH, 4000V / 20°C, and 50 µA current per strip to cause migration of proteins to their respective isoelectric points. IEF ran approximately 7.5 hours. Strips were drained onto a clean paper towel

and placed in lanes of a clean IEF storage tray. The tray was wrapped in Saran wrap and stored at -80°C until ready to perform the second dimension.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS: 2ND DIMENSION

SE600 vertical slab gel units were assembled in dual gel casting stands using glass pieces and 1.5-mm spacers that were pre-cleaned with 100% ethanol. The second dimension slab gel (15%) was prepared according to the Hoefer Electrophoresis Guide (Hoefer Scientific Instruments, San Francisco, CA). The slab gel solution contained 30 mL monomer solution (30% Acrylamide/Bis solution, 29:1 (3.3% C); Bio-Rad), 4X running gel buffer (1.5 M Tris-HCl Buffer, pH 8.8; Bio-Rad), 600 µL 10% SDS, and 14.1 mL ddH_2O. After degassing the mixture, 300 µL ammonium persulfate and 20 µL TEMED were added, and the mixture was pipetted into the slab gel molds. A layer of water-saturated butanol was added atop the gel surface to smooth the surface of the gel and prevent dehydration while polymerizing. Once polymerization was complete, the butanol was removed, and the gel surface was washed twice with ddH_2O and drained before being filled with upper tank buffer (1X Tris-glycine-SDS buffer).

IPG strips containing proteins separated by their isoelectric points were thawed exactly 15 minutes at room temperature. Second dimension equilibration buffer (6 M urea, 0.375 M Tris, 2% SDS, 20% glycerol, TBP) was prepared by adding 10 µL TBP per mL of equilibration buffer needed (2 mL equilibration buffer needed per IPG strip). After thawing, strips were incubated in this buffer for 20 minutes while on a shaker. Afterward, end tabs were removed from each strip, and strips were placed into wells and positioned flush against the second-dimension denaturing polyacrylamide gel (SDS-PAGE). The gel units were then placed into a Hoeffer electrophoresis chamber containing Tris-Glycine-SDS (TGS) running buffer (1X TGS buffer). Once assembled,

electrophoresis proceeded using 30 µA per gel constant current (Consort EV265 power supply) and was stopped when the dye front migrated about 7-9 cm from the top of the resolving gel.

Upon completion of SDS-PAGE, the second-dimension gels were fixed with a 40% methanol / 10% acetic acid solution for at least 30 minutes. The fixative was removed, and the gels were washed with ddH_2O (3 x 10 minutes) to remove the fixative and SDS within the gel, ensuring adequate staining by Coomassie blue G-250. The separated proteins were visualized by staining with colloidal Coomassie G-250 blue-silver stain (10% HPO_3, 10% NH_4SO_4, 20% CH_3OH, 0.12% Coomassie G-250).(79-80) The gels were stained for a minimum of 6.5 h with gentle shaking. The gels were destained with ddH_2O until the background was clear.

PROTEIN SPOT ANALYSIS

The gels were digitally scanned using HP PrecisionScan Pro 3.1 software. All were scanned on the same day to ensure consistency of variables within the scanner program. Original scanned images (64-bit color) were saved without adjustments to preserve the original image of each gel. An additional color image (64-bit color) of each gel was saved after slight adjustments to maximize visualization of the spots and maintain consistent spot densities between the gel images. The digitized images were then converted to grayscale (64-bit grayscale). Once maximal spot visibility was obtained, the grayscale images were saved. The spot densities (mm^2) were measured, and differences determined, by Phoretix 2D Analysis software (version 6.01). Spot density, or spot volume, is based on the pixel intensity within a given protein spot minus the most frequently occurring pixel intensity of the area outside of each spot, the latter of which is considered the background intensity.

Spot selection involved analyzing patterns and averages of protein changes within and between each treatment arm, taking into account standard deviations and standard errors. Spots

were not considered unless proteins changed in the same direction in at least four of the six subjects in the treatment group and exhibited greater than a 1.5-fold difference between the treatment and control groups. Some spots with less than a 1.5-fold difference were also chosen if patterns of protein change indicated an effect of zinc supplementation. Spots were analyzed separately unless they were positioned too close together on second-dimension gels to distinguish the edges of each spot.

To determine true spot intensity, data was normalized by subtracting the background intensity from the total intensity of each spot. The data were then analyzed, and spots showing significant variance according to the aforementioned criteria were selected and manually excised from 2D gels. A gel containing a spot to be excised was selected if it contained the most representative spot compared to other gels. We specifically avoided choosing spots with an extremely low spot volume to ensure enough protein was available for analysis by mass spectrometry. A protein content of < 50 µg is not sufficient for subsequent staining procedures or proper identification. Excised spots were placed in clean, 1.5-mL microcentrifuge tubes. The gel plugs were sent to the Proteomics Core Laboratory at the Medical College of Georgia for in-gel tryptic digestion, mass spectrometry analysis and protein identification using GPS Explorer to compare the spectra to sequence information found in the standardized databases Protein Prospector, ProFound, and Mascot. The top protein, out of 10 possibilities, was considered as the protein identity for each spot.

STATISTICAL ANALYSES

Statistical analyses were performed using Microsoft® Office Excel® 2007 and PASW Statistics 18. Differences between baseline characteristics were determined using independent t-tests assuming equal variances, as indicated by non-significant *f*-statistics. Two-tailed, paired t-

tests were used to calculate within-group differences in baseline and four-week protein spot volumes. Independent t-tests were used to analyze between-group differences. F-tests were used to determine which t-tests, assuming equal or unequal variances, were appropriate for each data set. Sample variances with a p-value > 0.05 were considered significantly different, and such data were analyzed using t-tests assuming unequal variances. If significant differences existed between mean group baseline spot volumes, analysis of covariance (ANCOVA) was used to determine differences between groups. ANCOVA was also used to calculate effect sizes.

CHAPTER 3

RESULTS

SUBJECT CHARACTERISTICS

Values of baseline characteristics are shown in Table 3.1. Subjects were matched according to baseline maturity offset (MO), baseline body weight (BW), and Tanner stage of maturation. To a lesser extent, efforts were made to match subjects with similar baseline ages and body-mass-index (BMI). None of these variables were significantly different at baseline. Values of *p* are shown in Table 3.2.

DIFFERENTIALLY EXPRESSED PROTEINS

Total protein concentrations and total protein contents of immunoaffinity-depleted serum samples are listed in Table 3.3. A total of 64 spots were observed and matched on two-dimensional gels. Representative zinc and control two-dimensional gels are shown in Figure 3.1. The relative locations of the 64 matched spots on two-dimensional gels are shown in Figure 3.2. Initial screening of 2D gels, spot volumes and spot patterns suggested that ten of these spots were differentially expressed between treatment groups. These ten spots were selected for identification by mass spectrometry (MS). Of these ten proteins, three exhibited > 1.5-fold difference, with one > 2-fold. The other six proteins exhibited > 1.0-fold difference ranging from 1.047- to 1.350-fold. The results of MS are shown in Table 3.4. Results are reported in the order of decreasing significance. Images of two-dimensional gels illustrating differences in protein expression for each spot were magnified and are located at the end of this chapter. Although no

spot change differences reached significance, there were other changes within each group that are of interest and may warrant future investigation. All spot volumes are reported in mm^2.

Due to the limited sample size of this study, statistical analysis was performed two ways: 1) analysis including all subjects in each group, regardless of directional change patterns in spot volumes (i.e., including those showing increases or decreases in a given protein); and 2) analysis including only those subjects showing changes in the same direction. Values derived from the former analysis will be referred to as "all-subject" values. Values derived from the latter analysis will be referred to as "trend-subject" values. One of the criteria for identifying differential protein patterns was that the majority of subjects (i.e., at least four of six) in the zinc supplementation group must exhibit spot volume changes in the same direction. The directional change identified for each spot is considered the spot's trend. Subjects in both groups showing spot volume changes for a given spot consistent with the spot's trend were included in trend-subject analysis. All values are reported in tables at the end of this chapter, but results of trend-subject analyses are only reported if the data suggest a substantial increase in significance.

IgM Kappa IIIB SON

Spot 48 was identified as IgM Kappa IIIB SON (IgMk3) (accession #gi_224377) by mass spectrometry. IgMk3 has an isoelectric point (p*I*) of 8.65 and a molecular weight of 13.9 kDa. This protein migrated about 4-5 cm below the top of the resolving gel. Overall, IgMk3 decreased in the majority of subjects.

Trend subjects: IgMk3 decreased in four of the six subjects in the treatment group (n=4) and in only three of the six subjects in the control group (n=3); the treatment group exhibited less variability in directional change. Baseline spot volumes were not significantly different between groups ($p = 0.393$). The treatment group exhibited a 1.676-fold larger decrease than the control

group trend subjects. Baseline and four-week spot volumes were significantly different in the treatment group ($p = 0.014$) but not in the control group ($p = 0.343$). In these subjects, zinc supplementation contributed to about 70.3% of the differences observed between groups, which is considered a large effect ($\eta p2 = 0.703$). This effect reached statistical significance ($p = 0.037$).

All subjects: At baseline, spot volumes between treatment and control groups were not significantly different ($p = 0.348$). The treatment group exhibited a 1.084-fold larger change in spot volume over the course of the study than the control group. The average volume change over four weeks was not significantly different between groups ($p = 0.876$). Spot volumes at four weeks did not significantly differ between the two groups ($p = 0.154$). Baseline spot volumes were not significantly different from those at four weeks within the treatment group ($p = 0.219$) or control group ($p = 0.978$). When comparing these changes between groups, univariate analysis reveals a small effect of zinc, with zinc contributing to about 14.7% of the difference ($\eta p2 = 0.147$).

All values for IgMk3 are shown in Tables 3.5 – 3.7 and are illustrated in Figures 3.3 and 3.4. The spot corresponding to IgMk3 on 2D gels is shown in Figure 3.5.

Proapolipoprotein and Chain E, Serum Amyloid Protein

Spot 84 was identified as Proapolipoprotein (Pal) (accession #gi_178775) using mass spectrometry. Pal has a p*I* of 5.45 and a molecular weight of 28.9 kDa. Spot 61 was identified as Chain E, Serum Amyloid P (E-SAP) (accession #gi_30582339), which has a p*I* of 6.1 and a molecular weight of 25.5 kDa. The following reported volumes include both spots, because they were too close together on 2D gels to be accurately analyzed as separate spots. These proteins migrated about 4-5 cm below the top of the resolving gel. Overall, decreases in spot volumes were observed.

Trend subjects: Spot volume decreased in five of the six subjects in the treatment group (n=5) and in all of the six subjects in the control group (n=6); the treatment group showed more variability in directional change. Baseline spot volumes were not significantly different between groups ($p = 0.509$). As all of the subjects in the control group had decreases in spot volume, all control subjects were included in all-subject and trend-subject analyses. The treatment trend subjects showed a change 1.137-fold higher than the control group subjects. Spot volume differences between groups at four weeks trended toward significance ($p = 0.097$). Among treatment trend subjects, baseline spot volumes were significantly different from those at four weeks ($p = 0.022$). Univariate analysis reveals that zinc supplementation had a moderate effect, contributing about 45.8% to the difference observed between groups ($\eta p2 = 0.458$). This effect did reach statistical significance ($p = 0.032$).

All subjects: At baseline, spot volumes between treatment and control groups were not significantly different ($p = 0.374$). The control group exhibited a 1.047-fold larger change in spot volume over the course of the study than the treatment group. The average volume change over four weeks was not significantly different between groups ($p = 0.904$), nor were spot volumes at four weeks ($p = 0.149$). Within the treatment group, baseline spot volumes were significantly different from those at four weeks ($p = 0.034$). These values were also significantly different within the control group ($p = 0.002$). Zinc supplementation had a relatively small effect, contributing a mere 13.8% to the difference observed between groups ($\eta p2 = 0.138$).

All values for Pal and E-SAP are shown in Tables 3.8 – 3.10 and are illustrated in Figures 3.6 and 3.7. The spot corresponding to E-SAP and Pal on 2D gels is shown in Figure 3.8.

Alpha-1-Antitrypsin and Keratin 1

Spot 69 was identified as Alpha-1-Antitrypsin (A1A) (accession #gi_6137432). A1A has a p*I* of 5.43 and a molecular weight of 44.4 kDa. Spot 99 was identified as Keratin 1, Type II cytoskeletal (K1) (accession #gi_7428712), which has a p*I* of 6.03 and a molecular weight of 65.6 kDa. Reported spot volumes include both spots, once again, due to the close proximity of the spots on 2D gels. These proteins migrated about 2 cm below the top of the resolving gel. Overall, we observed an increasing pattern in the zinc supplementation group and a decreasing pattern in the control group.

Trend subjects: Spot volume increased in four of the six subjects in the treatment group (n=4) but decreased in all of the six subjects in the control group (n=6); the treatment group exhibited more variability in directional change. As all of the subjects in the control group had decreases in spot volume, all control subjects were analyzed in all-subject and trend-subject analyses. At baseline, spot volumes between treatment and control groups were significantly different for trend subjects ($p = 0.032$). The control group showed a change 1.371-fold larger than the treatment group trend subjects. Within the treatment group, differences between baseline and four-week spot volumes trended toward significance ($p = 0.068$), while differences were significant within the control group ($p = 0.010$). When controlling for baseline differences in spot volume, univariate analysis reveals that zinc supplementation contributed to about 51.6% of the difference observed between groups, which is considered a moderate to large effect ($\eta p2 = 0.516$). Additionally, when baseline values were normalized, the difference between group changes did reach significance in trend-subject analysis ($p = 0.029$).

All subjects: At baseline, spot volumes between treatment and control groups were not significantly different ($p = 0.356$). The treatment group exhibited a 1.231-fold larger change in

spot volume over the course of the study than the control group. The average volume change over four weeks was not significantly different between groups ($p = 0.672$). Spot volumes at four weeks did not significantly differ between the two groups ($p = 0.762$). Within the treatment group, baseline spot volumes were not significantly different from those at four weeks ($p = 0.732$). Within the control group, baseline values were significantly different from those at four weeks ($p = 0.010$). Univariate analysis reveals that zinc supplementation had virtually no effect on the difference observed between groups ($\eta p2 = 0.011$).

All values for A1A and K1 are shown in Tables 3.11 – 3.13 and are illustrated in Figures 3.9 and 3.10. The spot corresponding to A1A and K1 on 2D gels is shown in Figure 3.11.

Hemopexin

Spot 19 was identified as Hemopexin (Hpx) (accession #gi_1321561) using mass spectrometry. Hpx has a p*I* of 6.55 and a molecular weight of 52.4 kDa. Reported volumes for Hpx include spots 19 and 20, as the two proteins were positioned too close together on 2D gels to be analyzed separately. One spot, Hpx, was chosen for further analysis, because it seemed to have the larger impact of the two proteins on the changes in total spot volume. These proteins migrated about 2 cm from the top of the resolving gel. Overall, we observed decreases in Hpx.

Trend subjects: Spot volume decreased in five of the six subjects in the treatment group (n=5) and in only four of the six subjects in the control group (n=4); the treatment group exhibited less variability in directional change. At baseline, spot volumes between treatment and control groups were not significantly different for trend subjects ($p = 0.544$). The treatment group exhibited a 3.071-fold larger change in spot volume over the course of the study than the control group. Though the average spot volumes at four weeks did not differ significantly between groups ($p = 0.495$), the average volume change between groups trended toward

significance (p = 0.081). Baseline spot volumes were significantly different from those at four weeks in the treatment group (p = 0.020) but not in the control group (p = 0.110). Univariate analysis reveals a small to moderate effect of zinc supplementation (ηp2 = 0.340), with zinc contributing to about 34% of difference between groups. Though a small to moderate effect size was observed, it was not large enough to reach significance (p = 0.129).

All subjects: At baseline, spot volumes between treatment and control groups were not significantly different for all subjects (p = 0.274). The treatment group exhibited a 2.590-fold larger change in spot volume over the course of the study than the control group. The average volume change over four weeks was not significantly different between groups (p = 0.119; 0.132). Two p-values are reported for all-subject data, because a t-test was performed for equal as well as for unequal variances; the f-statistic indicated borderline significant variance among samples (f = 5.048, p = 0.050). Spot volumes at four weeks did not significantly differ between the two groups (p = 0.746). Within the treatment group, baseline spot volumes were significantly different from those at four weeks (p = 0.030), whereas they were not significantly different within the control group (p = 0.564). Univariate analysis of all-subject data reveals a smaller effect of zinc supplementation on spot volume changes, with zinc contributing about 23.3% to the difference observed between groups (ηp2 = 0.233).

All values for Hpx are shown in Tables 3.14 – 3.16 and are illustrated in Figures 3.12 and 3.13. The spot corresponding to Hpx on 2D gels is shown in Figure 3.14.

Alpha 2-HS Glycoprotein

Spot 24 was identified as Alpha 2-HS Glycoprotein (A2HS) (accession #gi_2521981) using mass spectrometry. A2HS has a p*I* of 5.2 and a molecular weight of 36.3 kDa.

Volumes reported for A2HS include a total of seven protein spots (spots 24-30) which are positioned very close together on 2D gels. These proteins migrated about 2 cm below the top of the resolving gel. Spots 25-30 were very small, were not observed in several gels, and showed no pattern of appearance separately for further analysis. Therefore, A2HS, the biggest spot and the one likely to have contributed to the majority of spot volume variance among subjects, was selected for further analysis. More details are provided in Chapter 2 under the section heading, "Protein Spot Analysis". Overall, we observed decreases in A2HS.

All subjects: At baseline, spot volumes between treatment and control groups were not significantly different ($p = 0.341$). The treatment group exhibited a 1.219-fold larger change in spot volume over the course of the study than the control group. The average volume change over four weeks was not significantly different between groups ($p = 0.530$), nor were spot volumes at four weeks ($p = 0.442$). Within the treatment group, baseline spot volumes were not significantly different from those at four weeks ($p = 0.316$). Similar observations were made within the control group ($p = 0.157$). Univariate analysis reveals that zinc supplementation virtually had no effect on the observed difference between groups ($\eta p2 = 0.001$).

Trend subjects: A2HS decreased in four of the six subjects in the treatment group (n=4) and in five of the six subjects in the control group (n=5); the treatment group exhibited more variability in directional change. Baseline spot volumes were not significantly different between groups ($p = 0.142$). Differences between baseline and four-week values trended toward significance in zinc-supplemented subjects showing decreases in A2HS ($p = 0.053$) and were significantly different among control trend subjects ($p = 0.004$). When comparing the changes between groups, the effect of zinc was small to moderate ($\eta p2 = 0.335$), yet insignificant ($p = 0.133$).

All values for A2HS are shown in Tables 3.17 – 3.19 and are illustrated in Figures 3.15 and 3.16. The spot corresponding to A2HS on 2D gels is shown in Figure 3.17.

Inter-alpha (globulin) inhibitor, H2 polypeptide

Spot 32 was identified as Inter-alpha (globulin) Inhibitor, H2 polypeptide (IH2) (accession #gi_55958063). IH2 has a p*I* of 6.56 and a molecular weight of 105.6 kDa. Reported volumes for IH2 include spots 31 and 32, as these two spots were positioned too close together on 2D gels for separate analysis. The two proteins migrated about 0.5 cm below the top of the resolving gel. Again, IH2 appeared to be the more variable of the two spots between groups, so it was selected for further analysis. Overall, we observed increases in IH2 in most subjects.

Trend subjects: IH2 increased in five of the six subjects in the treatment group (n=5) and in only three of the six subjects in the control group (n=3); the treatment group showed less variability in directional change. At baseline, spot volumes between treatment and control groups were not significantly different for trend subjects ($p = 0.357$). The control group exhibited a 2.047-fold larger change in spot volume over the course of the study than the treatment group. The average volume change over four weeks was not significantly different between groups ($p = 0.114$). Spot volumes at four weeks did not significantly differ between the two groups ($p = 0.262$). Baseline spot volumes were not significantly different from those at four weeks within the treatment group ($p = 0.121$), while they were significantly different within the control group ($p = 0.002$). Univariate analysis reveals a small effect of zinc supplementation on changes in spot volume among trend subjects, with zinc contributing to about 21.2% of the difference observed between groups ($\eta p2 = 0.212$).

All subjects: Baseline spot volumes were not significantly different between groups when all subjects were included in analysis ($p = 0.931$). Differences between baseline and four-week

spot volumes were also not significant in the zinc-supplemented subjects when all subjects were included in the analysis ($p = 0.159$), while differences within the control group trended toward significance ($p = 0.084$). The effect of zinc was much smaller when all subjects were considered ($\eta p2 = 0.011$).

All values for IH2 are shown in Tables 3.20 – 3.22 and are illustrated in Figures 3.18 and 3.19. The spot corresponding to IH2 on 2D gels is shown in Figure 3.20.

Vitamin D Binding Protein precursor

Spot 17 was identified as Vitamin D Binding Protein precursor (VDBPp) (accession #gi_32483410) using mass spectrometry. VDBPp has a p*I* of 5.32 and molecular weight of 54.5 kDa. This protein migrated about 2 cm from the top of the resolving gel. Overall, spot volumes for VDBPp decreased.

All subjects: At baseline, spot volumes between treatment and control groups were significantly different ($p = 0.021$). The treatment group exhibited a 1.814-fold larger change in spot volume over the course of the study than the control group. The average volume change over four weeks was not significantly different between groups ($p = 0.310$). When controlling for baseline volumes, differences remained insignificant ($p = 0.698$), and the effect size was small ($\eta p2 = 0.017$). Spot volumes at four weeks also did not significantly differ between groups ($p = 0.466$). Baseline spot volumes were not significantly different from those at four weeks in the treatment group ($p = 0.150$) or the control group ($p = 0.727$). Zinc supplementation did not contribute to the differences seen between groups.

Trend subjects: Spot volumes decreased in five of the six subjects in the treatment group (n=5) and in only three of the six subjects in the control group (n=3); the treatment group exhibited less variability in directional change. Baseline spot volumes were not significantly

different between groups (p = 0.309). Baseline and four-week spot volumes were significantly different among zinc-supplemented subjects that showed decreases in VDBPp (p = 0.034). However, in the control group, these values did not significantly differ among trend subjects (p = 0.372). However, univariate analysis reveals very little effect of zinc supplementation on the difference between groups (ηp2 = 0.020).

All values for VDBPp are shown in Tables 3.23 – 3.25 and are illustrated in Figures 3.21 and 3.22. The spot corresponding to VDBPp on 2D gels is shown in Figure 3.23.

Human Retinol Binding Protein with Transthyretin

Spot 83 was identified as Chain D, human retinol binding protein with transthyretin (RBP-TTr) (accession #gi_7428712). RBP-TTr has a p*I* of 6.03 and a molecular weight of 65.6 kDa. This protein migrated about 6-7 cm below the top of the resolving gel. Overall, we observed decreases in RBP-TTr in both treatment groups.

All subjects: At baseline, spot volumes between treatment and control groups were not significantly different (p = 0.123). The treatment group exhibited a 1.350-fold larger change in spot volume over the course of the study than the control group. The average volume change over four weeks was not significantly different between groups (p = 0.448; 0.455). Two p-values are reported for all-subject data, because a t-test was performed for equal as well as for unequal variances; the f-statistic indicated borderline significant variance among samples (f = 5.058, p = 0.050). Spot volumes at four weeks did not significantly differ between the two groups (p-value = 0.068). Within the treatment group, baseline spot volumes were not significantly different from those at four weeks (p = 0.123), but changes trended toward significance within the control group (p = 0.083). Zinc supplementation only contributed to about 12.6% of the difference observed between groups. This effect size is considered small (ηp2 = 0.126).

Trend subjects: RBP-TTr decreased in five of the six subjects in the treatment group (n=5) as well as in five of the six subjects in the control group (n=5); the two groups exhibited equal variability in directional change. Baseline spot volumes were significantly different between groups ($p = 0.042$). Spot volumes at four weeks differed significantly between groups when only subjects showing decreases in RBP-TTr within each group were considered ($p = 0.046$). Baseline and four-week values significantly differed among subjects in the zinc group ($p = 0.042$) as well as among trend subjects in the control group ($p = 0.008$). When comparing changes between groups while controlling for baseline differences in spot volume, univariate analysis reveals that zinc contributed to about 19% of the difference observed. This effect size is considered small ($\eta p2 = 0.190$).

All values for RBP-TTr are shown in Tables 3.26 – 3.28 and are illustrated in Figures 3.24 and 3.25. The spot corresponding to RBP-TTr on 2D gels is shown in Figure 3.26.

TABLES

Table 3.1 **Subject baseline characteristics.** [a] Number of pills remaining in pill bottle at four weeks; [b] Self-reported compliance by subject/guardian; [c] A value of 1.5 was assigned for the reported value of Tanner Stage as 1-2; a value of 2.5 was assigned for the value 2-3. These values were assigned simply for statistical purposes; [d] For subjects with no available pill count data reporting supplement use, data from other subjects with similar reported intake were averaged to assign a value to those with missing data for statistical purposes only.

Zinc Subjects	Maturity Offset	Body Weight	Age	Body-mass-index	Tanner Stage	Pill Count[a]	Reported Compliance[b]
1	-1.63	43.9	9.2	21.2	2	3	Daily
2	-1.47	36.5	10.5	19.2	2	2	Almost daily
3	-1.21	35.7	10.8	17.7	2	1	Daily
4	-0.90	43.4	10.3	19.5	2	5	Almost daily
5	-0.72	46.9	11.3	20.0	2.5[c]	1.7[d]	Daily
6	-0.28	42.2	11.5	18.4	2.5[c]	2	Daily
Placebo Subjects							
7	-1.53	44.6	9.2	20.3	1.5[c]	4	Daily
8	-1.56	37.7	10.3	19.2	2	1.7[d]	Daily
9	-1.34	36.4	10.4	17.5	1.5[c]	4	Almost daily
10	-0.93	42.4	10.4	19.3	2	3	Almost daily
11	-0.72	46.7	10.9	22.4	2	1	Daily
12	-0.14	42.1	11.8	16.4	2.5[c]	3.5[d]	Almost daily
Average	-1.04	41.5	10.6	19.3	2.0	2.7	N/A
Standard Deviation	± 0.50	± 4.0	± 0.8	± 1.6	± 0.3	± 1.3	N/A
Standard Error	0.15	1.2	0.2	0.5	0.1	0.4	N/A

Table 3.2 Differences in baseline subject characteristics.

Characteristic	Zinc	Placebo	*p*-value
Mean Maturity Offset (MO) (years to peak height velocity)	-1.035	-1.306	0.996
Mean Body Weight (BW) (kg)	41.4	41.7	0.930
Mean Body-Mass-Index (kg/m^2)	19.3	19.2	0.883
Mean Age (years)	10.6	10.5	0.840
Mean Tanner Stage	2.1	1.9	0.209

Table 3.3 Baseline protein concentration and total protein content.

Subject	[BL protein], µg/µL	BL Protein Content, µg	[4-wk protein], µg/µL	4-wk Protein Content, µg
1	0.4370	421.0	0.6652	648.0
2	0.4098	383.0	0.3626	353.0
3	0.3273	278.1	0.2628	232.8
4	0.2931	266.2	0.4903	557.6
5	0.3957	338.0	0.4149	369.5
6	0.3466	301.6	0.4634	370.4
7	0.1912	166.2	0.2542	347.4
8	0.3847	354.2	0.6934	649.0
9	0.3778	348.7	0.3738	350.3
10	0.7839	705.5	0.6016	557.6
11	0.6654	591.1	0.5758	489.4
12	0.6678	591.5	0.4755	402.1
Average	0.4400	395.4	0.4695	443.9
Standard Deviation	± 0.1747	± 157.4	± 0.1447	± 133.2
Standard Error	0.0504	45.50	0.0148	38.50

Table 3.4 **Proteins identified by mass spectrometry.** [a] C.I.% = Protein score percent confidence interval.

Spot #	Protein Description	Accession #	MW	p*I*	C.I.% [a]
48	IgM kappa IIIB SON (IgMk3)	gi_224377	13870.0	8.65	98.035
61	Chain E, Serum amyloid P (E-SAP)	gi_30582339	25485.2	6.10	100
84	Proapolipoprotein (Pal)	gi_178775	28943.9	5.45	100
69	Alpha-1-antitrypsin (A1A)	gi_6137432	44355.8	5.43	100
99	Keratin 1, type II cytoskeletal (K1)	gi_7428712	65568.5	6.03	100
19	Hemopexin (Hpx)	gi_1321561	52384.6	6.55	99.997
24	Alpha 2-HS glycoprotein (A2HS)	gi_2521981	36268.1	5.20	99.941
32	Inter-alpha (globulin) inhibitor, H2 polypeptide (IH2)	gi_55958063	105606.2	6.56	99.922
17	Vitamin D binding protein precursor (VDBPp)	gi_32483410	54479.5	5.32	100
83	Chain D, human retinol binding protein w/transthyretin (RBP-TTr)	gi_4558178	12994.5	5.53	100

Table 3.5. Between-group spot volume differences for IgMk3. The effect size (ηp2) and the significance of the effect of zinc supplementation (*p*) are shown in the last column.

	Mean Baseline Spot Volume	*p*	Mean 4-Week Spot Volume	*p*	Mean Change in Spot Volume	*p*	ηp2 (*p*)
ZINC *All*	0.467 ± 0.193 *SE = 0.079*	0.348	0.207 ± 0.322 *SE = 0.131*	0.154	0.451 ± 0.206 *SE = 0.084*	0.876	0.147 (0.244)
PLACEBO *All*	0.674 ± 0.467 *SE = 0.191*		0.666 ± 0.653 *SE = 0.267*		0.416 ± 0.484 *SE = 0.198*		
ZINC *Trend*	0.533 ± 0.209 *SE = 0.104*	0.393	0.000 ± 0.000 *SE = 0.000*	0.100	0.533 ± 0.209 *SE = 0.104*	0.479	0.703 (0.037)
PLACEBO *Trend*	0.791 ± 0.511 *SE = 0.295*		0.367 ± 0.218 *SE = 0.126*		0.424 ± 0.596 *SE = 0.344*		

Table 3.6. Zinc group spot volume changes for IgMk3.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
ZINC *All*	0.467 ± 0.193 *SE = 0.079*	0.207 ± 0.322 *SE = 0.131*	0.219
ZINC *Trend*	0.533 ± 0.209 *SE = 0.104*	0.000 ± 0.000 *SE = 0.000*	0.014

Table 3.7. Placebo group spot volume changes for IgMk3.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
PLACEBO *All*	0.674 ± 0.467 *SE = 0.191*	0.666 ± 0.653 *SE = 0.267*	0.978
PLACEBO *Trend*	0.791 ± 0.511 *SE = 0.295*	0.367 ± 0.218 *SE = 0.126*	0.343

Table 3.8. **Between-group spot volume differences for Pal and E-SAP.** The effect size (ηp2) and the significance of the effect of zinc supplementation (*p*) is shown in the last column.

	Mean Baseline Spot Volume	*p*	Mean 4-Week Spot Volume	*p*	Mean Change in Spot Volume	*p*	ηp2 (*p*)
ZINC *All*	4.372 ± 1.394 *SE = 0.569*	0.374	3.059 ± 0.608 *SE = 0.248*	0.149	1.334 ± 1.077 *SE = 0.440*	0.904	0.138 (0.260)
PLACEBO *All*	5.068 ± 1.191 *SE = 0.486*		3.672 ± 0.743 *SE = 0.303*		1.397 ± 0.606 *SE = 0.248*		
ZINC *Trend*	0.219 ± 0.466 *SE = 0.208*	0.509	0.789 ± 0.637 *SE = 0.285*	0.097	0.569 ± 0.648 *SE = 0.290*	0.700	0.458 (0.032)
PLACEBO *Trend*	5.068 ± 1.191 *SE = 0.486*		3.672 ± 0.743 *SE = 0.303*		1.397 ± 0.606 *SE = 0.248*		

Table 3.9. **Zinc group spot volume changes for Pal and E-SAP.**

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
ZINC *All*	4.372 ± 1.394 *SE = 0.569*	3.059 ± 0.608 *SE = 0.248*	0.034
ZINC *Trend*	0.219 ± 0.466 *SE = 0.208*	0.789 ± 0.637 *SE = 0.285*	0.022

Table 3.10. **Placebo group spot volume changes for Pal and E-SAP.**

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
PLACEBO *All / Trend*	5.068 ± 1.191 *SE = 0.486*	3.672 ± 0.743 *SE = 0.303*	0.002

Table 3.11. Between-group spot volume differences for A1A and K1. The effect size (ηp2) and the significance of the effect of zinc supplementation (*p*) are shown in the last column.

	Mean Baseline Spot Volume	*p*	Mean 4-Week Spot Volume	*p*	Mean Change in Spot Volume	*p*	ηp2 (*p*)
ZINC *All*	6.332 ± 2.828 *SE = 1.155*	0.356	5.792 ± 1.915 *SE = 0.782*	0.762	2.576 ± 2.397 *SE = 0.978*	0.672	0.011 (0.757)
PLACEBO *All*	7.587 ± 1.451 *SE = 0.592*		5.494 ± 1.345 *SE = 0.549*		2.093 ± 1.271 *SE = 0.519*		
ZINC *Trend*	4.874 ± 1.878 *SE = 0.939*	0.032	6.401 ± 1.975 *SE = 0.987*	0.408	1.527 ± 1.091 *SE = 0.546*	0.488	0.516 (0.029)
PLACEBO *Trend*	7.587 ± 1.451 *SE = 0.592*		5.494 ± 1.345 *SE = 0.549*		2.093 ± 1.271 *SE = 0.519*		

Table 3.12. Zinc group spot volume changes for A1A and K1.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
ZINC *All*	6.332 ± 2.828 *SE = 1.155*	5.792 ± 1.915 *SE = 0.782*	0.732
ZINC *Trend*	4.874 ± 1.878 *SE = 0.939*	6.401 ± 1.975 *SE = 0.987*	0.068

Table 3.13. Placebo group spot volume changes for A1A and K1.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
PLACEBO *All / Trend*	7.587 ± 1.451 *SE = 0.592*	5.494 ± 1.345 *SE = 0.549*	0.010

Table 3.14. Between-group spot volume differences for Hpx. The effect size (ηp2) and the significance of the effect of zinc supplementation (*p*) are shown in the last column.

	Mean Baseline Spot Volume	*p*	Mean 4-Week Spot Volume	*P*	Mean Change in Spot Volume	*p*	ηp2 (*p*)
ZINC *All*	3.092 ± 1.300 *SE = 0.531*	0.274	1.791 ± 1.226 *SE = 0.500*	0.746	1.305 ± 1.052 *SE = 0.430*	0.119	0.233 (0.133)
PLACEBO *All*	2.205 ± 1.357 *SE = 0.554*		2.029 ± 1.246 *SE = 0.508*		0.503 ± 0.468 *SE = 0.191*	0.132	
ZINC *Trend*	3.361 ± 1.252 *SE = 0.560*	0.544	1.798 ± 1.370 *SE = 0.613*	0.495	1.563 ± 0.940 *SE = 0.420*	0.081	0.340 (0.129)
PLACEBO *Trend*	2.892 ± 0.848 *SE = 0.424*		2.383 ± 0.960 *SE = 0.480*		0.509 ± 0.452 *SE = 0.226*		

Table 3.15. Zinc group spot volume changes for Hpx.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
ZINC *All*	3.092 ± 1.300 *SE = 0.531*	1.791 ± 1.226 *SE = 0.500*	0.030
ZINC *Trend*	3.361 ± 1.252 *SE = 0.560*	1.798 ± 1.370 *SE = 0.613*	0.020

Table 3.16. Placebo group spot volumes changes for Hpx.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
PLACEBO *All*	2.205 ± 1.357 *SE = 0.554*	2.029 ± 1.246 *SE = 0.508*	0.564
PLACEBO *Trend*	2.892 ± 0.848 *SE = 0.424*	2.383 ± 0.960 *SE = 0.480*	0.110

Table 3.17. Between-group spot volume differences for A2HS. The effect size (ηp2) and the significance of the effect of zinc supplementation (*p*) are shown in the last column.

	Mean Baseline Spot Volume	*p*	Mean 4-Week Spot Volume	*p*	Mean Change in Spot Volume	*p*	ηp2 (*p*)
ZINC *All*	5.383 ± 2.065 *SE = 0.043*	0.341	4.610 ± 2.377 *SE = 0.971*	0.442	1.515 ± 0.925 *SE = 0.378*	0.530	0.001 (0.932)
PLACEBO *All*	4.402 ± 1.227 *SE = 0.501*		3.628 ± 1.836 *SE = 0.750*		1.243 ± 0.401 *SE = 0.164*		
ZINC *Trend*	6.072 ± 1.648 *SE = 0.824*	0.142	4.357 ± 2.438 *SE = 1.219*	0.426	1.716 ± 1.103 *SE = 0.552*	0.437	0.335 (0.133)
PLACEBO *Trend*	4.410 ± 1.372 *SE = 0.618*		3.200 ± 1.686 *SE = 0.754*		1.210 ± 0.439 *SE = 0.196*		

Table 3.18. Zinc group spot volume changes for A2HS.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
ZINC *All*	5.383 ± 2.065 *SE = 0.043*	4.610 ± 2.377 *SE = 0.971*	0.316
ZINC *Trend*	6.072 ± 1.648 *SE = 0.824*	4.357 ± 2.438 *SE = 1.219*	0.053

Table 3.19. Placebo group spot volume changes for A2HS.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
PLACEBO *All*	4.402 ± 1.227 *SE = 0.501*	3.628 ± 1.836 *SE = 0.750*	0.157
PLACEBO *Trend*	4.410 ± 1.372 *SE = 0.618*	3.200 ± 1.686 *SE = 0.754*	0.004

Table 3.20. Between-group spot volume differences for IH2. The effect size (ηp2) and the significance of the effect of zinc supplementation (*p*) are shown in the last column.

	Mean Baseline Spot Volume	*p*	Mean 4-Week Spot Volume	*p*	Mean Change in Spot Volume	*P*	ηp2 (*p*)
ZINC *All*	0.213 ± 0.417 *SE = 0.170*	0.931	0.657 ± 0.654 *SE = 0.267*	0.776	0.505 ± 0.601 *SE = 0.245*	0.810	0.011 (0.760)
PLACEBO *All*	0.191 ± 0.464 *SE = 0.189*		0.763 ± 0.595 *SE = 0.243*		0.593 ± 0.630 *SE = 0.257*		
ZINC *Trend*	0.219 ± 0.466 *SE = 0.208*	0.357	0.789 ± 0.637 *SE = 0.285*	0.262	0.569 ± 0.648 *SE = 0.290*	0.114	0.212 (0.298)
PLACEBO *Trend*	0.003 ± 0.005 *SE = 0.003*		1.167 ± 0.099 *SE = 0.057*		1.165 ± 0.095 *SE = 0.055*		

Table 3.21. Zinc group spot volume changes for IH2.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
ZINC *All*	0.213 ± 0.417 *SE = 0.170*	0.657 ± 0.654 *SE = 0.267*	0.159
ZINC *Trend*	0.219 ± 0.466 *SE = 0.208*	0.789 ± 0.637 *SE = 0.285*	0.121

Table 3.22. Placebo group spot volume changes for IH2.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
PLACEBO *All*	0.191 ± 0.464 *SE = 0.189*	0.763 ± 0.595 *SE = 0.243*	0.084
PLACEBO *Trend*	0.003 ± 0.005 *SE = 0.003*	1.167 ± 0.099 *SE = 0.057*	0.002

Table 3.23. Between-group volume differences for VDBPp. The effect size (ηp2) and the significance of the effect of zinc supplementation (*p*) are shown in the last column.

	Mean Baseline Spot Volume	*p*	Mean 4-Week Spot Volume	*p*	Mean Change in Spot Volume	*p*	ηp2 (*p*)
ZINC *All*	6.874 ± 1.282 *SE = 0.523*	0.021	4.965 ± 2.692 *SE = 1.099*	0.466	2.589 ± 1.679 *SE = 0.685*	0.310	0.017 (0.698)
PLACEBO *All*	4.381 ± 1.722 *SE = 0.703*		3.996 ± 1.600 *SE = 0.653*		1.427 ± 2.067 *SE = 0.844*		
ZINC *Trend*	6.674 ± 1.401 *SE = 0.627*	0.309	4.029 ± 1.580 *SE = 0.707*	0.693	2.645 ± 1.871 *SE = 0.837*	0.623	0.020 (0.762)
PLACEBO *Trend*	5.371 ± 1.949 *SE = 1.125*		3.560 ± 1.497 *SE = 0.865*		1.812 ± 2.752 *SE = 1.589*		

Table 3.24. Zinc group spot volume changes for VDBPp.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
ZINC *All*	6.874 ± 1.282 *SE = 0.523*	4.965 ± 2.692 *SE = 1.099*	0.150
ZINC *Trend*	6.674 ± 1.401 *SE = 0.627*	4.029 ± 1.580 *SE = 0.707*	0.034

Table 3.25. Placebo group spot volume changes for VDBPp.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
PLACEBO *All*	4.381 ± 1.722 *SE = 0.703*	3.996 ± 1.600 *SE = 0.653*	0.727
PLACEBO *Trend*	5.371 ± 1.949 *SE = 1.125*	3.560 ± 1.497 *SE = 0.865*	0.372

Table 3.26. Between-group spot volume differences for RBP-TTr. The effect size (ηp2) and the significance of the effect of zinc supplementation (*p*) are shown in the last column.

	Mean Baseline Spot Volume	*p*	Mean 4-Week Spot Volume	*p*	Mean Change in Spot Volume	*p*	ηp2 (*p*)
ZINC *All*	1.262 ± 0.630 *SE = 0.257*	0.123	0.841 ± 0.369 *SE = 0.101*	0.068	0.548 ± 0.402 *SE = 0.164*	0.448 0.455	0.126 (0.284)
PLACEBO *All*	0.737 ± 0.203 *SE = 0.083*		0.433 ± 0.320 *SE = 0.131*		0.406 ± 0.179 *SE = 0.073*		
ZINC *Trend*	1.361 ± 0.650 *SE = 0.291*	0.042	0.780 ± 0.377 *SE = 0.168*	0.046	0.581 ± 0.440 *SE = 0.197*	0.490	0.190 (0.241)
PLACEBO *Trend*	0.737 ± 0.227 *SE = 0.101*		0.311 ± 0.130 *SE = 0.058*		0.426 ± 0.192 *SE = 0.086*		

Table 3.27. Zinc group spot volume changes for RBP-TTr.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
ZINC *All*	1.262 ± 0.630 *SE = 0.257*	0.841 ± 0.369 *SE = 0.101*	0.123
ZINC *Trend*	1.361 ± 0.650 *SE = 0.291*	0.780 ± 0.377 *SE = 0.168*	0.042

Table 3.28. Placebo group spot volume changes for RBP-TTr.

	Mean Baseline Spot Volume	Mean 4-Week Spot Volume	*p*-value of difference
PLACEBO *All*	0.737 ± 0.203 *SE = 0.083*	0.433 ± 0.320 *SE = 0.131*	0.083
PLACEBO *Trend*	0.737 ± 0.227 *SE = 0.101*	0.311 ± 0.130 *SE = 0.058*	0.008

FIGURES

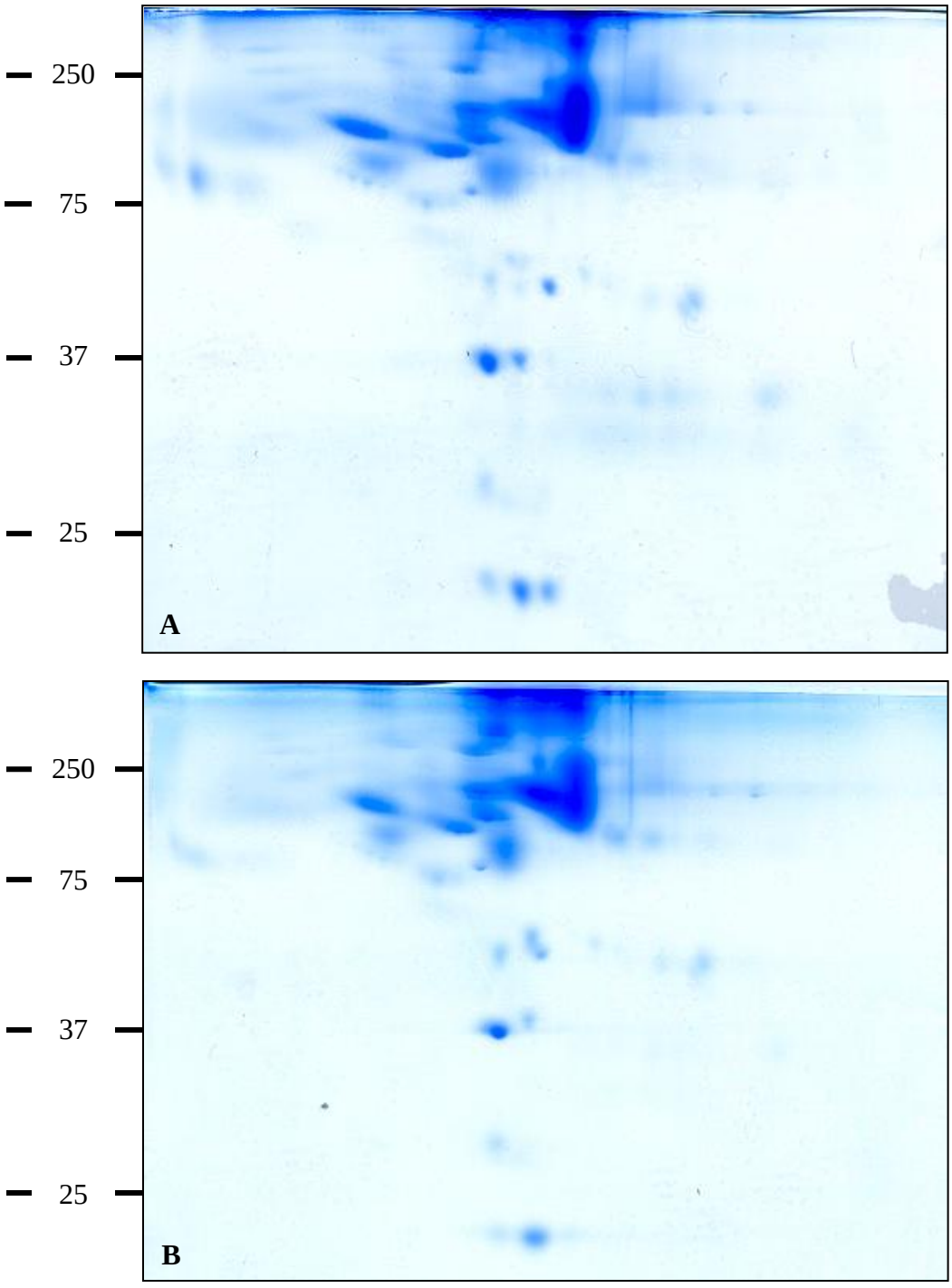

Figure 3.1. **Representative two-dimensional gels.** Image A represents a four-week gel from a subject in the treatment group. This gel was the reference gel chosen by the Phoretix© 2D software. Image B represents a four-week gel from a subject in the placebo group.

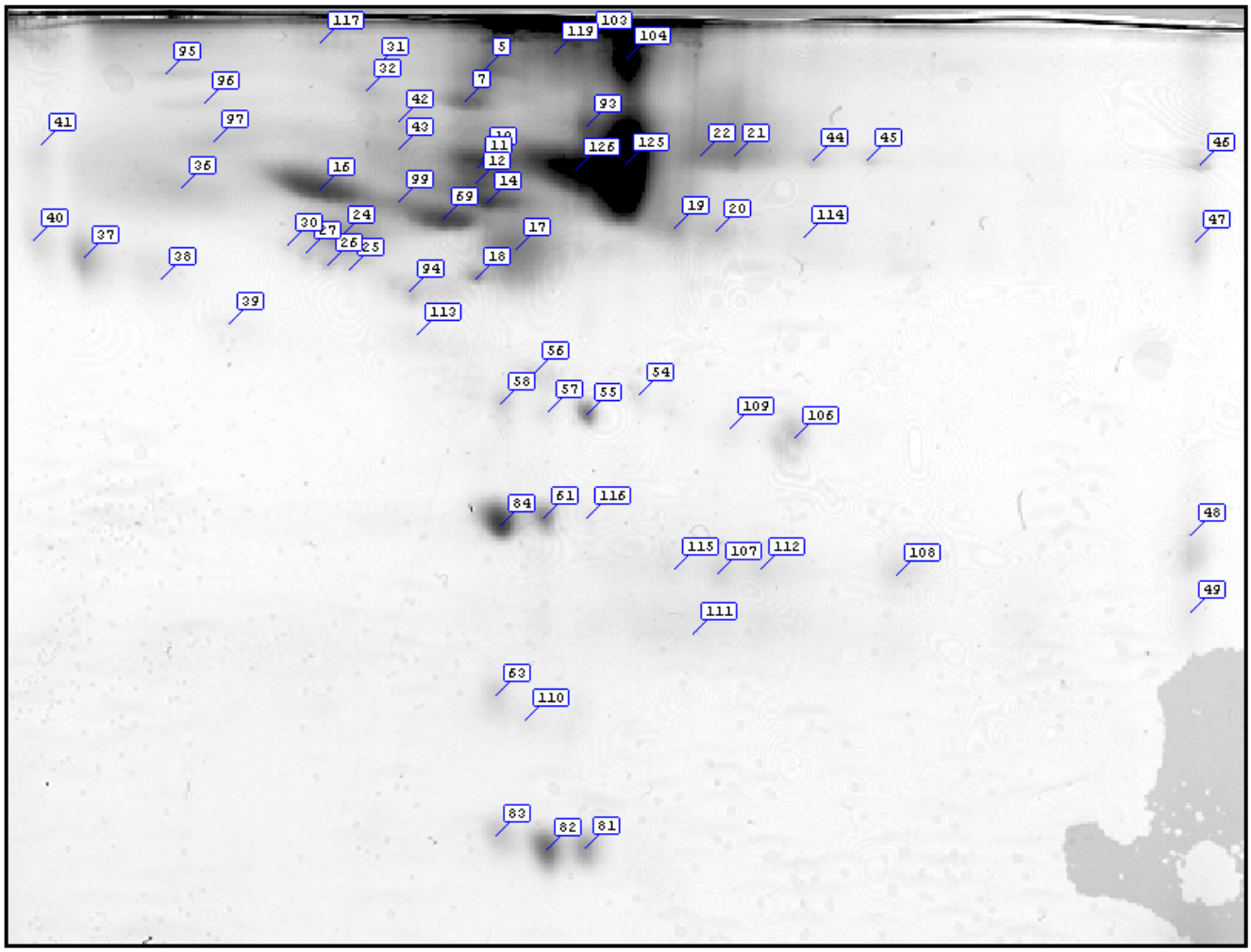

Figure 3.2. **Relative location of matched protein spots.** This image is the reference gel chosen by the Phoretix™ 2D software. Sixty-four protein spots were matched on 2D gels, and this image shows the relative location of each of these spots.

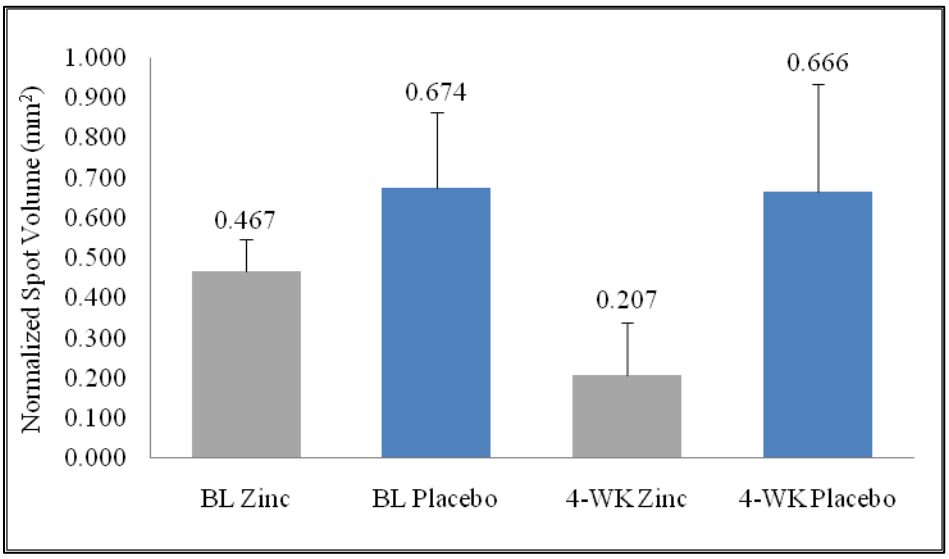

Figure 3.3. **Spot volumes for IgMk3 at baseline and four weeks.** Both groups exhibited similar patterns of directional change in IgMk3, with the zinc group (Δ 0.451 ± 0.206, SE=0.084) showing a larger change than the placebo group (Δ 0.416 ± 0.484, SE=0.198). The difference between average volume change was not statistically significant (p=0.876). **BL**=Baseline; **4-WK**=4-Week.

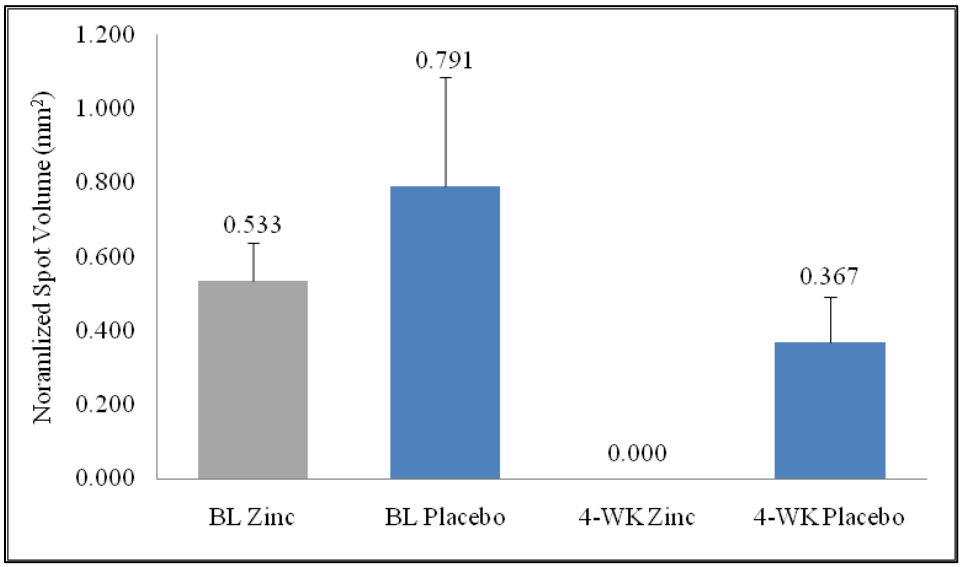

Figure 3.4. **Trend-subject spot volumes for IgMk3 at baseline and four weeks.** Both groups exhibited similar patterns of directional change in IgMk3, with the zinc trend subjects (n=4, Δ 0.533 ± 0.209, SE=0.104) showing a larger change than the placebo trend subjects (n=3, Δ 0.424 ± 0.596, SE=0.344). The difference between average volume change was not significant (p=0.479), yet a large, significant effect size was found (ηp2=0.703, p=0.037). **BL**=Baseline; **4-WK**=4-Week.

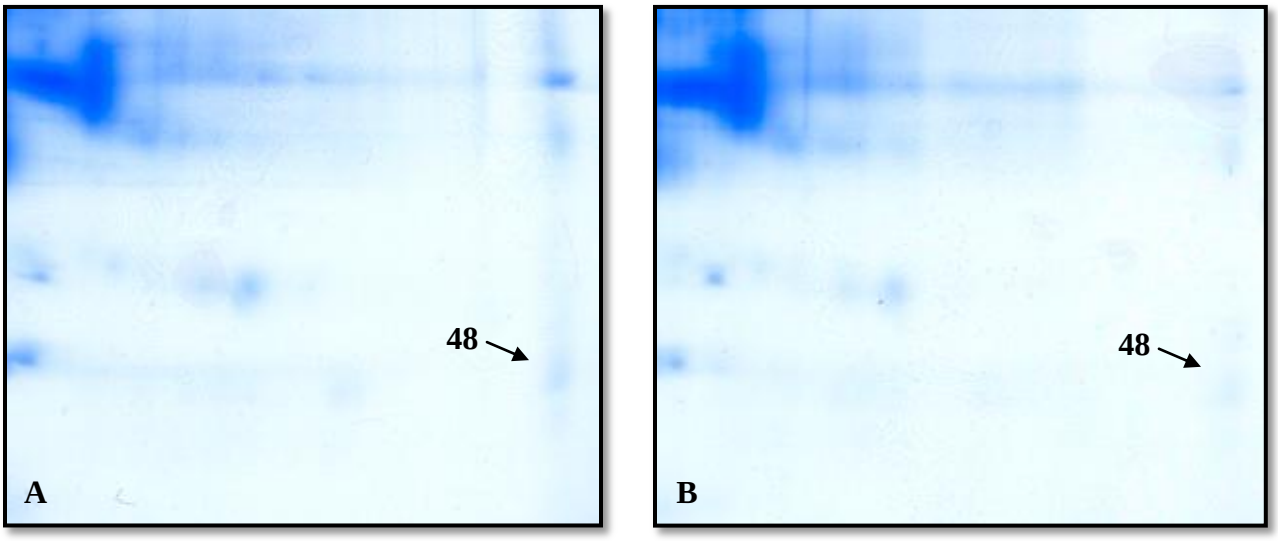

Figure 3.5. **Two-dimensional gels showing IgMk3.** Spot 48 corresponds to IgMk3. Image A represents the spot at baseline in one subject, and image B represents the spot at four weeks in the same subject. IgMk3 decreased from baseline to four weeks, as illustrated by the presence of the spot in image A and its absence in image B. The spot was consistently absent from 2D-gels of all zinc subjects showing decreases in spot volume.

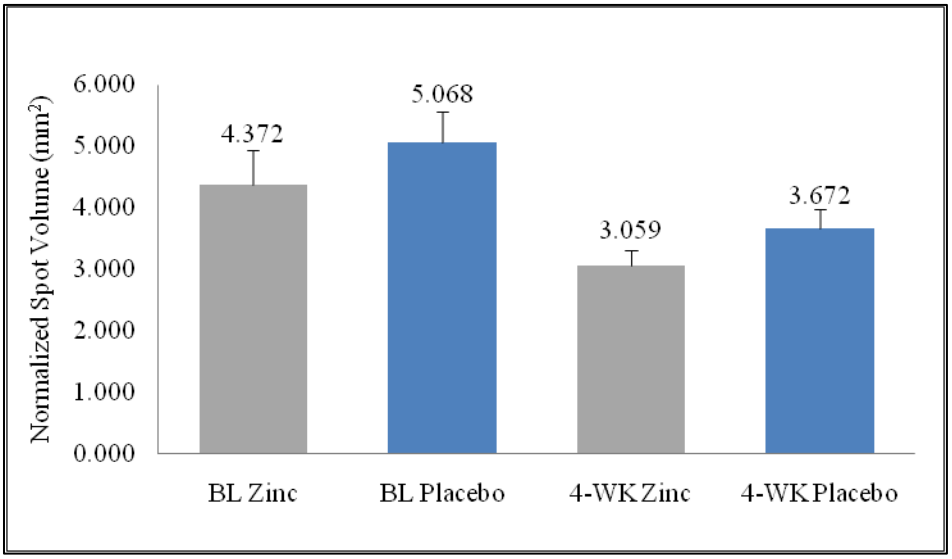

Figure 3.6. **Spot volumes for Pal and E-SAP at baseline and four weeks.** Both groups exhibited similar patterns of directional change in Pal / E-SAP, with the placebo group (Δ 1.397 ± 0.606, SE=0.248) showing a larger change than the zinc group (Δ 1.334 ± 1.077, SE=0.440). The difference between average volume change was not statistically significant (p=0.904). **BL**=Baseline; **4-WK**=4-Week.

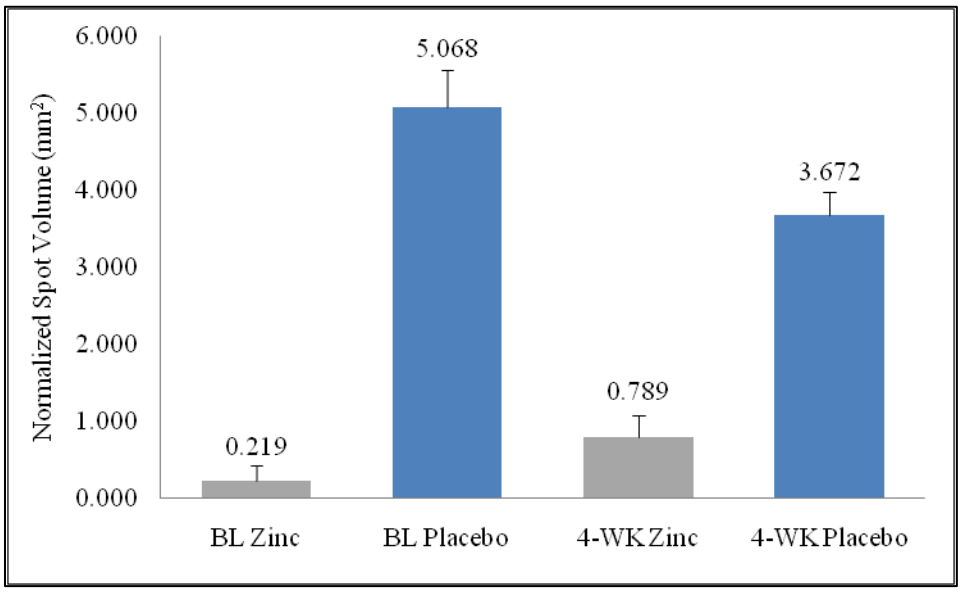

Figure 3.7. **Trend-subject spot volumes for Pal and E-SAP at baseline and four weeks.** Group trend subjects exhibited opposite patterns of directional change in Pal / E-SAP, with the placebo trend subjects (n=6, Δ 1.397 ± 0.606, SE=0.248) showing a larger change than the zinc trend subjects (n=5, Δ 0.569 ± 0.648, SE=0.290). The difference between average volume change was not significant (p=0.700), yet a moderate, significant effect size was found (ηp2=0.458, p=0.032). **BL**=Baseline; **4-WK**=4-Week.

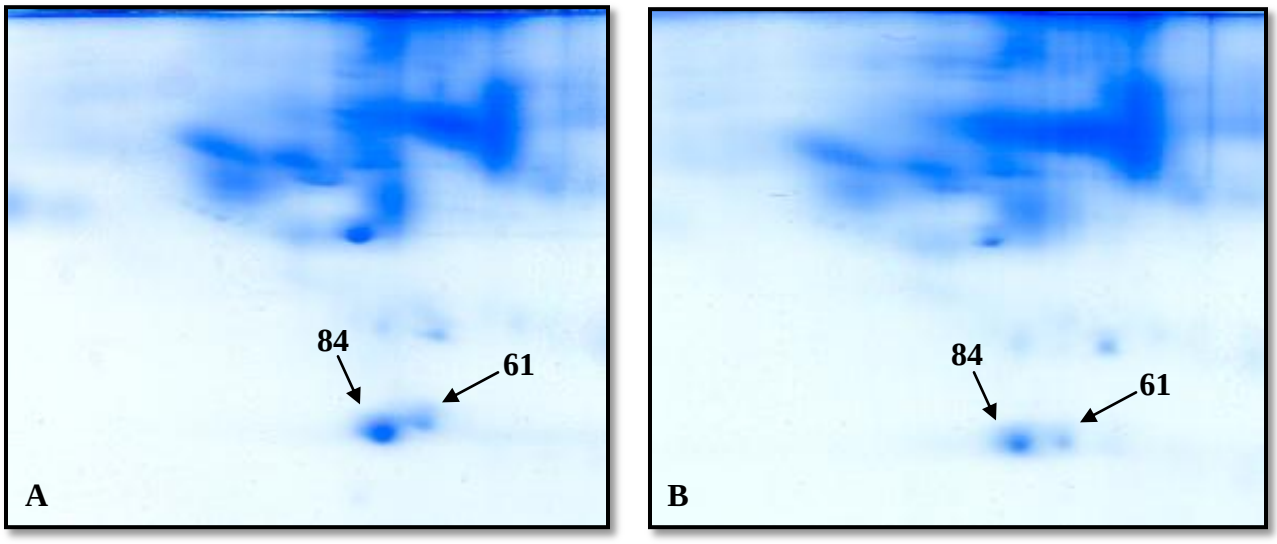

Figure 3.8. **Two-dimensional gels showing Pal and E-SAP.** Spot 61 corresponds to E-SAP, and spot 84 corresponds to Pal. Image A represents the spot at baseline in one subject, and image B represents the spot at four weeks in the same subject. The 2D-gels for this subject show the two spots separately, unlike some of the other gels. The spots appear larger and darker in image A.

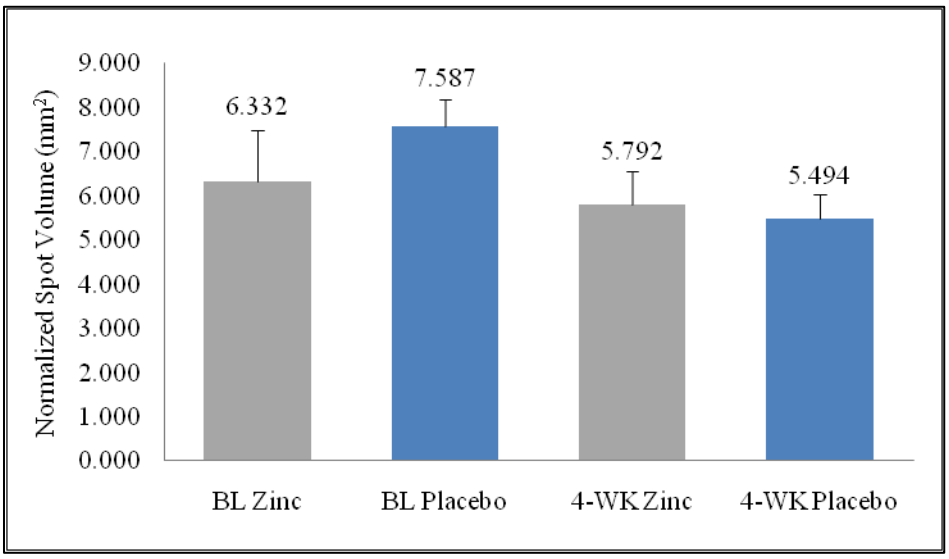

Figure 3.9. **Spot volumes for A1A and K1 at baseline and four weeks.** Both groups exhibited similar patterns of directional change in A1A/K1, with the zinc group (Δ 2.576 ± 2.397, SE=0.978) showing the larger change (Δ 2.093 ± 1.271, SE=0.519). The difference between average volume change was not statistically significant (*p*=0.672). **BL**=Baseline; **4-WK**=4-Week.

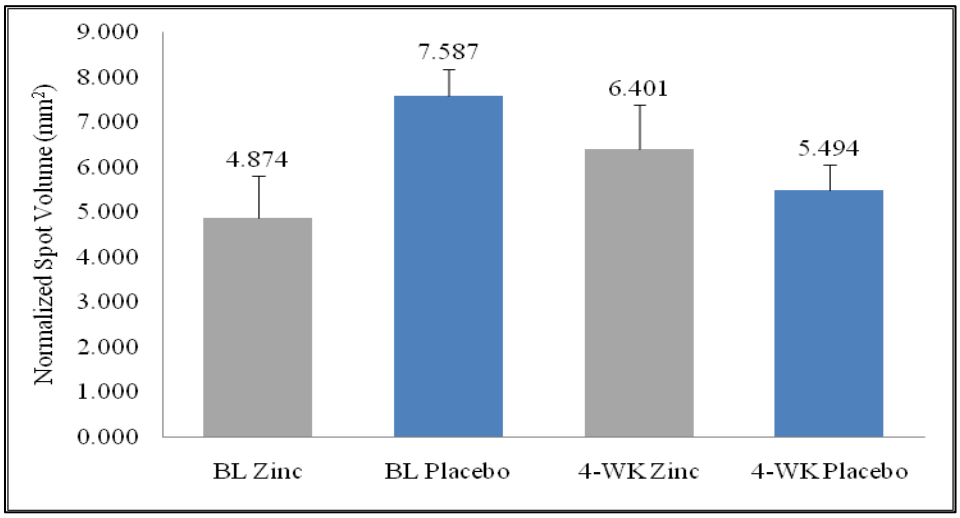

Figure 3.10. **Trend-subject spot volumes for A1A and K1 at baseline and four weeks.** Group trend subjects exhibited opposite patterns of directional change in A1A/K1, with the placebo trend subjects (n=6, Δ 2.093 ± 1.271, SE=0.519) showing a larger change than the zinc trend subjects (n=4, Δ 1.527 ± 1.091, SE=0.546). The difference between average volume change was not significant (*p*=0.488), yet a moderate, significant effect size was found (ηp2=0.516, *p*=0.029). **BL**=Baseline; **4-WK**=4-Week.

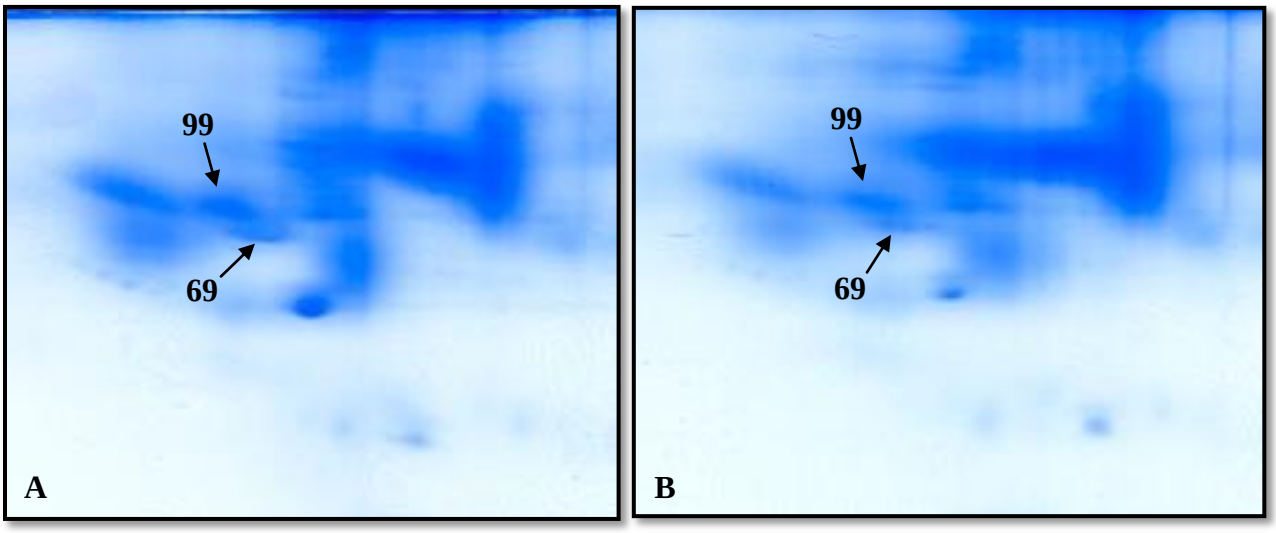

Figure 3.11. Two-dimensional gels showing A1A and K1. Spot 69 corresponds to A1A, and spot 99 corresponds to K1. Image A represents the spots at baseline in one subject (darker), and image B represents the spots at four weeks in the same subject. These images represent the decrease seen when all subjects were included. These 2D-gels were specifically chosen, because they show the two spots separately, whereas they appeared as a single spot on some gels.

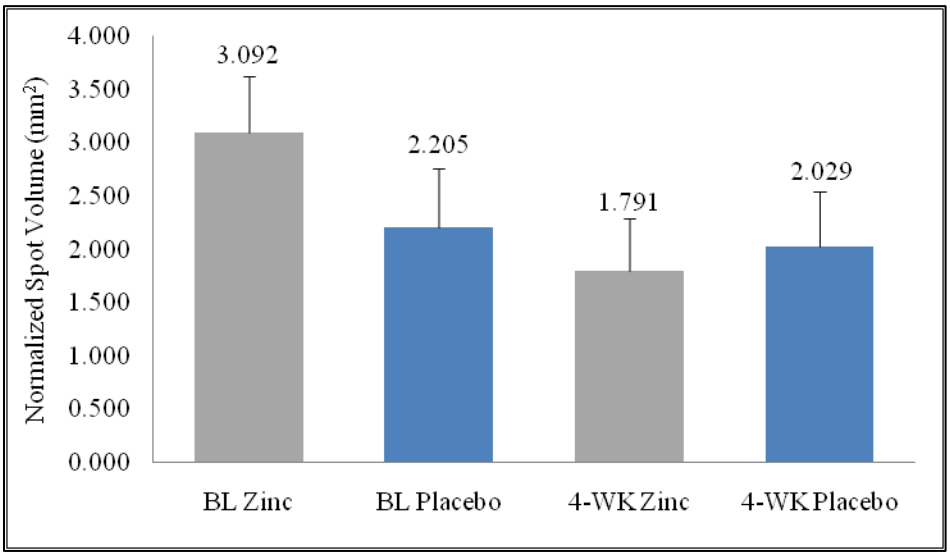

Figure 3.12. Spot volumes for Hpx at baseline and four weeks. Both groups exhibited similar patterns of directional change in Hpx, with the zinc group (Δ 1.305 ± 1.052, SE=0.430) showing a larger change than the placebo group (Δ 0.503 ± 0.468, SE=0.191). The difference between average volume change was not statistically significant (p=0.119, 0.132). Two p-values are reported as previously described. **BL**=Baseline; **4-WK**=4-Week.

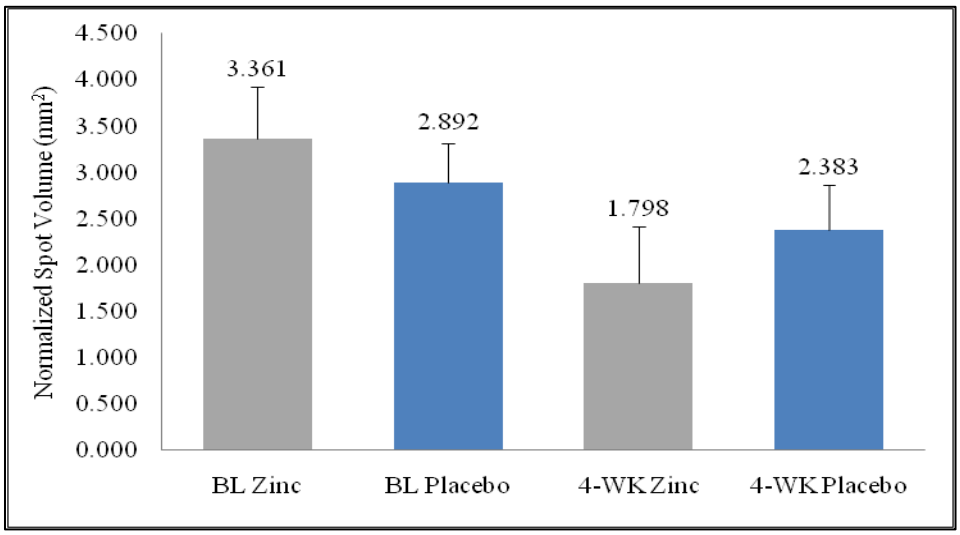

Figure 3.13. Trend-subject spot volumes for Hpx at baseline and four weeks. Both groups exhibited similar patterns of directional change in Hpx, with the zinc trend subjects (n=5, Δ 1.563 ± 0.940, SE=0.420) showing a larger change than the placebo trend subjects (n=4, Δ 0.509 ± 0.452, SE=0.226). The difference between average volume change trended toward significance (*p*=0.081). **BL**=Baseline; **4-WK**=4-Week.

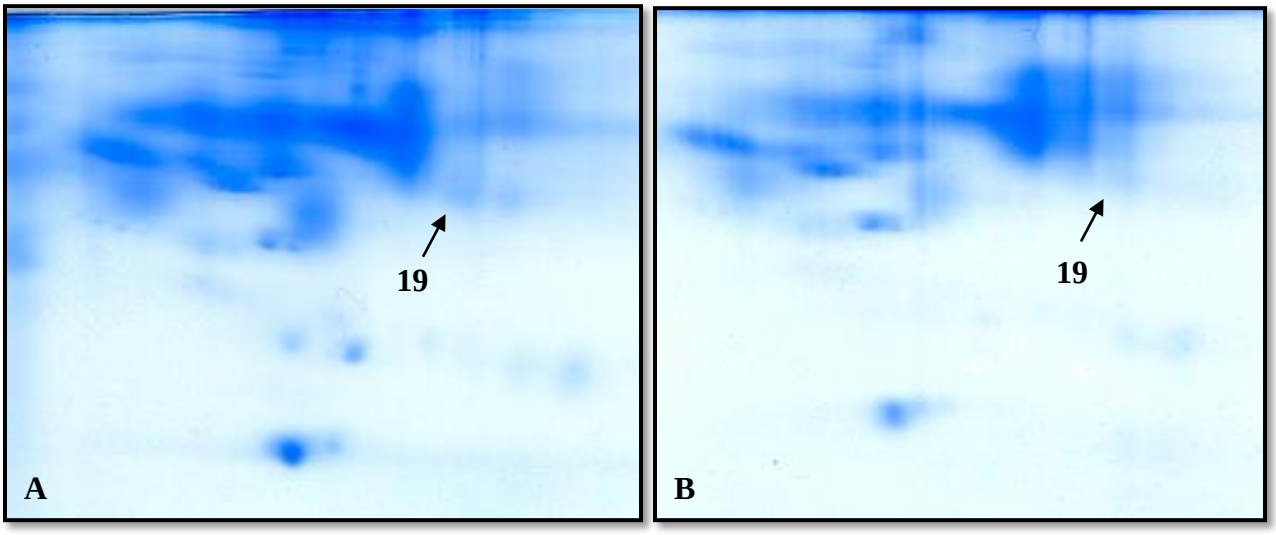

Figure 3.14. Two-dimensional gels showing Hpx. Spot 19 corresponds to Hpx. Image A represents the spot at baseline in one subject, and image B represents the spot at four weeks in the same subject. The spot is more defined in image A and appears slightly darker and larger than the corresponding spot in image B.

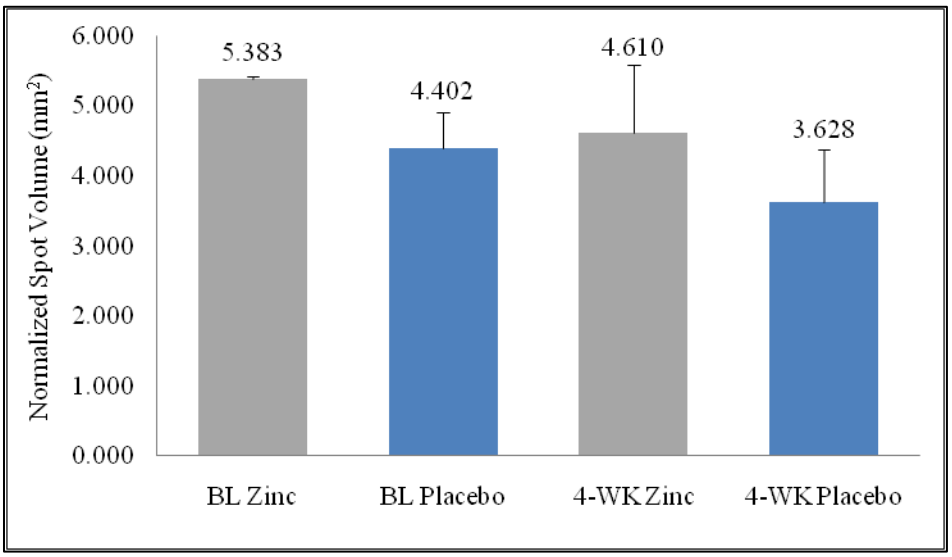

Figure 3.15. Spot volumes for A2HS at baseline and four weeks. Both groups exhibited similar patterns of directional change in A2HS, with the zinc group (Δ 1.515 ± 0.925, SE=0.378) showing a larger change than the placebo group (Δ 1.243 ± 0.401, SE=0.164). The difference between average volume change was not statistically significant (*p*=0.530). **BL**=Baseline; **4-WK**=4-Week.

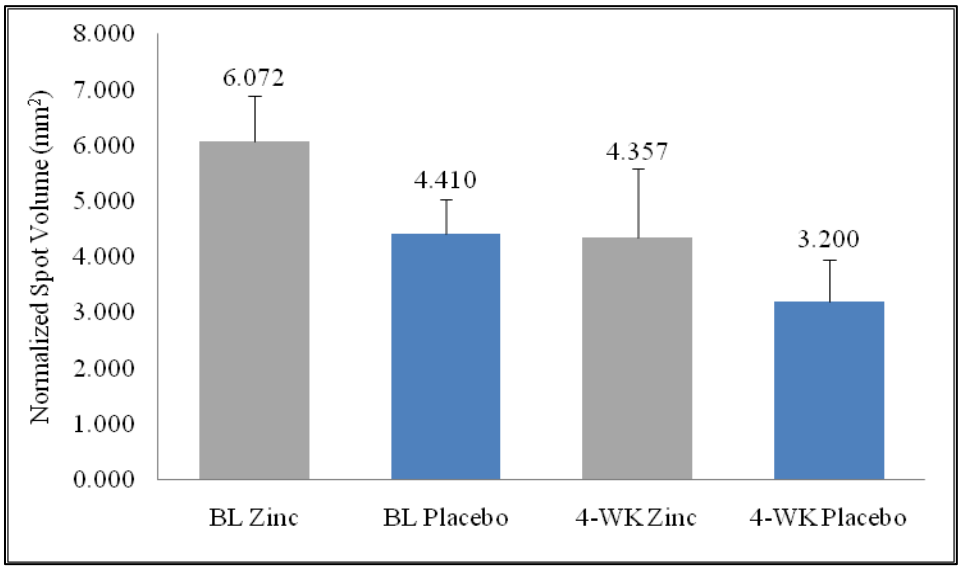

Figure 3.16. Trend-subject spot volumes for A2HS at baseline and four weeks. Both groups exhibited similar patterns of directional change in A2HS, with the zinc trend subjects (n=4, Δ 1.716 ± 1.103, SE=0.552) showing a larger change than the placebo trend subjects (n=5, Δ 1.210 ± 0.439, SE=0.196). The difference between average volume change was not significant (*p*=0.437). **BL**=Baseline; **4-WK**=4-Week.

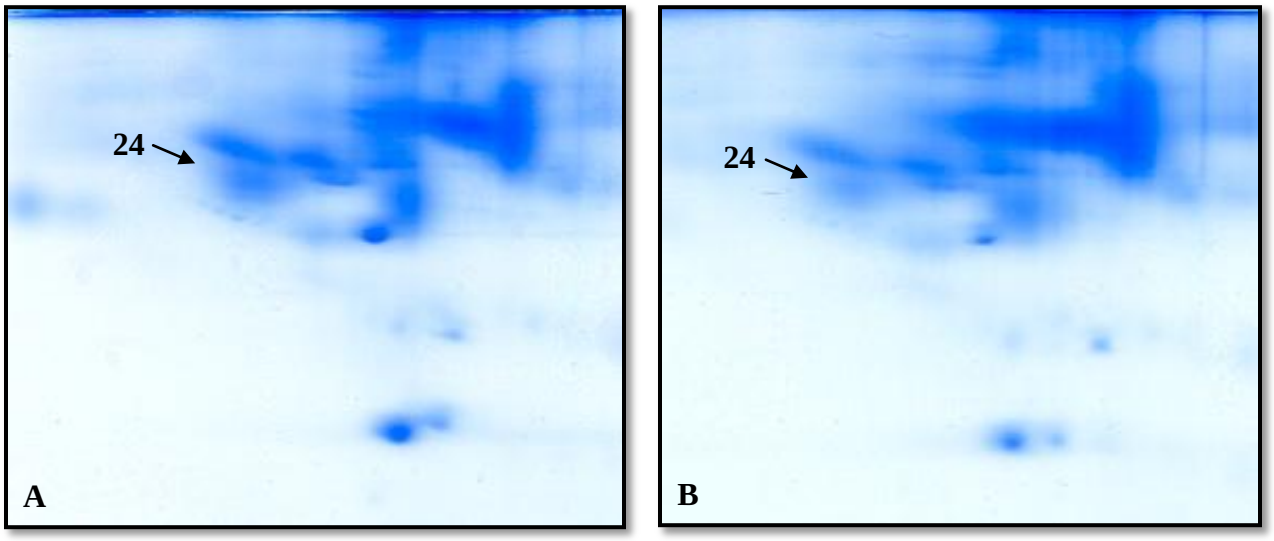

Figure 3.17. Two-dimensional gels showing A2HS. Spot 24 corresponds to A2HS. Image A represents the spot at baseline in one subject, and image B represents the spot at four weeks in the same subject. The spot appears larger and more dense in image A.

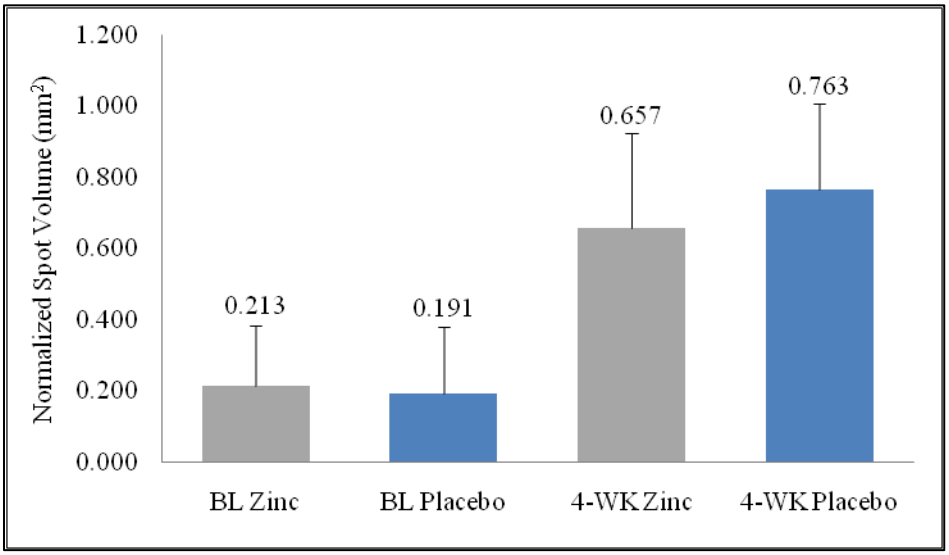

Figure 3.18. Spot volumes for IH2 at baseline and four weeks. Both groups exhibited similar patterns of directional change in RBP-TTr, with the placebo group (Δ 0.593 ± 0.630, SE=0.257) showing a larger change than the zinc group (Δ 0.505 ± 0.601, SE=0.245). The difference between average volume change was not statistically significant (p=0.810). **BL**=Baseline; **4-WK**=4-Week.

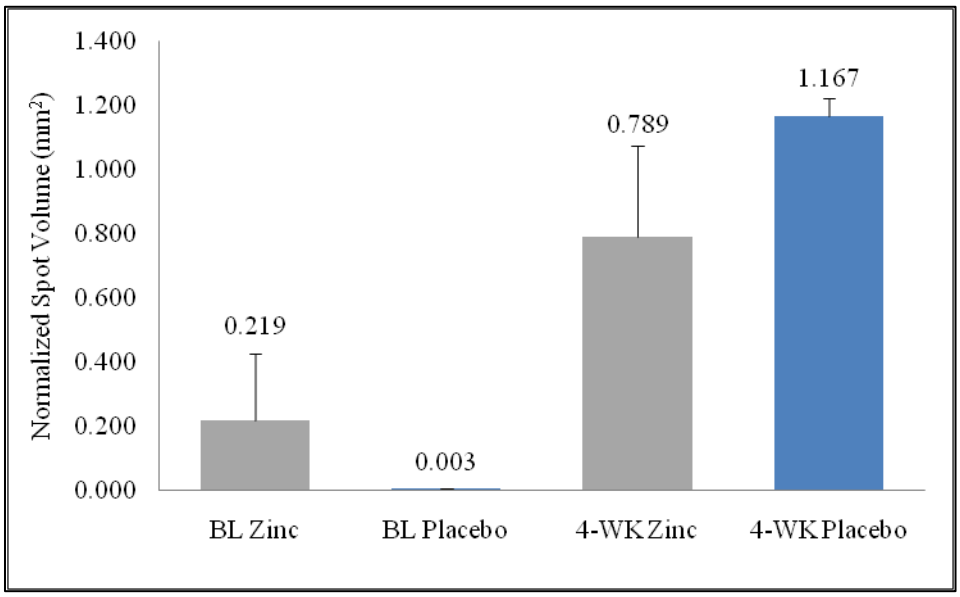

Figure 3.19. Trend-subject spot volumes for IH2 at baseline and four weeks. Both groups exhibited similar patterns of directional change in IH2, with the placebo trend subjects (n=3, Δ 1.165 ± 0.095, SE=0.055) showing a larger change than the zinc trend subjects (n=5, Δ 0.569 ± 0.648, SE=0.290). The difference between average volume change was not significant but was much closer than for all subjects (*p*=0.114). **BL**=Baseline; **4-WK**=4-Week.

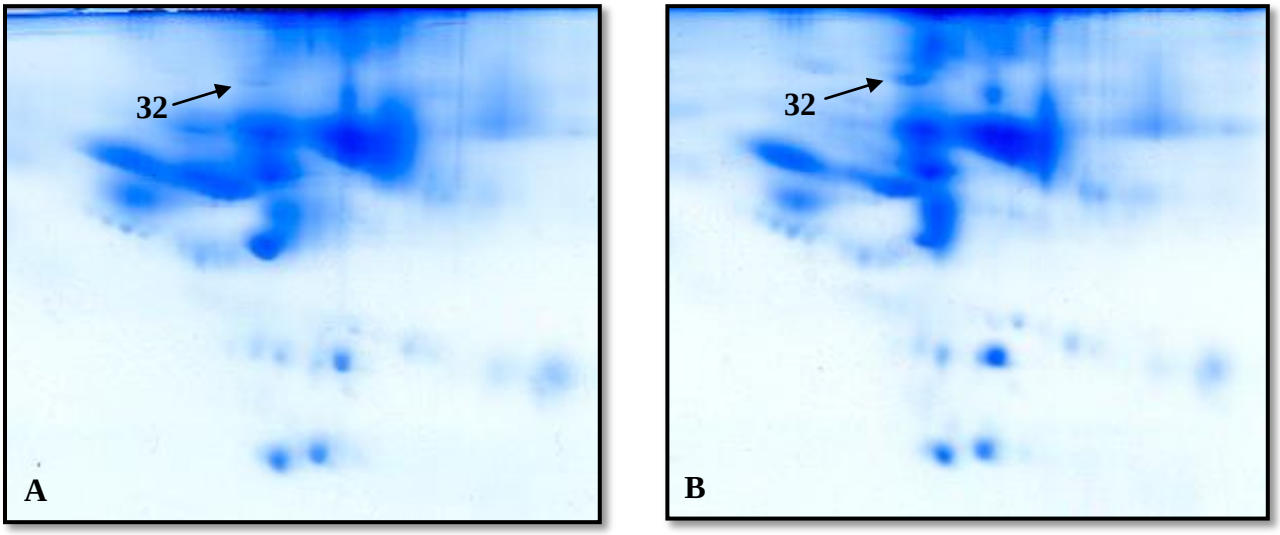

Figure 3.20. Two-dimensional gels showing IH2. Spot 32 corresponds to IH2. Image A represents the spot at baseline in one subject, and image B represents the spot at four weeks in the same subject. IH2 increased from baseline to four weeks, as illustrated by the darker spot shown in image B.

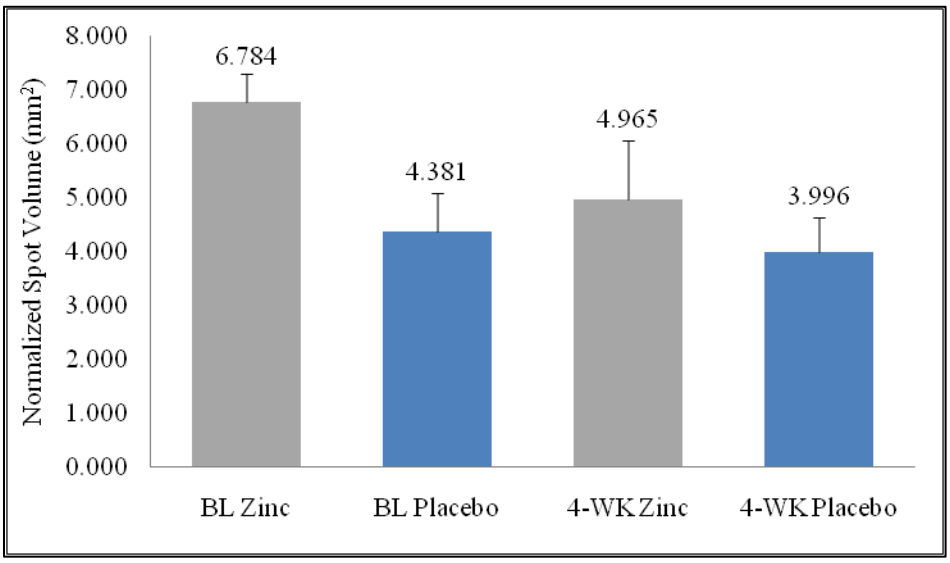

Figure 3.21. Spot volumes for VDBPp at baseline and four weeks. Both groups exhibited similar patterns of directional change in VDBPp, with the zinc group (Δ 2.589 ± 1.679, SE=0.685) showing a larger change than the placebo group (Δ 1.427 ± 2.067, SE=0.844). The difference between average volume change was not statistically significant (p=0.310). **BL**=Baseline; **4-WK**=4-Week.

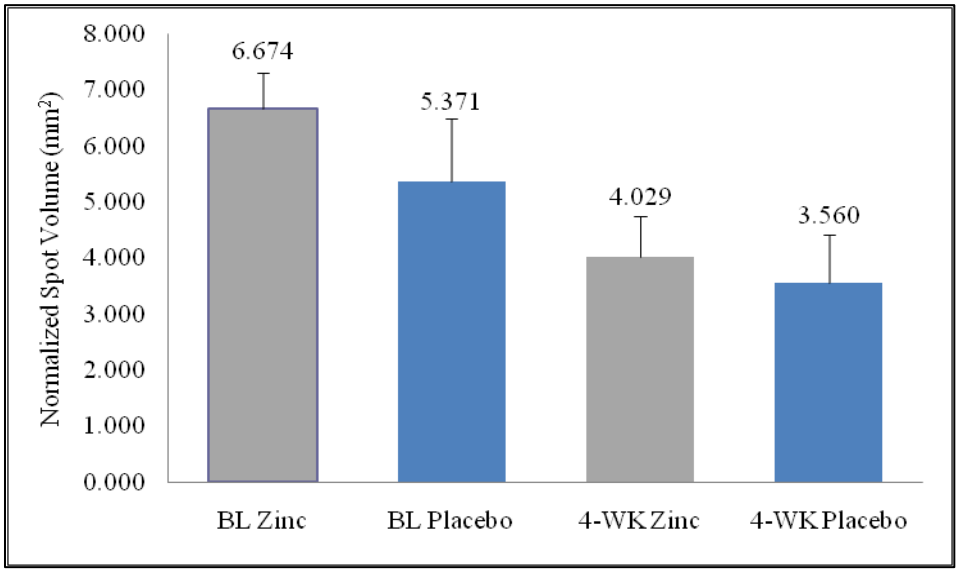

Figure 3.22. Trend-subject spot volumes for VDBPp at baseline and four weeks. Both groups exhibited similar patterns of directional change in VDBPp, with the zinc trend subjects (n=5, Δ 2.645 ± 1.871, SE=0.837) showing a larger change than the placebo trend subjects (n=5, Δ 1.812 ± 2.752, SE=1.589). The difference between average volume change was not statistically significant (p=0.623). **BL**=Baseline; **4-WK**=4-Week.

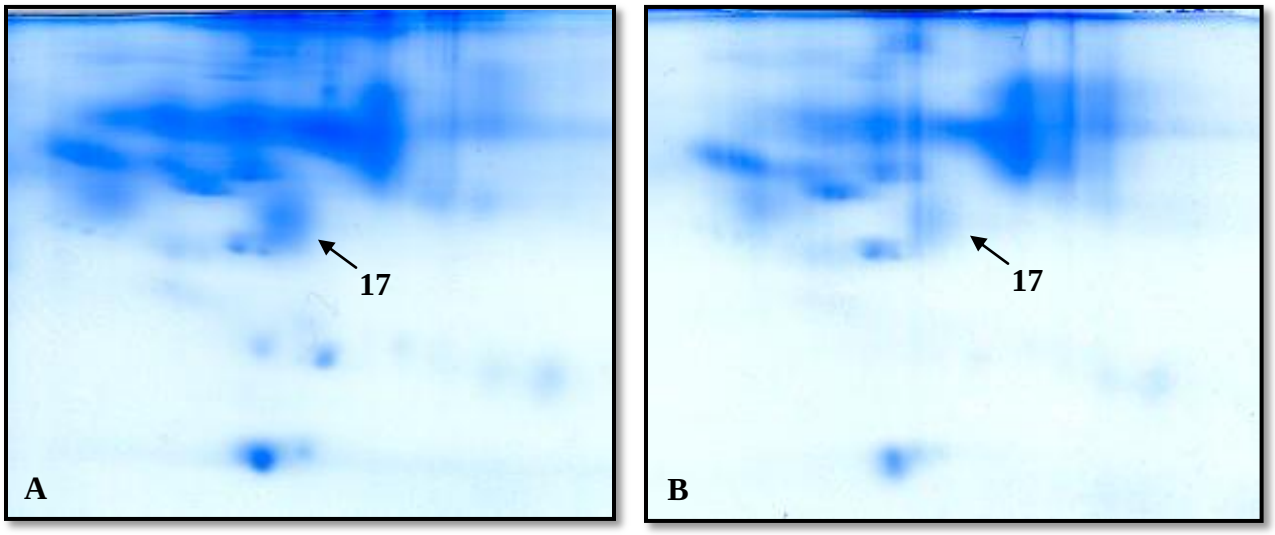

Figure 3.23. Two-dimensional gels showing VDBPp. Spot 17 corresponds to VDBPp. Image A represents the spot at baseline in one subject, and image B represents the spot at four weeks in the same subject. Although the spots appear to be the same size, the spot in image A is more intense (darker).

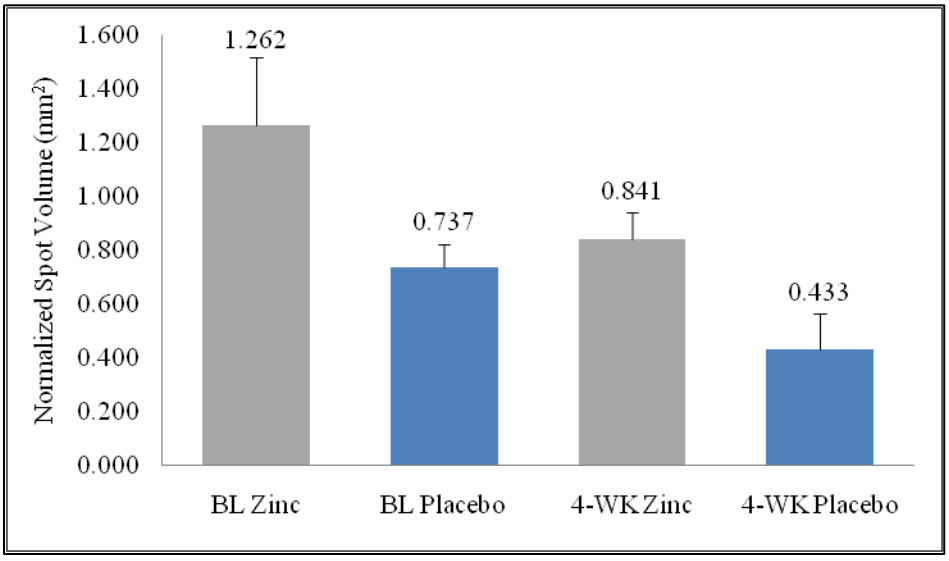

Figure 3.24. Spot volumes for RBP-TTr at baseline and four weeks. Both groups exhibited similar patterns of directional change in RBP-TTr, with the zinc group (Δ 0.548 ± 0.402, SE=0.164) showing a larger change than the placebo group (Δ 0.406 ± 0.179, SE=0.073). The difference between average volume change was not statistically significant (p=0.448, 0.455). Two p-values are reported as described earlier. **BL**=Baseline; **4-WK**=4-Week.

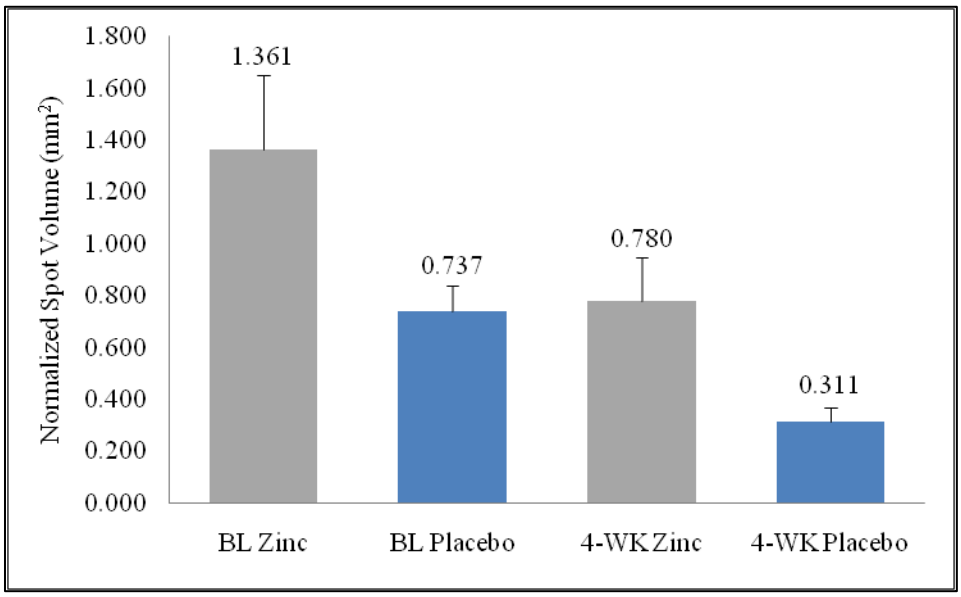

Figure 3.25. Trend-subject spot volumes for RBP-TTr at baseline and four weeks. Both groups exhibited similar patterns of directional change in RBP-TTr, with the zinc trend subjects (n=5, Δ 0.581 ± 0.440, SE=0.197) showing a larger change than the placebo trend subjects (n=5, Δ 0.426 ± 0.192, SE=0.086). The difference between average volume change was not significant (*p*=0.490). **BL**=Baseline; **4-WK**=4-Week.

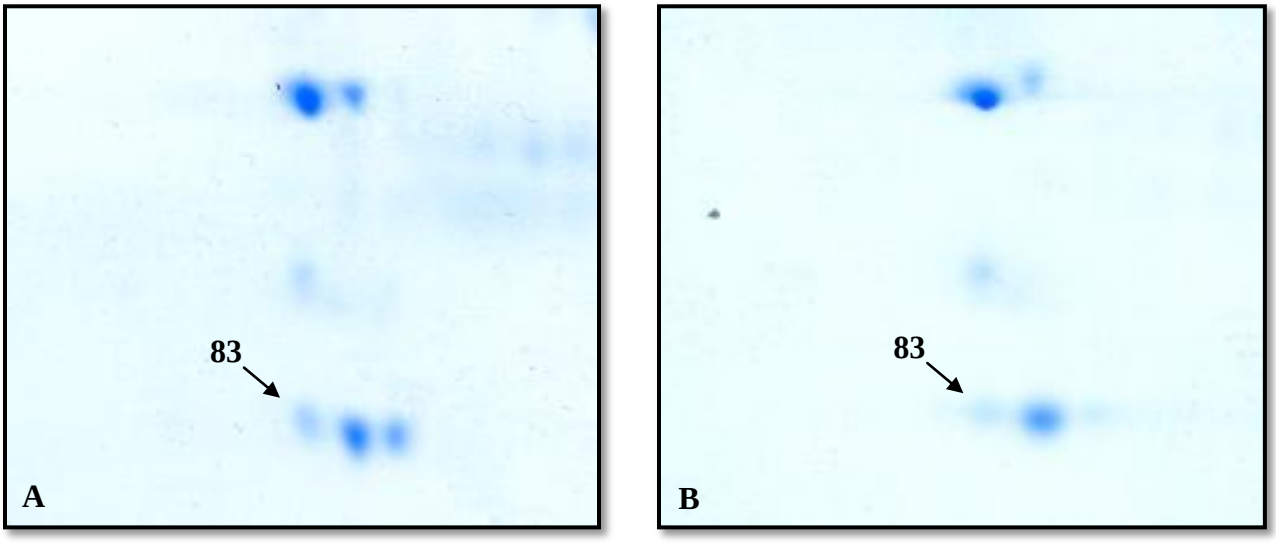

Figure 3.26. Two-dimensional gels showing RBP-TTr. Spot 83 corresponds to RBP-TTr. Image A represents the spot at baseline in one zinc-supplemented subject, and image B represents the spot at four weeks in the same subject. The spot in image A is slightly larger and more dense.

CHAPTER 4

DISCUSSION

SUBJECT MATCHING

Subjects were matched according to baseline maturity offset (MO), baseline body weight (BW), and Tanner stage of maturation. To a lesser extent, efforts were made to match subjects with similar baseline ages and body-mass-index (BMI). None of these variables were significantly different at baseline, indicating that matching was successful and that the ranges defining similarity among subjects were appropriate. Subjects were matched for two main reasons: matching eliminated some degree of variability within the limited sample size; and, matching controlled for some variables that could very well influence protein behavior. Although several variables were accounted for, there are sure to be other confounders, such as amount of physical activity, composition of diet, or degree of health status. Eliminating these variables was essential to analyze protein response to this intervention, because it is unknown to what degree these variables affect protein behavior.

PROTEIN CONTENT

Total protein content and total protein concentration at baseline did not differ between groups, but baseline levels of some of the proteins identified did. Baseline levels of a given protein may very well determine the degree of change the individual will experience following an intervention that increases or decreases levels of that protein. For example, if the ideal amount of a certain protein is 3.125 µg, an individual with a baseline level of 2.555 µg will probably experience a smaller change than an individual with a baseline level of 0.333 µg. Since this

relationship has previously been documented, we controlled for baseline values to see if any differences between groups would reach significance.(71) In the present study, baseline spot volume did not appear to affect the level of change in the proteins identified to any significant degree. However, future studies that aim to accurately quantify protein behavior should take this potential confounder under consideration.

DIFFERENTIALLY EXPRESSED PROTEINS

Six of the ten proteins identified exhibited decreases (IgMk3, Pal, Hpx, A2HS, VDBPp, RBP-TTr), while two proteins increased (E-SAP, IH2). Two proteins increased in zinc subjects and decreased in control subjects (A1A, K1). Decreases in serum proteins can occur through various mechanisms, which are summarized in Table 4.1. One site of regulation of serum protein levels is post-translational modifications, as the addition of polysaccharides and protein packaging, for example, mediate many of these mechanisms.

Small to moderate effects of zinc were found for several the proteins showing differential trends between treatment arms, and a few of these effects were significant. For some of the other proteins, zinc either had a slight effect on serum levels of these proteins, or the study period was of insufficient length to elicit sizeable effects. The proteins we identified are described below in addition to discussions of potential causes for the patterns observed.

IgM Kappa IIIB SON

IgM Kappa IIIB SON (IgMk3) decreased in four of six subjects in the zinc group and in only three of six subjects in the placebo group. In the zinc group, all four subjects with decreases had undetectable levels at four weeks, suggesting a decreased use of this protein in the serum in the presence of zinc. Overall, we found a large and significant effect of zinc supplementation on levels of IgMk3 in trend subjects after normalizing baseline spot volumes.

To understand the protein identified in our study, it helps to understand a little more about its various parts. Kappa III is a subgroup of light molecular weight protein chains that contribute to the overall structure of monoclonal immunoglobulins (IgM), which are B lymphocytes that function as autoantibodies to regulate responses to endogenous antigens. The "B" of kappa IIIB denotes a subclass within this group, constituting about ½ of the kappa III chains and about 15% of all kappa chains normally produced in an individual. Kappa IIIB chains are incorporated into certain IgMs. In the present study, the kappa IIIB chains were incorporated into the IgM, SON.(81)

Much evidence supports a role of IgM antibodies in the development of autoimmune disorders, such as rheumatoid arthritis.(82) In studies of IgM knockout mice (IgM ¯/¯), fewer IgM ¯/¯ mice acquired the autoimmune disorder induced compared to wild-type mice. The IgM ¯/¯ mice that did develop the disorder did so much later than wild-type mice, and their symptoms were much less intense.(83) These data could support the argument that the decrease in serum IgMk3 seen in the present study offers a degree of immuno-protection. However, there is at least one report that suggests a protective role of IgM autoantibodies.(84) Further conclusions cannot be made without further investigation.

The IgM autoantibody, SON, recognizes and binds apo-beta-lipoproteins, which are components of lipoprotein molecules involved with transporting dietary fatty acids to tissues but not HDL.(85-86) It is possible that SON regulates levels of apo-beta-lipoproteins to limit excess circulating levels of fat. It could also be protecting an individual from abnormal delivery of fats by binding apo-beta-lipoproteins with structural mutations. As discussed earlier, zinc may raise HDL levels while lowering total cholesterol and triglycerides.(87) Such changes in blood lipids should result in decreases of LDL, VLDL and IDL, lowering the need for apo-beta-lipoproteins.

With lower levels, there would be a lower need for SON, and thus kappa IIIB, to bind these lipoproteins. This sequence of events can explain the differential observations between the zinc and placebo groups. All in all, our data suggests a decreased need for SON and IgMk3 in the presence of zinc.

It appears that IgMs may very well have some protective roles, but zinc may modulate these effects and redirect IgM metabolism by positively affecting the immune system. Zinc deficiency is reported to impair the transition from the IgM- to the IgG-mediated immune response, which is part of the usual response progression. Adequate synthesis of IgG requires proper functioning of both B and T lymphocytes of the immune system and an appropriate interaction between the two cell types. B lymphocytes were not altered significantly in zinc-deficient mice. The study could not conclude whether zinc deficiency affected T cell development or the function of either or both cell types.(88) Since this study took place, several others have reported the negative effects of zinc deficiency on organs that produce immune cells, specifically the marrow, thymus and spleen, and its impact on the immune response.(89-96) The mechanism by which zinc regulates levels of lymphocytes is now believed to involve increased levels of early lymphocyte apoptosis.(97)

Additionally, our observations could be explained by the interrelated roles of zinc and B lymphocytes in apoptosis. B lymphocytes respond to inflammation by increasing in number.(98) The death of follicular cells preceding pubertal development is accompanied by such inflammation, thus stimulating proliferation of IgMs.(99) A gene known as Egr-1 encodes a zinc-finger transcription factor and is up-regulated in proliferating B lymphocytes. Upon investigation, lymphocytes prepared from Egr-1$^{-/-}$ mice were not able to proliferate compared to

normal cells.(98) This zinc-finger transcription factor appears to be involved with regulating the cell cycle.

There seem to be several mechanisms by which zinc may regulate serum levels of IgM kappa IIIB SON. Yet, IgMs seem to have many physiological roles, meaning they likely have many regulatory controls as well. The evidence available is promising that researchers are getting relatively close to revealing the intricacies of IgM metabolism. It is probable that future study will also elucidate the role of zinc in regulating levels of serum IgMs and their constituents. With such information, the feasibility of IgMk3 as a biomarker of zinc status can be established. Future research is indicated by the fact that we observed a large and significant effect of zinc supplementation on levels of IgMk3. It is interesting that the significant findings only occurred in zinc subjects that showed decreases in IgMk3.

Proapolipoprotein A-I and Chain E, Serum Amyloid P

Proapolipoprotein A-I (Pal) and Chain E of serum amyloid P (E-SAP) were analyzed together because of their relative positions on two-dimensional gels. The total spot volume including these two proteins decreased in five of six subjects in the zinc group and in all subjects in the placebo group. Not only did more control group subjects show decreases in spot volume, but the average decrease was larger as well. It seems that zinc supplementation could function to maintain protein levels. However, in all-subject analysis, zinc contributed very little to the differential responses observed between the two groups.

No visible pattern is detected in the changes of the two separate proteins. In other words, neither protein consistently increased or decreased in a particular treatment arm nor appeared to respond to zinc. Yet, the total area was differentially altered, suggesting that other factors are affecting these proteins. Over the course of the study, Pal exhibits decreasing patterns, while E-

SAP exhibits increasing patterns. There is a very good possibility that zinc does have an effect on a different protein that then affects the two discussed here. We did observe a moderate effect size that reached statistical significance.

Pal is a precursor to apolipoprotein A-I, which is the major protein incorporated into high-density lipoproteins (HDL). In fact, apolipoprotein A-I is required for synthesis of HDL particles.(100) As such, apolipoprotein A-I is reported to decrease with the level of HDL particles or with certain changes in its physical characteristics. Decreases in apolipoprotein A-I may not be apparent unless HDL levels or particles are also analyzed.(101) Recently, apolipoprotein A-I has been given credit for much of HDL's ability to remove cholesterol from tissues and deposits.(102) Considering this chain of events, synthesis of Pal could have decreased in the study population in order to limit cholesterol removal, resulting in the decreased serum levels observed. In the midst of growth, cholesterol is needed at tissues all over the body for incorporation into cell membranes and for synthesis of hormones.(103)

Pal is made in the liver and intestines and then secreted into the bloodstream where it is converted to the functional apolipoprotein A-I.(104) The enzyme responsible for this conversion is currently unknown, but it is thought to be a metalloenzyme and to carry out the forward and reverse conversion reactions.(104-105) Therefore, increases in one result in transient decreases in the other. The metalloenzyme that converts the proprotein into its active form cleaves Pal between two acidic residues, leading some to hypothesize that this enzyme could be a zinc-dependent protease, as these enzymes often cleave proteins between acidic amino acids.(105) If this theory holds true, it could serve to explain the observation of control subjects potentially showing larger decreases in the protein, as zinc supplementation would result in higher protease activity, and reduced levels of apoliprotein A-I and Pal, ultimately.

One study reported concomitant decreases in apolipoprotein A-I with increased levels of sex hormone-binding globulin (SHBG). SHBG levels were highest in subjects with the most height gain, suggesting either a role of SHBG in growth or simply in functions that occur during the same maturational period.(106) No increases in SHBG were found among female subjects in the study, but the age range was 12 to 15 years old. The researchers also report decreases in SHBG levels in the boys as they got older and gained weight. The girls did not experience weight gain and had only small increases in height over the study period. From this information, we deduce that SHBG levels may be increased in females at a younger age than in males, as the timing of pubertal events is typically earlier in females.(107) In this case, the subjects in our study may very well have increased levels of SHBG as well as decreased levels of apolipoprotein A-I and Pal, which could explain the decreasing patterns observed in both treatment groups.

It is questionable as to whether Pal is influenced by inflammation.(104) Chau et al. (2007) reports decreased conversion of Pal to apolipoprotein A-I during inflammation as a result of increases in the protease inhibitor, α-macroglobulin.(167) Following the decreased conversion, serum Pal levels would increase transiently before renal excretion was enhanced to lower levels back to normal. Synthesis of Pal in the liver and intestines could also decrease in response to the decreased demand for Pal in the serum. Inflammation is a very common biological process, which makes it difficult to measure or anticipate when designing a study and even more difficult to measure when assessing the status of a nutrient. The complexity of interactions results in having to measure several variables in addition to the biomarker, and these variables depend on the study population and the relationship being tested. If Pal is indeed influenced by inflammation, analytical methods and conclusions should be evaluated carefully.

In addition to inflammation, levels of Pal are also influenced by the gene which codes for its synthesis. The APOAI gene is highly polymorphic, introducing a world of variability to the mix.(108) As such, many variations in Pal structure and regulation exist within the population as a whole. Genetic factors are not always easy to characterize or control for in experimental settings, so they often end up confounding the relationship between the variables being tested. In addition to the variation introduced from genetic mutation, APOAI is also regulated by other elements, such as estrogen.(100)

Pal is already a candidate biomarker for some cancers, so studies are underway to characterize the influences that other molecules have on this protein.(104) Pal levels may or may not be influenced by zinc. We observed a moderate effect of zinc supplementation on protein patterns in trend subjects, but both groups exhibited significant changes in serum levels over the course of the study, suggesting a maturational effect. Yet, the evidence of a potential role for zinc in regulating the converting metalloenzyme counters the evidence of a potentially independent effect of maturational stage on serum levels of Pal.(105-106) Future proteomic studies are needed to uncover the structure and regulatory mechanisms associated with the converting enzyme.

Like several of the other proteins we have identified, E-SAP is an acute-phase protein. It has the ability to bind to foreign invaders, possibly tagging them for proper identification by the immune system. By tagging certain molecules, E-SAP signals for their accelerated excretion.(109) The larger decrease experienced by subjects taking zinc supplements could be explained by a heightened immune defense to keep out invaders, which could result from zinc's supportive roles in immunity. Overall, this would lower the need for E-SAP.

E-SAP also binds to cells undergoing apoptosis, where it is thought to facilitate the breakdown of the cell's DNA, accelerating the death process, and somehow signal macrophages to phagocytize the cell.(110) Prepubertal females experience apoptosis of follicular cells up until levels of leptin, and possibly other molecules, limit apoptosis so that follicles can mature.(99) Therefore, apoptosis in our study population may have naturally been decreasing in response to leptin over the course of the study, which would explain the decreases in E-SAP observed in all of our subjects. There are other mediators of leptin and the onset of puberty, such as BMI, but we did make efforts to eliminate the influence of major variables.(111-112) Plus, BMI and MO were not different between groups at baseline. Thus, we cannot explain why differences were observed between groups based on this information. Future studies examining similar populations should consider measuring leptin levels and controlling for variables that alter leptin levels and pubertal onset.

Certain functions of E-SAP are regulated by the presence of calcium, which is necessary for the binding of ligands, such as apoptotic cells, lipoproteins, and cells of the immune system, to name a few. E-SAP is reported to correlate positively with serum levels of very-low-density lipoproteins (VLDL) and negatively with serum levels of HDL.(101, 113) Such correlations lead many to believe E-SAP is involved with the atherogenic process. In fact, E-SAP associates with and builds up near the lesions that precede atherosclerotic plaques, especially when calcium levels in these areas increase. Localization of E-SAP at these lesions may potentially skew results of serum measurements.(113)

Zinc does not appear to directly interact with E-SAP, yet some effects of zinc on levels of blood lipids have been documented. In individuals with diabetes, a condition accompanied by alterations in zinc metabolism, large doses of zinc supplementation led to decreases in

triglyceride levels and total cholesterol levels and increases in HDL molecules.(87) Increases in HDL often accompany decreases in LDL and, thus, VLDL, which would explain the larger decreases in serum E-SAP observed in the zinc group, but the conclusions of a recent meta-analysis should also be considered. After analyzing differences among the study populations, it appears that zinc only benefits the lipid profile in the presence of existing aberrations in zinc metabolism. In healthy individuals, zinc had relatively little effect. In some cases, zinc negatively impacted HDL levels. Also, a large range of zinc doses have been used in the studies testing the effects of zinc on lipid profiles, starting as low as 15 mg per day and reaching to 300 mg per day.(114) There does not seem to be a dose-response relationship. Decreases in HDL were noted at both ends of the intake range, while no changes were found with doses of 50 mg per day.(114) The study population probably serves as an intervening variable in this relationship, though there may be other factors that are unknown at this time. Considering the healthy population of the present study, zinc may or may not have had an effect on E-SAP levels.

Whether or not measurements of serum E-SAP levels can be indicative of zinc nutriture cannot yet be determined. E-SAP is a candidate biomarker that will remain so until there is evidence to conclude its ultimate usefulness. At this time, there seems to be several factors complicating relationships between E-SAP and zinc status, but the exclusion these factors is not beyond our current research abilities and will become even easier to exclude in the future. However, our data suggests a very small effect of zinc supplementation on serum E-SAP levels in all subjects and a moderate effect of zinc in trend subjects.

Alpha-1-Antitrypsin and Keratin 1

Alpha-1-antitrypsin (A1A) and keratin 1 (K1) were also analyzed together. Spot volumes increased in four of six zinc-supplemented subjects but decreased in all subjects in the control

group. The opposing patterns suggest that zinc could be required for synthesis or maintenance of serum A1A levels. The two proteins are discussed separately below, but keep in mind that both proteins may have contributed to the changes in spot volumes observed to varying degrees. Unfortunately, we cannot quantify the individual effects of each protein in the present study. Based on our results, zinc supplementation had virtually no effect on the differences observed between groups.

A1A belongs to a family of serine protease inhibitors.(115) Enzyme inactivation is the main underlying function of A1A. A1A is mostly made in the liver but is also synthesized to some degree in other tissues, namely intestinal cells, macrophages, neutrophils and alveolar cells. Normally, A1A is secreted into the serum where it functions to inactivate inflammatory molecules. With reduced secretion, the serum loses much of its ability to regulate the production such molecules.(116) Reduced secretion occurs when A1A is synthesized improperly. When the protein builds up within hepatocytes, it can cause harm to the liver itself, resulting in major dysfunction.(117)

A1A is a positive acute phase reactant, and serum levels increase greatly with infection or inflammation. Since none of the subjects in the control group showed increases in A1A, it is possible that the subjects were not experiencing abnormal levels of inflammation. The increase observed in the four zinc-supplemented subjects is, therefore, mostly likely due to some other reason. A1A controls levels of cytokine production during inflammation.(116) It is regulated, perhaps down-regulated, by IL-6 binding at its promoter region, so conditions affecting IL-6 levels indirectly affect A1A synthesis.(117-118) Pregnancy and oral contraceptives are also though to affect circulating levels of A1A, but these variables did not likely confound the results of the present study given the maturation status of the study population.(117)

Although rare, there are individuals that suffer from A1A deficiency. These individuals often contract emphysema at a young age, which permanently damages lung tissue.(119-120) Often, they suffer from severe respiratory distress later in life similar to that experienced with chronic obstructive pulmonary disease (COPD). Multiple studies have documented that A1A protects lung tissue by preventing hyperactivity of the enzyme, elastase.(116) When uncontrolled, elastase damages lung cells and causes apoptosis.(121) A1A inhibits elastases and may also inhibit the death of lung cells and reduce oxidative damage to lung tissue.(120)

To date, few relationships between A1A and zinc have been reported. Alam et al. (1994) reports decreased excretion of A1A following zinc supplementation. A1A excretion was positively associated with increased protein excretion, such as that experienced with chronic diarrhea, and negatively associated with nitrogen absorption. An increase in nitrogen absorption was also observed following zinc supplementation, which confounded the relationship between zinc and A1A.(122) Zinc supports cell regeneration, so it is possible that zinc may also be involved with maintaining the health of lung tissue.(123) Instead of controlling A1A synthesis or function, it is more likely that zinc would increase to aid cell regrowth when A1A levels are insufficient. Serum levels are not always predictive of intracellular A1A accumulation or function. A1A may accumulate in cells at functional levels despite normal or very small increases in serum A1A.(120) If a role for zinc is identified in A1A physiology, serum A1A levels may or may not be a sensitive biomarker.

Two genes are responsible for the production of A1A, allowing for a high degree of variability among individuals. In fact, multiple mutations have been identified in these genes, some resulting in clinical A1A deficiency.(117) In light of the quickly developing technology in genetic research, future studies may soon be able to control for the influence of genotype.

Considering this large variability and the number of factors regulating A1A functioning, this protein will not serve as a useful biomarker anytime soon. In addition, we did not find zinc to affect A1A levels with all-subject analysis. In trend-subject analysis, however, we observed a moderate to large, significant effect of zinc supplementation on the difference observed between groups when controlling for differences in baseline spot volume.

Keratins belong to a structural class of proteins involved with forming intermediate filaments of the cytoskeleton. These filaments form complex networks via disulfide bonding and offer structural stability to the cell with possible roles in cytoplasmic organization, shuttling of intracellular components, and cell signaling.(124) Keratin 1 (K1), specifically, is most abundant in cells comprising the outer layers of the epidermis and is commonly found in hair and nails. Cells containing keratin 1 protect organisms from invasion of foreign bodies and physical trauma. As keratinized cells are sloughed off of the body's surface as a result of normal activity, keratinocytes deep within the epidermal layer undergo mitosis to produce new keratinocytes. These cells are pushed toward the surface to replace the cells that were lost. Once at the surface, keratinocytes are cut off from their nutrient supply and die.(10, 124) These dead keratinocytes serve the protective role mentioned earlier. They also serve as the site of the first step in the conversion of 7-dehydrocholesterol to vitamin D. With ultraviolet light serving as a catalyst, 7-dehydrocholesterol is first converted to previtamin D_3 and then to vitamin D_3 in the presence of heat.(124)

K1 is usually found intracellularly and, in line with its function, is only reported to be associated with tissues exposed to the atmosphere.(10) It is not fully clear why keratin was detected in the serum of the subjects in this study. K1 could enter the serum upon lysis of keratinocytes, but we are not sure what would cause such lysis in all our subjects. Increases were

only observed in subjects receiving zinc supplements, which could suggest an increase in cell lysis. Ultimately, we are unable to explain these patterns at this time.

Zinc is not known to be involved directly with keratin synthesis or function, though the protein did show different trends between groups. Like A1A, K1 works in some of the same biological processes as zinc, such as vitamin D metabolism and cell growth.(124) Thus, zinc supplementation and changes in K1 could occur simultaneously.

Overall, K1 has potential for a biomarker of zinc nutriture. There is little evidence of factors or conditions that affect serum K1 levels other than certain skin diseases, but the possibility that moderator variables exist remains.(125) Additional studies testing the effects of zinc deficiency, repletion and supplementation on serum levels of K1 will hopefully reveal more information. Overall, when analyzing all subjects, we observed no effect of zinc supplementation on K1, but a few significant trends were present. When analyzing trend subjects, a moderate to large effect of zinc was found that did reach statistical significance.

Hemopexin

Hemopexin (Hpx) was not detected in one control subject at baseline or four weeks, but decreased in five of six subjects receiving zinc supplements and in four of six subjects receiving placebos. The only increase in the zinc group was very slight, at a mere 0.007% of the subject's baseline value. Baseline levels of Hpx did not differ between groups, nor did four-week values or levels of change, yet subjects receiving zinc did have larger decreases overall. All-subject analysis revealed a 2.590-fold larger decrease in the zinc groups, and trend-subject analysis revealed a 3.071-fold larger decrease. Although the difference in the degree of change did not reach significance, the *p*-value was < 0.12 for all subjects and equaled 0.08 for trend subjects. A

large sample variance within the zinc group is believed to contribute to the lack of significance. Overall, zinc supplementation had a small to moderate effect on Hpx levels.

Hpx is a glycoprotein which is mainly synthesized within hepatic parenchymal cells. Hpx binds heme in the plasma with a relatively high affinity and facilitates heme entry into the liver for iron recycling. Binding heme protects cells from the oxidizing tendencies of free, unbound heme. Once in the liver, Hpx is stripped of its heme moiety by the inducible enzyme, heme monoxygenase, and returned to the plasma.(126)

Increases in plasma heme concentration are often caused by hemolysis and tissue damage.(126) It is reasonable to assume that conditions with altered iron metabolism, such as diabetes, would also have altered Hpx levels.(127) For example, increased levels of Hpx have been found in diabetic patients accompanying an expected decrease in serum iron.(128) Some of these conditions have also been found to correlate with altered zinc metabolism.(87) Decreases in Hpx can occur as a result of decreased synthesis in the liver, iron malabsorption or conditions associated with hemolysis (e.g., hemolytic anemia, hemorrhagic conditions, and decreases in haptoglobin, such as that which occurs when inflammation accompanies hemolysis), and muscle catabolism when myoglobin is release into the bloodstream.(126)

A decrease in iron status could affect the association between increased hemolysis and decreases in serum Hpx. In the presence of low iron status, it is possible that less hemoglobin, and thus heme, would be released into the bloodstream when cells are lysed. When plasma heme concentrations exceed 6 mg/L, Hpx concentrations begin to fall, but may not decrease by the degree expected. A lower iron status may also increase the effect of zinc on various metalloproteins, as the two metals sometimes exert opposing effects.(129) Therefore, iron status may confound the interpretation of Hpx concentrations. As iron status and metabolism largely

directs the functioning of Hpx, excluding these factors from sample analysis does not allow one to accurately explain any alterations in serum Hpx. In the present study, iron, heme and Hpx concentrations were not measured directly, so it is unknown whether serum iron levels were high, medium or low. All that is known is that most subjects exhibited a decrease in Hpx over the course of the study, with zinc-supplemented subjects exhibiting a larger decrease than subjects taking the placebo. At best, this information suggests our subjects may have had alterations in iron recycling.

Hpx domains are found within matrix metalloproteinases, such as collagenases and elastases, which are regulated by several metal ions including zinc.(129) Any upregulation of metalloproteinases caused by zinc could, in turn, cause serum Hpx levels to decrease as more Hpx molecules are incorporated into developing enzymes. Although changes in Hpx were not significantly different between groups, changes from baseline to four-weeks within the zinc-supplemented group were significant while those in the placebo group were not. This indicates that zinc could have slightly modulated the biological use of Hpx.

During the acute phase response, Hpx levels increase slightly due to increases in interleukin-6 (IL-6), which is heavily involved in inflammatory and immune responses.(126, 128) The increase in Hpx, though moderate (about 2-fold), may compromise the suitability of Hpx as a nutrient biomarker until we devise sound methods of characterizing and quantifying levels of inflammation and immune system activation. At this time, we can measure certain inflammatory molecules, such as IL-6 and C-reactive protein, which may improve our ability to interpret serum levels of Hpx if they are also measured.

Regarding sample collection, steps were taken to avoid hemolysis of serum samples, and samples with obvious signs of hemolysis were not chosen for the present study. However, any

hemolysis occurring during collection would not have an effect on Hpx concentrations, as Hpx is not contained within erythrocytes. Serum concentrations of Hpx mainly decrease when heme-Hpx complexes are taken up into the liver, an effect that is normally transient.

Despite the large variability in changes in serum Hpx levels, zinc supplementation did have a small to moderate effect; the zinc group did show significant decreases in spot volume, while the placebo group did not. Additionally, these decreases were relatively larger than those observed in the control group, and the difference between groups trended toward significance. Based on these observations, Hpx may be useful as a biomarker of zinc status if future research can characterize the effects of variables that affect serum levels in addition to zinc.

Alpha 2-HS Glycoprotein

α2-Heremans Schmid glycoprotein (A2HS), or fetuin-A, decreased in four out of six subjects receiving zinc supplements and in five out of six subjects receiving placebos. Average changes in A2HS levels were not significant when considering all subjects. Yet, trend-subject analysis reveals that the average change was larger in zinc-supplemented subjects but only significant in the subjects taking a placebo. This observation is likely due to differences in the number of trend subjects in each group, because the level of change in the zinc group did not differ significantly from the change observed in the control group. Zinc appears to have had only a small effect on A2HS levels, possibly causing larger decreases in A2HS to occur. Furthermore, four-week values were also not significant, suggesting that zinc does not have a sizeable effect over a four-week period in this population. Statistical analysis revealed virtually no effect.

A2HS belongs to a cytastatin superfamily consisting of serine proteases. A2HS binds calcium in the crystalline form of apatite, impeding calcium's ability to form hydroxyapatite crystals with phosphorus. A2HS is expressed in several tissues where calcification does not

normally occur, such as the kidney, liver, brain, skin, and gastrointestinal tract, as well as within extracellular spaces.(127, 130) Its affinity of binding calcium is even greater than that of serum albumin, which is a major transporter of calcium in the plasma. These findings suggest calcium binding as a major role of A2HS.(130) A2HS also binds calcium-phosphate complexes.(131)

Early stages of bone growth do not involve mineralization, yet minerals are mobilized to set the stage for the process. In the growing body, calcium is mobilized from non-osseous tissues to be incorporated into bone.(10) Release of calcium from non-osseous tissues and subsequent uptake into bone can create a need for more A2HS in bone tissue, resulting in a decrease in serum levels.(131) As mineralization should not occur immediately during ossification, A2HS in bone serves to limit premature calcification of the bone matrix. Ossification occurs as a tightly controlled sequence of events, and A2HS acts as a calcium/phosphate reservoir for bone tissue, releasing ions when and where mineralization is needed. Osteoblasts decrease binding of A2HS to calcium and phosphate by neutralizing the protein.(10) This mechanism for decreased serum A2HS levels is a logical explanation for the results observed in the present study population experiencing rapid linear growth rates. Also, A2HS can be disassembled to allow for synthesis of other glycoproteins, like those involved with calcification in bone tissue.(132-133)

There is no evidence available to suggest or deny a role for zinc in the regulation of serum A2HS levels. Therefore, it is not possible at this time to explain differences seen between the supplement and placebo groups. Zinc may have some influence over A2HS levels due to its involvement with bone growth. A lack of serum A2HS has been reported to cause inadequate collagen mineralization.(132) Additional studies of longer duration are needed to further develop this construct. The significant differences observed in the present study support further investigation.

A2HS is a negative acute phase reactant, thus decreasing following injury or infection.(134) During inflammation, transcription of the gene encoding A2HS is down-regulated in the liver. The official name of this gene is the same as the protein name, but the abbreviation is AHSG. Transcriptional regulators of this gene such as interleukin-1β (IL-1β), bind to negative regulatory regions in its promoter region.(135-136) In addition, IL-1β and other pro-inflammatory molecules decrease serum levels by increasing the uptake of A2HS into circulating macrophages. This process sets the stage for the calcification seen in atherosclerosis, as less A2HS is available in the serum to bind calcium and prevent its deposition.

The A2HS gene is typically abbreviated as AHSG. This gene is highly polymorphic. Studies have shown associations between certain alleles and relative levels of serum A2HS.(137) These associations could confound certain experiments, especially if these alleles turn out to affect regulatory processes in addition to serum levels. Also, this issue highlights the limitation of any cross-sectional data based on serum A2HS levels, as only comparisons between changes in serum levels could possibly remove the influence of genetic polymorphism to allow for accurate analysis.

Levels of A2HS determined by protein detection methods can be misleading, because the protein's high degree of hydrophobicity makes it particularly susceptible to proteolysis.(134) If proteolysis is not limited or if only intact molecules are measured, levels of A2HS may be underestimated. Although measures were taken to reduce proteolysis, such as limiting freeze-thaw cycles, keeping samples on ice or frozen at -80°C, and processing samples quickly after collection, some proteolysis may still have occurred. We did not identify any A2HS metabolites by mass spectrometry, so we cannot confirm whether or not they were present.

A2HS is influenced by several factors, many of which have yet to be fully characterized as to how they exert their influence and to what degree they affect serum levels. There is no current evidence of an interaction of A2HS with zinc, so it is doubtful that zinc has a direct effect on serum A2HS levels. Yet, no evidence was found to conclude that the two are not associated, so the relationship between them requires further study. Indirect effects are often more complex and are plagued with interference by other biological molecules and processes. In effect, any existing relationship may not be very obvious. In addition, methods used to measure levels of A2HS lack accuracy, which greatly limits the use of A2HS as a biomarker.

In conclusion, much more research is needed to clarify the regulatory mechanisms associated with A2HS before its full potential as a biomarker of zinc status can be evaluated. Our observations indicate changes in A2HS are likely related to normal maturation or growth, as both zinc and placebo trend subjects showed significant decreases in serum levels.

Inter-alpha (globulin) Inhibitor, H2 polypeptide

Inter-alpha (globulin) inhibitor, specifically the H2 polypeptide (IH2), increased in five of six subjects in the zinc group and in only three of six subjects in the placebo group. Seven subjects had undetectable levels of IH2 at baseline, whereas only three had undetectable levels at four weeks, one in the treatment group and two in the control group. The H2 polypeptide (IH2) is one of the heavy chains associated with multiple serine protease inhibitors known as inter-alpha inhibitors (IAi). These proteins are relatively abundant in serum and are not typically absent. It may be that certain types of IAis that contain IH2 were down-regulated to very low levels in some of our subjects. Another explanation for the undetectable levels observed is that IAis are taken up into other tissues depending on need.(138) We do not have the data needed to

differentiate between these two possible causes of the patterns observed in this study population. Overall, zinc supplementation had a small effect on IH2 levels.

The synthesis, and thus regulation, of the IAi protein family is complicated by the involvement of four separate genes that encode various subunits. IH2 is a part of several different proteins, so synthesis of IH2 is affected by the metabolism and physiological need of those proteins.(139) IH2 is made in the liver and brain, and many have reported it to be down-regulated in response to inflammation.(140-143) In the inflammatory response, IAis are involved with inactivating proteins, particularly metalloproteinases, which are very active during this process.(138, 144) Specifically, levels of IAi proteins have shown increases in tissues in the presence of inflammation occurring in the cervix and ovaries, such as that which occurs in the prepubertal period.(145-147) We observed an increase in IH2, often from a baseline spot volume of zero. Our subjects may have indeed been experiencing inflammation in the ovaries or cervix prior to and at baseline, and IH2 uptake may have been increased during this time. Increased uptake would decrease serum IH2 levels. During the four-week supplementation trial, the inflammation could have decreased, which provides a possible explanation for the increases we observed.

A direct role for zinc in regulating levels of IH2 or IAi proteins is not documented. Yet, similar changes in levels of zinc-finger proteins and IAis have been found in skeletal muscle.(148) Again, this suggests that zinc and IAis are involved with similar biological functions. An increase in zinc intake may indirectly cause an increase in IAi synthesis or uptake, but no studies have been designed to test this association. Indeed, IAi metabolism could influence zinc uptake or mobilization from storage.

The potential for IH2 as a biomarker cannot yet be fully evaluated, because there are many unknowns that warrant further investigation. Furthermore, studying the inflammatory and intercellular pathways in which IAis and zinc metalloproteins are involved will help identify if any true associations exist between them. Our observations that the control group had significant increases in IH2 levels whereas the zinc group did not warrants further investigation of this candidate biomarker.

Vitamin D Binding Protein precursor

Vitamin D binding protein precursor (VDBPp) decreased in five of six subjects receiving zinc supplements and in three of six subjects receiving placebos. When including all subjects in the data analysis, baseline levels of VDBPp differed significantly between groups, with the treatment group having a higher average baseline spot volume. Subjects were similar in age, body weight and maturity. Therefore, there is a high degree of variability in VDBPp levels within this study population and, possibly, additional moderators. Although baseline values were different, neither changes over the study period nor levels at four weeks differed significantly when variations in baseline spot volume were controlled. It seems that baseline levels of this protein did not have a major change on its response to zinc supplementation. All in all, four weeks of zinc supplementation does not appear to affect VDBPp levels. Supplementation may have an effect if administered for a longer duration or at a different stage of maturity, since changes among zinc-supplemented subjects followed a more consistent pattern. No conclusions can be made without further investigation to test for additional moderator variables and determine why baseline differences existed in this study population.

Baseline values did not differ significantly among trend subjects, so comparing protein changes and final volumes using trend-subject data should carry less bias. The larger fold

difference of 1.814 found when analyzing all-subject data likely overestimates the true amount of change in spot volume related to zinc supplementation. Trend-subject analysis revealed only a 1.460-fold change, which may be a truer representation of zinc's effect on VDBPp in this study population. Yet, this effect of zinc is small, and the average change in VDBPp levels between groups was insignificant. These observations again suggest that zinc had relatively little impact on serum levels of VDBPp. Statistical analysis implies that VDBPp could have changed simply due to maturation, though the larger consistency seen in the zinc supplementation group sustains interest in VDBPp as a candidate biomarker.

VDBPp is a precursor to Vitamin D Binding Protein (DBP), which is also known as vitamin D-binding protein, Gc-globulin, vitamin D-binding alpha-globulin and group-specific protein.(149-150) Decreases in VDBPp, as found in the present study, could indicate either an increased synthesis of DBP without a subsequent increase in transcription of the precursor, or decreased transcription of the precursor due to a lower rate of conversion to DBP. It is not possible to determine which scenario occurred, since DBP was not identified for further analysis in this study. In addition, because each collected sample offers merely a snapshot of whole body metabolism and protein levels, the decrease in VDBPp could have been a transient state while synthesis rates were increasing.

DBP belongs to the albumin superfamily of proteins, because it is encoded by the same gene as albumin. Similar to albumin, DBP transports many different molecules, mainly vitamin D metabolites and G-actin, through the blood to various tissues.(151) Due to the small number of studies investigating the effects of vitamin D supplementation in children and adolescents, exact roles of vitamin D in bone metabolism are not yet clear, but it is likely active during periods of heightened bone formation and resorption.(152-154) With increases in vitamin D mobility, more

DBP may be needed to transport vitamin D and its metabolites to sites of activation and function. In addition, DBP forms a complex with macrophages that promotes bone resorption.(151) Since bone resorption and formation occur in concert in healthy individuals, an increase in resorption in this study population may possibly be setting the stage for the bone formation to follow. Zinc deficiency negatively impacts the immune system, which can result in the death of lymphocytes and, ultimately, insufficient bone formation.(89, 155-156) Lymphocytes stimulate the binding of DBP to macrophages.(151) If a person is deficient in zinc, less complexes would form, resulting in inadequate bone resorption and aberrant bone formation. This sequence of events may or may not be accompanied by decreases in VDBPp synthesis. Additional studies could reveal relationships among VDBPp synthesis, DBP-macrophage complexes, and bone metabolism.

There are several factors other than active bone growth that can alter levels of DBP, potentially providing viable explanations for the observed decreases in serum VDBPp levels. The DBP-macrophage complexes described earlier also activate macrophages at sites of injury or inflammation when signaled by B- and T-cells of the immune system.(151) The level of inflammation or severity of injury at which these complexes are activated is not known. Thus, even minor bouts of inflammation, such as those resulting from regular physical activity, could alter DBP metabolism. Neither inflammatory markers nor immune system variables were measured or considered in the selection of participants or sample analysis, so these theories cannot be substantiated at this time. Future studies may need to consider inflammation a moderator. Zinc repletion has been documented to stimulate T-cell function, which could relate to an increased formation of active DBP-macrophage complexes compared to that found in individuals exhibiting zinc insufficiency.(155, 157-158)

DBP also inhibits angiogenesis.(159). During periods of growth, DBP may increase to regulate cell and tissue growth and development. Angiogenesis requires the molecule, G-actin, to help form new capillaries. By sequestering G-actin in the plasma and facilitating actin excretion, DBP inhibits the angiogenic process.(127, 151, 159) As part of a system to regulate cell growth, DBP may prevent the cell cycle from accelerating too quickly or continuing past the point of growth needs. Zinc-finger proteins have been identified as regulators of the cell cycle, and zinc does support tissue and cell growth.(122, 160-161) Zinc supplementation may stimulate the cell cycle and result in an increased need of DBP to inhibit excessive growth, which could explain the larger decrease in serum VDBPp observed in the treatment group.

Another possibility relates to DBP's involvement with G-actin transport in the serum. The protein, actin, forms part of the cytoskeleton and is released into the blood when cells are lysed. DBP binds and sequesters actin in the blood, regulating its availability to tissues and for altering blood clotting proteins.(151, 162-163) During periods of growth, cells may die as replacement cells are being made, releasing actin into the blood. Also, synthesis of actin may be stimulated to support cell and tissue growth. Either way, an increased amount of actin may require increases in levels of circulating DBP to regulate its uptake into tissues and prevent the toxic buildup of actin.(162, 164) Again, this could result in the increased conversion of VDBPp to DBP, ultimately decreasing the level of the precursor in the blood, assuming an increase in VDBPp synthesis does not occur.

Furthermore, a decrease in DBP can cause decreased synthesis of its precursor. Decreased DBP serves as a mechanism to prevent vitamin D toxicity by increasing vitamin D uptake into the liver and, thus, increasing the vitamin's conversion to excretory metabolites. The decrease in DBP in this scenario may very well be accompanied by a decrease in VDBPp

synthesis, which could explain our findings. Yet, serum levels of VDBPp may appear elevated until a new steady state is achieved. Activation of this protective mechanism could be a biological indicator of an increased need or use for vitamin D. With increased protection from toxicity, a person could consume more vitamin D without harm.(150) This theory will remain unsubstantiated until more studies are conducted to test the effects of vitamin D supplementation in children and adolescents.

Vitamin D and zinc may also simply play similar roles in affecting bone metabolism, and, thus, levels may change together accordingly. When administered separately or together, zinc and vitamin D increase the activity of alkaline phosphatase and bone DNA levels, both of which are indicators of bone growth.(165-166) Vitamin D receptors have been identified as zinc-finger proteins, suggesting that zinc's effect may be mediated through its effects on the interaction between vitamin D and its receptor.(167) Inhibiting protein synthesis, perhaps influencing the levels of these receptors, the effects of zinc on bone growth are inhibited.(165, 168)

Vitamin D levels are also affected by many factors, such as sun exposure, dietary intake of calcium and vitamin D, and calcium metabolism, which could impact DBP metabolism to some degree. Also, Laing and Fraser (2007) report that energy or protein restriction results in decreased levels of DBP in rats.(119) It is unlikely that subjects in this study were consuming such diets, as they all had a healthy BW and BMI and provided dietary recalls. The other factors cannot yet be ruled out as confounding variables.

The overall potential of VDBPp as a biomarker of zinc status is low given the lack of statistical significance between changes in treatment groups and the large number of variables that can alter serum DBP and VDBPp levels. However, zinc-supplemented subjects showing an increase in VDBPp did have significant differences between baseline and four-week spot

volumes, suggesting a slight effect of zinc. Additional studies should control for the variables identified here to eliminate their influence on VDBPp levels or characterize their effects. To determine specific mechanisms, additional compounds, such as DBP and vitamin D, should be measured.

Retinol Binding Protein with Transthyretin

Chain D of retinol binding protein with transthyretin (RBP-TTr) decreased in five subjects in both groups, with the zinc group having the larger average change. This also holds true when only trend subjects are considered. No significant results were found after performing all-subject analysis, but baseline spot volumes between groups were statistically similar. In trend subjects, baseline spot volumes were significantly different, as were volumes at four weeks, but the levels of change were very similar between groups. In other words, trend subjects started out with different baseline levels and had similar changes in serum RBP-TTr levels, ending up with different levels. Even though four-week values were significantly different, the changes were not. Considering these results, it is not clear whether or not zinc had any effect on serum RBP-TTr levels during this study. After controlling for baseline differences in spot volume, statistical analysis revealed only a small effect of zinc supplementation.

Serum retinol binding protein (RBP) bound to transthyretin (TTr), or prealbumin, has a molecular weight of 13 kDa. Serum RBP and TTr form a complex that binds and transports retinol, the hydrophobic, alcohol form of vitamin A, in the plasma. Vitamin A functions to enhance immunity, support reproduction, maintain visual function, and regulate cell proliferation and differentiation.(20, 169) Formation of the RBP-TTr complex regulates the serum retinol-binding capacity by preventing RBP from premature renal excretion, thereby maintaining the 1:1 ratio of RBP to TTr needed for binding.(170) At the extracellular surface, the enzyme,

lipoprotein lipase, cleaves retinol from the complex, allowing for retinol to enter the cell while the RBP-TTr complex remains in the plasma.(20) The two-protein carrier complex adds an additional level of specificity to the efficacy of retinol transport and function. In other words, RBP and TTr must be available to bind and form the complex and must remain bound throughout transport, or resulting conformational changes will release retinol from both proteins.(20) Altered protein concentrations, binding conformation, or association/dissociation constants of the complex can alter retinol metabolism and related biological processes.(171) The two proteins have been identified together using 2-DE and mass spectrometry in other studies, indicating the strength of the interaction between the two proteins.(172)

Vitamin A from the diet is typically consumed in the form of retinyl esters. Within the intestinal lumen, bile salts facilitate ester solubilization and activation of the enzymes, cholesterol esterase and retinyl ester hydrolase, that cleave retinyl esters to form free retinol. Eventually, retinol enters liver parenchymal cells, the major site of RBP synthesis. RBP is not secreted into the bloodstream until bound intracellularly by retinol.(173) At this stage of vitamin A metabolism, aberrations at any level of retinol absorption (e.g., gut function, enzyme levels, presence of bile salts, dietary intake) or transport to the liver (e.g., CM availability, cleavage activity) could ultimately impact how much RBP is secreted into circulation. None of these variables were considered in the present study, so they cannot be ruled out as explanations for our observations. We observed decreased serum levels of the RBP-TTr complex in most subjects, likely indicating decreased RBP secretion due to decreased need for retinol transport.

Although RBP binds the retinol form of vitamin A, serum levels of RBP can be influenced by the activity of other forms of vitamin A as well. Retinol is converted to other forms of vitamin A as needed. Within liver cells, the metalloenzyme, cellular retinol binding

protein II (CRBPII), binds retinol and determines whether it is stored, converted to retinyl-phosphate for effects on cell differentiation, or bound to RBP for secretion into the plasma.(20) Increased shuttling of retinol to the first two pathways would decrease secretion of RBP-retinol into the plasma. Therefore, variables such as increased activity of LRAT, the enzyme responsible for sequestering retinol intracellularly, impact serum levels of RBP. As there are numerous steps in vitamin A metabolism that can cause decreases in serum RBP complexes, the specificity of the RBP-TTR complex appears weak.

RBP receptors are located on several tissues including the retinal pigment epithelium (RPE) , intestines, testis and placenta.(174) Vitamin A is generally involved with cell proliferation and differentiation.(169) Studies focusing on the regulation of such processes in osteoblast progenitor cells report that retinoic acid indirectly decreases osteoblast proliferation by increasing the mRNA expression and synthesis of certain proteins involved with bone metabolism. Overall, these events inhibit bone formation. The participants in the present study are expected to be undergoing increased bone formation related to their maturational stage. It is possible that vitamin A levels decrease during this stage of development to allow bone formation to occur. Such a decrease in vitamin A could decrease levels of RBP in the serum; levels have been shown to decrease in vitamin A deficiency due to decreased secretion from the liver.(20)

A major function of retinoic acid is the regulation of gene expression. Retinoic acid binds to specific intracellular receptors, known as retinoid receptors. When retinoic acid binds, the receptors can bind to promoter sequences, descriptively termed retinoic acid response elements (RAREs), and alter gene expression according to the needs of the cell. Retinoid receptors are zinc-finger proteins, requiring zinc molecules to stabilize the structure of the DNA-binding motif.(20) Increasing intake of zinc would allow for increased activity of these transcription

factors and would, thus, create a need for additional intracellular vitamin A in the form of retinoic acid. It is sensible that retinol is pushed intracellularly in order to be converted to retinoic acid. Decreases in serum retinol levels may relate to decreases in serum RBP.

Synthesis of RBP is reported to decrease in zinc deficiency, possibly leading one to expect higher levels of the RBP-TTr complex in the zinc-supplemented group than in the placebo group.(175) The opposite finding is reported from our data. We observed decreases in most subjects, but these associations were not significant. This pattern suggests the possibility that many of our subjects may have been deficient to some degree. If an inadequate zinc status is cause of the decreases in RBP-TTr, supplementation with 24 mg zinc sulfate was, therefore, not sufficient to improve zinc status to this functional level. A few subjects showed increases in the RBP-TTr complex, suggesting that these subjects may have had sufficient zinc levels. Since there is no biomarker as of yet to assess the presence or degree of mild or moderate zinc deficiency, this observation cannot be validated. Future experimental designs will be greatly improved by excluding such confounding variables once a biomarker of zinc status is identified.

Our observations that changes in RBP-TTr levels were significantly different between groups in subjects exhibiting protein decreases suggests an effect of zinc may exist. Although we cannot conclude if RBP-TTr would serve as a biomarker of zinc status based on our data, data from future studies that measure additional variables will allow for a proper assessment of any relationships existing between zinc and RBP-TTr. Ultimately, our results indicate only a slight effect of zinc supplementation on levels of RBP-TTr.

LIMITATIONS

The present study has limited external validity due to the homogenous nature of the study population. However, without complete knowledge of differences in zinc metabolism existing

within other populations, we could not have included males or Non-Hispanic black females without substantially increasing the sample size. We were successful at matching our subjects on several variables designed to reduce sample variability, yet a substantial degree of variability seemed to still exist with our study population. This indicates there are other factors at work.

In addition, we did not assess any additional measures, such as inflammatory markers or indicators to verify administration of the zinc supplements. Although we hypothesized as to what proteins would respond to zinc, we did not anticipate that the majority of the proteins we would find would be acute phase reactants. Yet, our observations do make sense, as the liver is a rapidly exchanging zinc pool and is the main site of synthesis for acute phase proteins.(176) Regardless, the main purpose of this study is to identify protein candidate biomarkers of zinc status for future investigation, not to definitively identify a biomarker.

Without substantial, relevant preliminary data and having a small sample size, we were uncertain as to whether a large enough effect size would be found to reach statistical significance. Considering these limitations, it is promising that we did find some significant results. We also reported relationships that trend towards significance in the event that one of these limitations is responsible for the low significance levels. Hopefully, this study will provide some preliminary data for other researchers searching for a zinc biomarker. Future studies should analyze a variety of populations, including the one analyzed here, but should use a larger sample size. A few relationships are thought to not have reached significance due to the degree of variability within the treatment group. With a larger sample, the variability should be more normalized. In addition, a four-week supplementation period may have been too short to observe significant changes in the levels of some proteins. Again, we reported trends that suggested that lengthening the supplementation period could result in further changes.

In addition to study design, the proteomic methods of protein separation used in the present study are known to have some limitations. Even though major high-abundance proteins were removed from samples, some low abundance proteins may still be masked on SDS-gels. In addition, depletion strategies that remove albumin often result in the removal of low abundance serum proteins, because albumin is a major transport protein in serum.(63, 177) It is possible that a number of potential biomarkers could have been removed in this way. Yet, this limitation is combated by using multiple elutions to release bound proteins into the filtrate, and the excessive washes are not reported to majorly impact results. Also, SDS-PAGE techniques may not fully separate proteins that have similar isoelectric points and molecular masses. In result, two proteins may occupy the same spot on 2D gels; an increase in one could mask and be masked by a decrease in the other. We only loaded 150 µg of protein onto 2D gels, which is much lower than typically used, so hopefully, there was a lower degree of concealment. Finally, much of the interpretation regarding the causes of protein changes is speculative due to the lack of measuring multiple variables in our study. Serum protein content is influenced by physiological health as well as the release of dead cells and subcellular components from other tissues, and these conditions were considered when analyzing and reporting the results.(178) Despite these limitations, the methods we used are considered valid among researchers in the field.(78) In addition, extreme caution was taken to ensure all samples were treated alike at each level of testing.

The amount of protein loaded onto 2D gels was determined by the capacity of the columns used for immunoaffinity prefractionation. At most, only 20 µL of sample could be loaded into the columns. We chose to run 150 µg protein per sample, because this value was the

original limiting protein content and yielded acceptable results after running preliminary pooled samples.

Resolving gels were prepared at a 15% concentration. As higher concentrations reduce the degree of separation of proteins, using 15% gels could have resulted in certain proteins remaining too close to be viewed as separate spots. Another limitation is that most samples were only prepared and run a single time, with the exception of one subject. There were no major, visible differences in the duplicated 2D gels. Duplicating one or all of the samples and electrophoresing them on gels of a lower concentration, such as 12.5%, would have strengthened our analysis. Comparing 12.5% and 15% 2D gels would identify the separation of any proteins that had migrated together with the higher concentration, indicating whether or not all samples should be duplicated on 2D gels of a lower concentration.

The circling of spots using the Phoretix™ 2D software is a subjective step within our data analysis. We were unable to find previous reports of methods or reasoning for circling spots in a particular manner. Finally, not all possible connections within our dataset were tested statistically, so more information could be revealed with a more complete data analysis.

STRENGTHS

The sample population of this study consisted of generally healthy, young females who did not present overt zinc deficiency. Most studies of zinc status and metabolism use populations that have varying levels of zinc deficiency. This approach limits similarity of treatment and control subjects in human studies but not necessarily animal studies, as it is not against research ethics to induce moderate or severe zinc deficiencies in animal models. In addition, it has been noted that the comparison of groups with large differences in zinc status (e.g., severely zinc-deficient and zinc-adequate) is muddled by symptoms of zinc deficiency such as decreased food

intake and related weight loss.(175) The present study sample does not make comparisons over large ranges of zinc status, as assumed by results of dietary recalls and absence of clinical deficiency or toxicity symptoms (unpublished data). Also, average body weights at baseline and four-weeks were virtually the same between the zinc and placebo groups, as well as were average changes in body weight over the course of the intervention.

The laboratory methods we used are currently considered the most accurate for proteomic studies.(179) The immunoaffinity columns employed to prefractionate our samples are highly effective at removing several of the high abundance proteins found in human serum.(72) Maximal sample concentration was loaded into the columns to maximize immunoaffinity reactions.(66) Also, the protein fragments identified by mass spectrometry were compared to multiple databases, reducing the risk of misidentification.(180) In addition, statistical analyses were performed twice in order to observe any differences between all-subject and trend-subject data. Finally, all results were reported accurately and discussed as objectively as possible. Of course, we tried to explain our observations, but an effort was made to consider all possibilities.

TABLES

Table 4.1. Mechanisms for changes in serum protein levels.

Decreases	Increases
↑ mRNA degradation	↓ mRNA degradation
↑ Protein degradation / excretion	↓ Protein degradation / excretion
↑ Protein metabolism to functional metabolites	↓ Protein metabolism to functional metabolites
↑ Tissue uptake	↓ Tissue uptake
↓ mRNA expression	↑ mRNA expression
↓ Protein synthesis	↑ Protein synthesis
↓ Secretion from liver or tissue where synthesized	↑ Secretion from liver or tissue where synthesized

CHAPTER 5

CONCLUSION

SUMMARY

The aim of this study was to identify protein candidate biomarkers of zinc nutriture. Overall, ten proteins were differentially expressed between subjects receiving zinc supplementation and subjects receiving a placebo and were subsequently identified: IgM kappa IIIB SON (IgMk3); proapolipoprotein A-I (Pal); serum amyloid P, chain E (E-SAP); alpha-1-antitrypsin (A1A); keratin 1, type II cytoskeletal (K1); hemopexin (Hpx); alpha 2-HS glycoprotein (A2HS); inter-alpha (globulin) inhibitor, H2 polypeptide (IH2); vitamin D binding protein precursor (VDBPp); and retinol binding protein with transthyretin (RBP-TTr). A few significant effects of zinc supplementation on serum levels of these proteins were observed. Many of these proteins are acute phase reactants and are influenced by a number of variables. With the research tools currently available, many of these variables can be controlled in future studies to encourage more significant results. More of our observations may have reached significance with a larger sample size or longer period of zinc supplementation. We hope the information presented here will initiate new avenues for researching zinc biomarkers and strengthen the design of investigations to come.

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APPENDIX

Immunoaffinity Chromatography

Recipes and Reagents

10X Dilution Buffer *(Sigma-Aldrich, #S4199)*
100 mM Tris-Buffered Saline
1 M Tris-HCl with 1.5 M NaCl, pH 7.4

10X Stripping Buffer *(Sigma-Aldrich, #S4324)*
1 M Glycine, pH 2.5

10X Neutralization Buffer *(Sigma-Aldrich, #S4449)*
1 M Tris-HCl, pH 8.0

*Prepare 1X Dilution Buffer, 1X Stripping Buffer, and 1X Neutralization Buffer as needed by diluting the respective 10X buffers 10-fold with di-deoxy water (ddH_2O).

Storage Buffer (100 mL)
Combine the following in a 125-mL side-arm flask:
10 mL 10X Dilution Buffer
90 mL ddH_2O
0.02 g Sodium azide

Materials

Seppro® IgY-14 Spin Columns *(Sigma-Aldrich, #S4699)*
2-mL Collection Tubes *(Sigma-Aldrich, #T7813)*
Empty Spin Columns *(Sigma-Aldrich, #S4574)*
Clean paper towel

Protein Assay

Recipes and Reagents

Reagent A' (prepare as needed, 127 µL per tube)
Add 5 µL DC Reagent S per every 250 µL DC Reagent A

Serum Bovine Albumin (1.46 µg/µL)
Prepare 5 dilutions using ddH_2O to final volumes of 25 µL and to final concentrations of 0, 0.146, 0.409, 0.730, and 1.46 µg/µL

Samples
Prepare dilutions as desired using ddH_2O to final volumes of 25 µL

Other
ddH_2O
DC Reagent A
DC Reagent S
RC Reagent I
RC Reagent II
DC Reagent B
Liquid Detergent

Materials

Microtube centrifuge (Eppendorf)
1.5-mL Microcentrifuge Tubes
Vortex
15-mL Syringe
Absorbance Cuvettes, disposable
Ice Bucket
Electronic Pipette
Pasteur Pipets
Clean Paper Towel
Spectrophotometer (Beckman-Coulter DU-800)
Computer with spectrophotometer software installed
Pipe Cleaner

Acetone Precipitation

Recipes and Reagents

ddH_2O
100% Acetone
Samples

Materials

Microtube Centrifuge (Eppendorf)
1.5-mL Microcentrifuge Tubes
2-mL Microcentrifuge Tubes

Protein Resolubilization

Recipes and Reagents

Protein solubilization buffer (PSB) (2 mL)

1g	PSB powder
1.1 mL	PSB diluents
20 µL	Tributylphosphene (TBP)
20 µL	Bio-Rad 3-10 Buffer, 100x, 1 mL, 20% Bio-Lyte ampholytes
5 µL	Bromophenol blue

Other
Mineral oil

Materials

Isoelectric Focusing (IEF) Tray and Lid
IEF Strips, 7 cm, pH 3-10
Tweezers, 2 pairs
Weigh Boat
Measuring Scale
Magnetic Stirrer
Stir Bar, small
2-mL Microcentrifuge Tubes
Electronic Pipette
Pasteur Pipets
Microtube Centrifuge (Eppendorf)
IEF Cell (Protean)
Clean Paper Towel
Vortex

Isoelectric Focusing

Recipes and Reagents

None

Materials

IEF Cell *(Protean)*
IEF Tray and Lid
Tweezers, 2 pairs
Paper Wicks *(2 per strip)*

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Recipes and Reagents

15% Polyacrylamide Gel

30 mL	30% Acylamide/Bis solution, 29:1 (3.3% C)
15 mL	1.5 M Tris-HCl Buffer, pH 8.8
600 µL	10% SDS
14.1 mL	ddH_2O
300 µL	10% Ammonium persulfate
20 µL	TEMED

Lower Tank Buffer (1 L)

100 mL	10X Tris-glycine-SDS buffer
900 mL	ddH_2O

Upper Tank Buffer (600 mL)

60 mL	10X Tris-glycine-SDS buffer
540 mL	ddH_2O

IEF Equilibration Buffer (2 mL)

Other
Water-Saturated Butanol
Tributyl Phosphene (TBP)

Materials

SE600 Vertical Slab Gel Units
Glass Plates
1.5-mm Spacers
Dual-gel Casting Stand
Side Clamps
Cams

Other
1-L Graduated Cylinder
1-L Fleaker
Electrophoresis Apparatus (Hoeffer)
Parafilm
Stir Bar
125-mL Side-Arm Flask
Rubber Stopper
Vacuum Pump
Vacuum Hose
Magnetic Stirrer
Orbital Shaker
Test Tube *(if needed to prepare IEF equilibration buffer)*
Clean Paper Towel
Scissors
Tweezers, 2 pairs
Power Supply
Water Circulator