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# Dietary zinc depletion and repletion affects plasma proteins: an analysis of the plasma proteome

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**Abstract** Zinc (Zn) deficiency is a problem worldwide. Current methods for assessing Zn status are limited to measuring plasma or serum Zn within populations suspected of deficiency. Despite the high prevalence of Zn deficiency in the human population there are no methods currently available for sensitively assessing Zn status among individuals. The purpose of this research was to utilize a proteomic approach using two-dimensional gel electrophoresis (2DE) and mass spectrometry to identify protein biomarkers that were sensitive to changes in dietary Zn levels in humans. Proteomic analysis was performed in human plasma samples ($n = 6$) obtained from healthy adult male subjects that completed a dietary Zn depletion/repletion protocol, current dietary zinc intake has a greater effect on fractional zinc absorption than does longer term zinc consumption in healthy adult men. Chung et al. (Am J Clin Nutr 87 (5):1224–1229, 2008). After a 13 day Zn acclimatization period where subjects consumed a Zn-adequate diet, the male subjects consumed a marginal Zn-depleted diet for 42 days followed by consumption of a Zn-repleted diet for 28 days. The samples at baseline, end of depletion and end of repletion were pre-fractionated through immuno-affinity columns to remove 14 highly abundant proteins, and each fraction separated by 2DE. Following staining by colloidal Coomassie blue and densitometric analysis, three proteins were identified by mass spectrometry as affected by changes in dietary Zn. Fibrin β and chain E, fragment double D were observed in the plasma protein fraction that remained bound to the immuno-affinity column. An unnamed protein that was related to immunoglobulins was observed in the immunodepleted plasma fraction. Fibrin β increased two-fold following the Zn depletion period and decreased to baseline values following the Zn repletion period; this

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protein may serve as a viable biomarker for Zn status in the future.

**Keywords** Fibrin β · Zn biomarker · Marginal Zn deficiency · Dietary Zn depletion and repletion · Two-dimensional gel electrophoresis · Hemostasis

## Introduction

Zinc (Zn) is an essential trace element necessary for the normal growth and development of all organisms. The average adult human body contains 2.3 g of Zn, an amount second only to iron (Coleman 1992). Over 300 enzymes require Zn as a structural component or within the active site (Coleman 1992). Zn finger transcription factors (Cys2 His2) are encoded by approximately 3 % of the human genome, comprising 709 genes. Another 48 genes encode the thyroid and steroid hormone nuclear receptor gene families (Cys4) (Klug 2010). Other Zn-binding motifs that have been identified include Cys3HisCys4 (RING finger) and Cys2HisCys5 (LIM domain) (Berg and Shi 1996). Zn is necessary for insulin metabolism (Emdin et al. 1980), and for insulin signaling (Jansen et al. 2009). Consequently, Zn plays a role in numerous biochemical and physiological pathways, including those involved in gene expression, signal transduction, and antioxidant protection (Song et al. 2009; Sakaguchi et al. 2002; Taylor and Bray 1991; Sullivan et al. 1980).

Inadequate consumption of Zn is a worldwide problem. There is a medium to high risk of Zn deficiency worldwide (International Zinc Nutrition Consultative Group 2004), with a significant number of Americans consuming less than the estimated average requirement for Zn. For children less than 5 years of age Zn deficiency is estimated to cause ~8,00,000 deaths from malaria, diarrhea, and pneumonia, with a loss of disability-adjusted life years of 28 million (Caufield and Black 2004).

Determining the degree of marginal zinc deficiency within different human populations, as well as the ability to diagnose marginal zinc status among individuals, is the goal. The current list of potential biomarkers lacks the sensitivity and specificity for individual assessment, making it difficult to interpret the relevance of reports of inadequate Zn intake in otherwise healthy individuals including infants and children (Hambidge 2000). Studies to investigate the responses of Zn transporter proteins have been reported. In mouse erythrocyte plasma membrane, SLC30A1 (ZnT1) and SLC39A10 (Zip10), responded to dietary Zn deficiency (Ryu et al. 2008). Humans consuming a severely Zn deficient diet for 10 days exhibited significant reductions in peripheral blood mononuclear cell metallothionein mRNA, and mRNA from members of the SLC30A Zn transporter family (ZnT1, ZnT4, ZnT5). Reticulocyte ZnT1 and Zip3 mRNA similarly responded to dietary Zn deficiency. In addition, at least 20 serum microRNA species were responsive to dietary Zn depletion for 10 days followed by repletion for 7 days (Ryu et al. 2011).

The objective of this study was to measure plasma proteins from individuals consuming a marginal Zn-depleted diet for 42 days followed by consumption of a Zn-repleted diet for 28 days. This protocol could be considered closer to what is experienced in the general population, the consumption of a marginally deficient diet for weeks to months. We hypothesize that specific circulating plasma proteins will exhibit measurable responses to changes in dietary zinc. The identified proteins may ultimately serve as biomarkers for marginal zinc status in humans upon validation in the future using significantly more subjects.

## Experimental

The plasma samples used in this study were from a previous Zn depletion/repletion study performed in healthy male volunteers. Details of subject recruitment, inclusion/exclusion criteria and subject characteristics can be found in Chung et al. (2008) and Song et al. (2009). The dietary protocol from Chung et al. (2008) is shown in Fig. 1. Briefly, during the acclimatization period, (days 1–13), an adequate zinc diet (11 mg Zn/day) was given for 13 days to ensure adequate zinc status. During the Zn depletion period, (days 14–55), the subjects were given a liquid diet containing 0.6 mg Zn/day for 7 days followed by a low zinc diet (4 mg Zn/day) for another 35 days. Finally, during the Zn repletion period, (days 56–83), the subjects consumed a zinc adequate (11 mg Zn/day) diet for 28 days, with supplemental zinc (20 mg/d zinc as zinc gluconate) administered for the first 7 days. The subjects consumed a daily multiple vitamin supplements (100 % RDA) without minerals (Long's Pharmacy, CA) throughout the study. Plasma

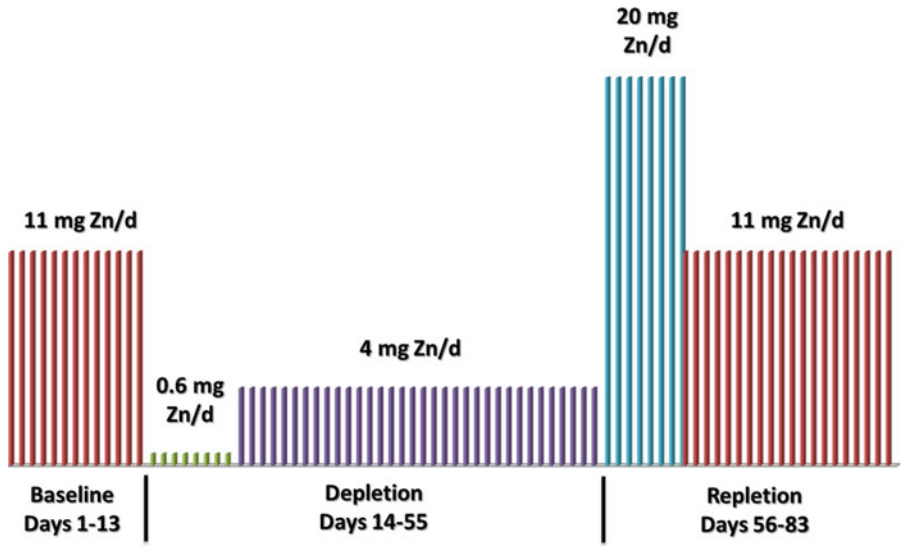


**Fig. 1** Zn depletion and repletion protocol. Subjects consumed 11 mg Zn/d for 13 days during the baseline period. The depletion period began with the consumption of 0.6 mg Zn/d for 8 days followed by 4 mg Zn/d for 34 days. The repletion period extended for 28 days starting with consumption of 20 mg Zn/d for 8 days, then 11 mg Zn/d for 20 days. (adapted from Chung et al. 2008)

from three time points was analyzed in the current study, day 13 (end of acclimation), day 55 (end of depletion), and day 83 (end of repletion). Plasma from 6 individuals from the previous study was pre-fractionated, and analyzed by two-dimensional gel electrophoresis (2DE) and mass spectrometry for the current study.

Fourteen high-abundance proteins were removed using the Seppro® IgY 14 immunoaffinity spin columns (Sigma). The columns contain antibodies against the following human proteins: albumin, α1-antitrypsin, α1-acid glycoprotein, α2-macroglobulin, apolipoproteins A-I and A-II, apolipoprotein B, complement C3, fibrinogen, haptoglobin, IgA, IgG, IgM, and transferrin (Bjorhall et al. 2005). Before pre-fractionating each sample, storage buffer (25 mM Tris, 25 mM NaCl, 0.01 % sodium azide; pH 7.5) was removed through centrifugation, and the columns were washed twice using 1× 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 s; 2,000×*g*). Each sample was prepared in duplicate, and both fractions were pre-fractionated simultaneously. A sample volume of 20 µl was combined with 480 µl of 1× 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 min; 10,000×*g*) 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. Both the immunodepleted plasma and the bound protein fractions were saved for analysis. The protein concentration of the both fractions was measured using a modified Lowry method (Lowry et al. 1951).

The bound (highly abundant) and immunodepleted (low/medium abundant) plasma protein fractions were separated in triplicate. 75 µg protein was taken from each fraction in order to load 225 µg protein on to immobilized pH gradient (IPG) strips. The protein in each sample was concentrated by acetone precipitation. Four volumes of acetone were added to each sample, and samples were frozen at −80 °C for at least 30 min. Following centrifugation (20 min; 10,000×*g*) the pellet was resolubilized in 125 µl protein solubilization buffer (Bio-Rad proprietary composition). The following was added to the samples containing protein solubilization buffer: 2 mM tributyl phosphine, 0.2 % ampholyte, 0.0002 % bromophenol blue. Samples were then pipetted into a clean isoelectric focusing (IEF) tray, and 3–10 IPG strips (Bio-Rad ReadyStrips™, 7 cm,) were laid on top. Mineral oil was applied atop the strips, 1.5 ml per lane. The solubilized sample mixture was adsorbed to the strips via active rehydration for approximately 15.5 h in a Bio-Rad Protean IEF cell set at 20 °C. IEF ran approximately 7.5 h on a rapid preset method with

the following settings: 20,000 VH, 4,000 V/20 °C, and 50 μA current per strip. 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.

IPG strips containing the proteins were thawed exactly 15 min at room temperature and equilibrated with second dimension equilibration buffer (6 M urea, 0.375 M Tris, 2 % sodium dodecyl sulfate, 20 % glycerol, 2 mM tributyl phosphine) for 20 min with gentle agitation. Strips were placed into wells and positioned flush against the polyacrylamide gel. The denaturing slab gels were composed of 15 % acrylamide and the second dimension was run using Tris–Glycine–sodium dodecyl sulfate buffer, with a constant current of 30 μA per gel. Gels were fixed with a 40 % methanol/10 % acetic acid solution for at least 30 min. After removing the fixative, gels were washed with $ddH_2O$ (3 × 10 min). Proteins were stained with colloidal Coomassie G-250 blue-silver stain (10 % phosphoric acid 10 % ammonium sulfate, 20 % methanol, 0.12 % Coomassie G-250) for a minimum of 6.5 h with gentle agitation (Candiano et al. 2004). The gels were destained with $ddH_2O$ until the background was clear.

The gels were digitally scanned using HP PrecisionScan Pro 3.1 software. The spot densities were measured, and differences determined, using Phoretix 2D Analysis software (V6.01c). The data were normalized by subtracting the background intensity from the total intensity of each spot. Spots showing significant variance were selected and manually excised from the gels. Excised spots were sent to the Proteomics Core Laboratory at the Georgia Health Sciences University 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. For each spot, the protein with the highest confidence interval of the ten possibilities, the closest matching in silico molecular weight and pI to the experimentally determined molecular weight estimated with the gel standards, was selected as the protein identity.

2DE was performed in triplicate for the plasma samples from each individual, and for each plasma protein fraction. The technical replicates from individual protein spots were averaged for each individual at each time point. The data from each protein spot is based on the biological replicate of six individuals at each time point. ANOVA was used to identify significant differences based on dietary treatment ($p \leq 0.05$). Post-hoc analysis to identify significant differences was determined by Fishers Least Significant Differences.

## Results and discussion

The subject characteristics from the original study and a follow-up study have been reported previously (Chung et al. 2008; Song et al. 2009). A summary of the subject characteristics, and the results from their plasma Zn analysis, follows. At the start of the original study (Chung et al. 2008), the age of the subjects was 38 ± 8 (SD) y. Their body mass index was between 20.8 and 25.9 $kg/m^2$. None of the subjects were anemic. Their dietary zinc intake at the beginning of the study was 12.9 ± 2.2 (SD) mg/d and their plasma zinc concentrations averaged 84.8 ± 8.5 (SD) μg/dL. Plasma Zn levels were measured at the end of the acclimatization period (day 13), the Zn depletion period (day 55) and the Zn repletion period (day 83). No change was observed between day 13 and day 55 (79 ± 9 (SD) μg/dL and 79 ± 10 (SD) μg/dL, respectively). However, there was a significant 13 % increase in plasma Zn measured on day 83 (86 ± 10 (SD) μg/dL; $p = 0.02$) compared to day 55 (Chung et al. 2008; Song et al. 2009).

In the current study, the plasma from baseline, Zn-depleted and Zn-repleted subjects was fractionated by immunoaffinity columns to remove 14 highly abundant proteins, resulting in bound and immunodepleted protein fractions. The proteins in these fractions were separated by 2DE. Figure 2 shows the electrophoretic pattern obtained from those proteins bound by the immunoaffinity column (Bound) and the immunodepleted plasma containing those proteins remaining in the effluent (Immunodepleted). The protein spots whose levels were significantly affected by dietary zinc are labelled by their accession number.

The identity of the protein spots was determined by MALDI-TOF mass spectrometry (Table 1). Two proteins, fibrin β (accession number gi|223002) and chain E, fragment double D from human fibrin (accession number gi|28373962), in the bound plasma protein fraction are byproducts of hemostasis; one protein, (accession number gi|34527413), in the

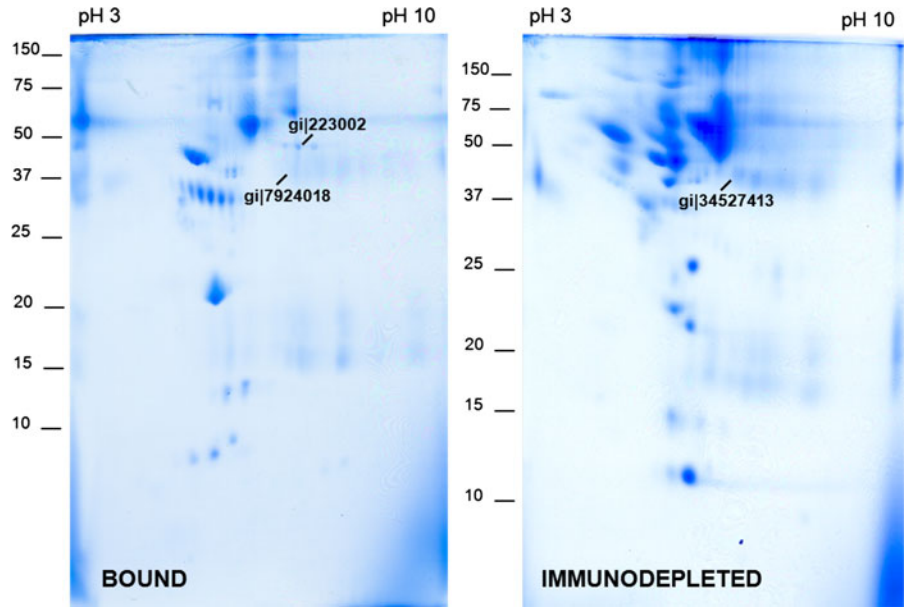


**Fig. 2** Representative 2DE gels showing the molecular wt. standards on the left side of each gel image, and the acidic and basic ends. Plasma was pre-fractionated prior to separation by 2DE; representative 2DE gels are shown from the BOUND (*immunoaffinity bound*) and IMMUNODEPLETED plasma proteins. The protein spot IDs are shown as accession numbers

immunodepleted fraction remains unnamed, though it is related to immunoglobulins. Each of the proteins identified by mass spectrometry exhibited an ion confidence interval (CI) of $> 99$ %.

The data indicate that hemostasis is affected by consumption of a Zn depleted diet. Figure 3 shows the responses that these three plasma proteins exhibited to changes in dietary Zn. Fibrin β increased approximately 2-fold by the end of the depletion period compared to baseline and decreased back to baseline levels by the end of the repletion period. No such change in plasma Zn levels was observed in the original study (Chung et al. 2008). This protein is a product of the coagulation cascade, catalysed by thrombin which cleaves fibrinopeptides α and β from fibrinogen resulting in clot formation (Nesheim 2003). Zn has been shown to reduce clotting times in a concentration-dependent manner by accelerating fibrin assembly (Marx and Eldor 1985; Marx and Hopmeier 1986; Marx et al. 1987). Dietary Zn deficiency is known to impair platelet aggregation and hemostasis (Tubek et al. 2008). Zn has been shown to inhibit the release of fibrinopeptide α during thrombin-induced clot formation (Marx and Hopmeier 1986). Conversely, one might expect that Zn deficiency may increase the release of fibrinopeptide α. Though an increase in fibrinopeptide α was not observed in the current proteomic analysis, the release of fibrinopeptide β occurs sequential to the release of fibrinopeptide α (Nesheim 2003); the resulting fibrin β may be biomarker for dietary Zn deficiency. However, the findings of the current study need to be validated using a much larger sample size.

Liver damage also affects liver fibrin levels in a rat model for cirrhosis induced by thio acetamide (Dashti et al. 1997). Zn supplementation was effective for reversing the liver damage. Cirrhotic changes in the liver increased liver fibrin, while Zn decreased fibrin

**Table 1** Identities of plasma protein spots exhibiting significant differences between treatments. Tryptic peptides from each protein spot were identified by mass spectrometry. The protein confidence intervals exceeded 94 % and the ion confidence intervals exceeded 99 %

| Protein score | Protein CI % | Protein name | Peptide count | Total ion score | Ion CI % | Accession number |
|---|---|---|---|---|---|---|
| 244 | 100 | Fibrin β | 14 | 208 | 100 | gi\|223002 |
| 83 | 99.929 | Chain E, fragment double-D from human fibrin | 6 | 75 | 100 | gi\|28373962 |
| 64 | 94.461 | Unnamed protein | 11 | 44 | 99.68 | gi\|34527413 |

in the liver (Dashti et al. 1997). The observed increase in plasma fibrin β in response to dietary Zn deficiency from this study may be related to increases in liver fibrin induced by Zn deficiency. Zn deficiency is pro-inflammatory through stimulation of NFκB DNA binding and inhibition of PPARα transactivation activity, which are also linked to coagulation and fibrinolysis pathways (Shen et al. 2008). The protein C pathway is another link between inflammation and hemostasis where Zn status may be an important component (Esmon 2003; Sen et al. 2010). Zn also stimulates the intrinsic (Shore et al. 1987; Bernardo et al. 1993), and inhibits the extrinsic (Pedersen et al. 1991, 2000), pathways of coagulation.

Another possible link between Zn and fibrin may be through the protein decorin (Tubek et al. 2008). The amino terminus of decorin binds to the fibrinogen D fragment in a Zn-dependent manner, and modulates fibrin assembly (Dugan et al. 2003, 2006). Fibrinogen-bound decorin is more susceptible to proteolysis by tissue-plasminogen activator; physiological concentrations of Zn decrease this interaction (Dugan et al. 2006). The observed increase and decrease in fibrin β with Zn depletion/repletion may be due to Zn-dependent changes in decorin binding to fibrinogen during normal hemostasis.

There was no significant change between baseline and the end of the depletion period in chain E, fragment double D from fibrin. The levels of this protein significantly increased more than 2-fold by the end of the repletion period. These fragments are generated during fibrinolysis, and are fibrin degradation products from the cleavage by plasmin of the α, β, and γ chains of fibrin at specific arginine and lysine residues (Nesheim 2003). The response of the chain E, fragment double D to dietary Zn may be the result of changes in the activity of thrombin-activatable fibrinolysis inhibitor. This metalloproteinase has 40 % amino acid homology to pancreatic carboxypeptidase B, and is inactivated by the Zn chelator *o*-phenanthroline (Eaton et al. 1991; Marx et al. 2002). The holo-metalloprotease is activated by thrombin and attenuates fibrinolysis (Redlitz et al. 1995). The opposite would occur with the loss of Zn, resulting in an increase in fibrin degradation products such as chain E, fragment double D. This fragment was shown to increase by the end of the repletion period, which may reflect a slow response to the changes in dietary Zn.

Alternatively, the activation of glu- and lys-plasminogen via the intrinsic pathway involving Factor XIIa was shown to be significantly enhanced by Zn (Schousboe 1997). The increase in chain E, fragment double D following dietary repletion of Zn may, therefore, be caused by an increase in the conversion of plasminogen to plasmin, increasing fibrolysis and the fibrin degradation products.

The levels of the unnamed protein, found among the low- to medium-abundant proteins in the immunodepleted plasma, did not significantly change between baseline and the end of the depletion period. Its amino acid sequence includes many regions associated with immunoglobulins (Entrez Protein: Sequence Database). However, the levels of this protein decreased significantly by the end of the repletion period when compared to the depletion

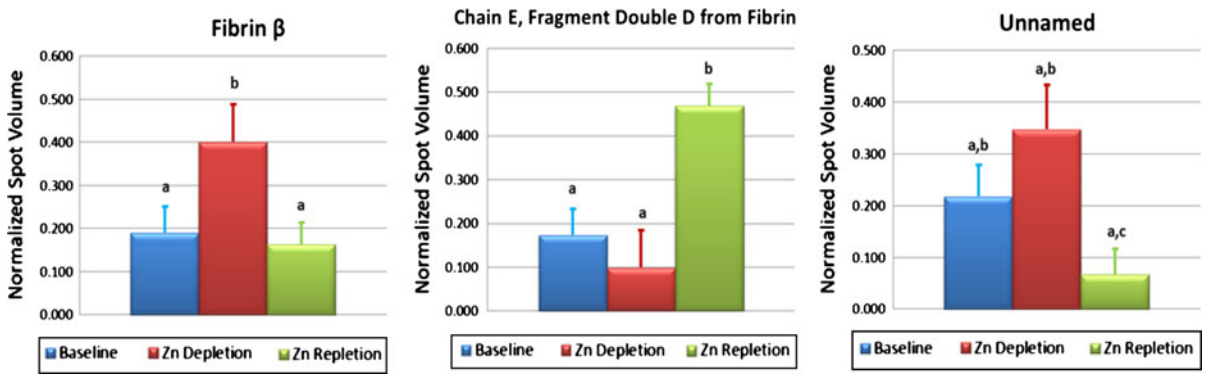


**Fig. 3** Average normalized spot volumes for fibrin β (accession #gi|223002), chain E fragment double D from fibrin (accession # gi|28373962), and unnamed protein (accession # gi|34527413). The bars represent the mean ± SEM; $n = 6$. Bars with different letters are significantly different from each other, $p \leq 0.05$

period. Its levels in the plasma were not significantly different from baseline, though.

## Conclusions

Immunoaffinity prefractionation of plasma was used to separate the plasma proteins into highly abundant and medium/low abundant protein fractions. The separation of each plasma protein fraction by 2DE resulted in the identification of three proteins that were responsive to dietary Zn. Fibrin β and chain E fragment double D from fibrin are involved with blood clotting, and were in the bound, highly abundant plasma protein fraction. Fibrin β was the most responsive to changes in dietary Zn, increasing during the depletion phase and decreasing to baseline levels during the repletion phase. Though sensitive to dietary zinc depletion, changes in fibrin β need to be specific for changes in zinc nutriture. Zinc is involved in numerous reactions associated with inflammation and hemostasis. Zinc deficiency is pro-inflammatory, and inflammation is linked to coagulation and fibrinolysis pathways, possibly through stimulation of NFkB DNA binding, and inhibition of PPARα transactivation activity. Zinc also enhances the intrinsic pathway, and inhibits the extrinsic pathway, of coagulation. Consequently, conditions that induce inflammation or affect hemostasis may also affect plasma fibrin β levels apart from zinc status; future research designed to test the hypothesis that plasma fibrin β levels are specific for assessing zinc nutriture will be important in this regard.

Chain E fragment double D from fibrin did not change during Zn depletion and increased during the repletion phase. An unnamed protein was identified in the medium/low abundant protein fraction. This protein has characteristics similar to immunoglobulins, but is not associated with any of the immunoglobulin classes. The levels of this unnamed protein did not change significantly during the Zn depletion phase of the study, but significantly decreased during the repletion phase. Previous studies in this study population have found that peripheral blood cell DNA strand breaks also increase with Zn depletion and is reversed with Zn repletion (Song et al. 2009). Although DNA damage may be highly sensitive to alterations in dietary zinc, its use as a specific Zn dietary assessment method is limited since alterations in DNA damage may also be affected by other environmental stresses, including other micronutrient deficiencies. Moreover, there is little standardization for quantifying absolute levels of DNA damage using single-cell electrophoresis as outlined in this previous study. However, these results indicate that fibrin β may be another candidate for consideration as a blood biomarker for assessing Zn status since it was responsive to changes in dietary Zn. The findings of this study are limited by the small sample size ($n = 6$); the Zn-dependent changes in plasma fibrin β need to be validated in more subjects in order to strengthen its consideration as a Zn biomarker. Zn deficiency is an important public health issue world-wide and there exists a critical need to develop sensitive and specific biomarkers for Zn deficiency in humans. The current study highlights the use of an unbiased proteomic approach to identify possible novel biomarkers for Zn staus in humans.

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