Genes Nutr (2015) 10:44
DOI 10.1007/s12263-015-0494-y

RESEARCH PAPER

Zinc-induced upregulation of metallothionein (MT)-2A is predicted by gene expression of zinc transporters in healthy adults

Anna Chu[1,2] · Meika Foster[1] · Sarah Ward[2] · Kamrul Zaman[2] · Dale Hancock[3] · Peter Petocz[4] · Samir Samman[1,2]

Received: 17 May 2015 / Accepted: 25 September 2015 / Published online: 7 October 2015

Abstract The usefulness of zinc transporter and metallothionein (MT) gene expressions to detect changes in zinc intake remains unclear. This pilot study aimed to determine the effects of zinc supplementation on zinc transporter and MT gene expressions in humans. Healthy adults ($n = 39$) were randomised to zinc treatment (ZT), receiving 22 mg Zn/day ($n = 19$), or no treatment (NT) ($n = 20$). Blood samples were collected on Days 0, 2, 7, 14, and 21. Plasma zinc and serum C-reactive protein concentrations were analysed. Gene expression of zinc transporters and MT in peripheral blood mononuclear cells was analysed using real-time PCR. Using repeated-measures ANOVA, *MT-2A* gene expression and fold change were found to be higher in the ZT group ($P = 0.025$ and $P = 0.016$, respectively) compared to the NT group, specifically at Day 2 (40 ± 18 % increase from baseline, $P = 0.011$), despite no significant increase in plasma zinc concentration. In a multiple regression model exploring the changes in gene expressions between Days 0 and 21, the change in *MT-2A* gene expression was correlated with changes in all zinc transporter expressions ($r^2 = 0.54$, $P = 0.029$); the change in *ZIP1* expression emerged as a univariate predictor ($P = 0.003$). Dietary zinc intake was predictive of zinc transporter and MT expressions ($P = 0.030$). Physical activity level was positively correlated with baseline *ZIP7* expression ($r = 0.36$, $P = 0.029$). The present study shows that *MT-2A* expression is related to changing expression of zinc transporter genes, specifically *ZIP1*, in response to zinc supplementation. The current report adds to our understanding of MT in the coordinated nature of cellular zinc homeostasis.

Keywords Zinc · Transporter · Metallothionein · Gene expression · Physical activity

Introduction

Marginal zinc deficiency has been suggested to influence multiple physiological systems due to the extensive involvement of zinc in numerous structural and biochemical functions of the body (Foster and Samman 2010); a significant proportion of the world's population may be at risk of marginal zinc deficiency (Samman 2007), a condition that contributes substantially to disease burden worldwide (Fischer Walker et al. 2009). Despite being redox-inert, zinc can act as a pro-antioxidant and modulate oxidative stress by maintaining cell membrane integrity and regulating the functions of metallothionein (MT), superoxide dismutases, and glutathione (Maret 2003; Oteiza 2012). Since the discovery of zinc deficiency in humans (Prasad et al. 1961), our knowledge of the physiological roles of zinc has extended beyond the initially proposed functions. Recent evidence

Electronic supplementary material The online version of this article (doi:10.1007/s12263-015-0494-y) contains supplementary material, which is available to authorized users.

✉ Samir Samman
samir.samman@otago.ac.nz

[1] Department of Human Nutrition, University of Otago, PO Box 56, Dunedin 9054, New Zealand

[2] Discipline of Nutrition and Metabolism, School of Molecular Bioscience, University of Sydney, Sydney, NSW 2006, Australia

[3] Discipline of Molecular Biology, School of Molecular Bioscience, University of Sydney, Sydney, NSW 2006, Australia

[4] Department of Statistics, Macquarie University, Sydney, NSW 2109, Australia

suggests that zinc ions participate in cellular signalling pathways as second messengers, for example propagating signals in the insulin and nitric oxide signalling pathways within the cell (Foster and Samman 2010). The involvement of zinc in cellular signalling can be categorised into early or late zinc signalling, with the latter primarily dependent on transcriptional changes in zinc transporter expressions (Fukada et al. 2011).

Recent advancements in molecular techniques have enabled the use of novel approaches in the study of zinc metabolism and homeostasis, especially within the cell. The cytoplasmic zinc concentration is regulated largely by two families of zinc transporters: the zinc transporter (ZnT; SLC30) and Zrt- and Irt-like protein (ZIP; SLC39) families (Lichten and Cousins 2009). Members from the ZIP family of transporters are responsible for increasing cytoplasmic zinc concentration by transporting zinc from intracellular organelles or the extracellular space, while ZnT proteins function to decrease cytoplasmic zinc by promoting cellular zinc efflux or sequestering zinc in subcellular compartments. Another key group of proteins involved in the regulation of cellular zinc homeostasis is MT. Within the cell, MT serves as a chaperone of zinc ions, mediating the movement and availability of zinc to other zinc-binding proteins. The chemical structure of MT, which contains seven zinc-binding sites with a range of affinities to zinc, complements the function of MT in buffering and shuttling zinc within the cytoplasm (Colvin et al. 2010).

A number of studies have explored the effect of zinc supplementation or depletion on the regulation of selected zinc transporters and MT in humans. The results predominately are consistent in showing changes at the gene expression level within blood cells in response to zinc depletion or supplementation; for instance, MT and ZnT1 expressions decreased significantly with a virtually zinc-free diet (0.3 mg/day) (Ryu et al. 2011) and increased during zinc supplementation (Aydemir et al. 2006; Ryu et al. 2011). Limited available evidence explores simultaneous changes in multiple zinc transporter and MT expressions during zinc supplementation in humans. The lack of a defined metabolic zinc store, in addition to the ubiquitous nature of zinc, complicates the assessment of zinc status in humans (King 2011). Multiple factors, such as oxidative stress, serum glucose concentration, and inflammatory cytokines, have been associated with the regulation of zinc transporters and MT in different cell types (Lichten and Cousins 2009; Foster and Samman 2012; Hennigar and Kelleher 2012), which may confound the delineation of the relationship between zinc status and the expression of zinc transporter and MT genes. The feasibility and usefulness of a range of zinc transporter and MT gene expressions to detect modest changes in dietary and/or supplemental zinc intake remain unclear.

The aim of the present study is to determine the effects of zinc supplementation on the gene expression of zinc transporters and MT. Furthermore, this study aims to explore the relationships between lifestyle characteristics, such as habitual physical activity levels, and expression of zinc transporter and MT genes to further understand cellular zinc homeostasis in the context of whole body metabolism.

Methods

Study design

Healthy adults, aged between 18 and 65 years, were recruited to participate in the present pilot study via advertising around the University of Sydney campus. Exclusion criteria were: diagnosis of chronic illness; use of prescription drugs (excluding oral contraceptive agents); use of micronutrient supplementation in the preceding 6 weeks; and women who were pregnant or breastfeeding. A screening questionnaire was used to determine eligibility and collect anthropometric and lifestyle data, such as height, weight, change in weight over the preceding 3 months, alcohol consumption, and smoking habits. Physical activity levels of the participants were assessed using the self-administered International Physical Activity Questionnaire (IPAQ)—short form (Craig et al. 2003) prior to study participation. Information gathered from the IPAQ was analysed according to the Guidelines for Data Processing and Analysis of the International Physical Activity Questionnaire (IPAQ Research Committee 2005).

Enrolled participants ($n = 39$; 18 females and 21 males) were randomised to zinc treatment (ZT group, $n = 19$) or no treatment (NT group, $n = 20$) by a computer-generated random-number sequence. The ZT group received an oral dose of 22 mg/day elemental zinc chelated with amino acid (Nature's Own, Sanofi-Aventis Healthcare Pty Ltd, Australia) for 21 days. The supplemental dose of 22 mg/day elemental zinc was chosen to provide a substantial increase in total zinc intake while minimising the risk of exceeding the upper daily limit (National Health and Medical Research Council 2006). Participants were advised to report any adverse effects that they experienced during the trial. During the final week of the study, height (to the nearest 0.1 cm) was measured with a stadiometer and body weight (to the nearest 100 g) was measured on an electronic calibrated scale. A survey was conducted at Day 21 to confirm that no nutritional supplements (other than the provided supplements) were consumed during the study period. Apparent compliance with the intervention was verified by counting of returned tablets at the end of the study. All eligible participants provided their written

informed consent to participate in the study. The Human Research Ethics Committee of the University of Sydney approved the study protocol, and the trial was registered at www.anzctr.org.au (ACTRN12613001200774).

Blood collection

Venous blood samples were collected from participants on Days 0 (baseline), 2, 7, 14, and 21. At each time point, blood was collected between 0700 and 0900 after an overnight fast of at least 10 h. Cell preparation tubes (CPT) with sodium citrate (Becton–Dickinson, Franklin Lakes, NJ) were used for the isolation of peripheral blood mononuclear cells (PBMC). Serum gel tubes (Becton–Dickinson) were used for C-reactive protein (CRP) analysis. EDTA tubes (Becton–Dickinson) were used for analysis of plasma zinc. Within two hours of blood collection, plasma and serum were separated from whole blood after centrifugation at 4 °C for 10 min at 1200×*g*. Plasma and serum samples were stored at −80 °C until analysis.

Biochemical analyses

Plasma zinc concentrations were determined by flame atomic absorption spectroscopy (SpectrAA220, Varian, Palo Alto, CA) in a single-batch run. Samples were diluted (1:4) with Milli-Q water and measured against a matrix-matched standard curve in triplicates, over five seconds of absorbance integration. Appropriate quality control samples were prepared and analysed throughout the run.

Serum CRP concentrations were analysed using an immunoturbidimetric assay kit (Cardiac CRP (Latex) High Sensitive Kit, Roche Diagnostics, Basel, Switzerland), adapted for the Cobas C311 auto-analyser (Roche Diagnostics). Quality control serum (Precinorm Protein, Roche Diagnostics) was analysed throughout a single-batch run.

Total RNA extraction and cDNA synthesis

PBMC were isolated from CPT after centrifugation at room temperature for 30 min at 1500×*g* within 2 h of blood collection. Extracted cells were washed twice with Dulbecco's PBS (Sigma-Aldrich, St. Louis, MO). Total RNA was prepared using the PARIS kit (Ambion, Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. The purity and yield of isolated RNA samples were checked using UV nanospectrophotometry, with all samples generating A260:A280 and A260:A230 ratios within the range of 1.7–2.1. The integrity of ribosomal RNA was checked by denaturing agarose gel electrophoresis (1 % agarose containing 2.2 M formaldehyde) (Lehrach et al. 1977). Total RNA was reverse transcribed into cDNA using the SuperScript VILO cDNA synthesis kit (Invitrogen, Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. cDNA samples were stored at −80 °C until real-time PCR analysis.

Quantitative real-time PCR

Relative quantification of zinc transporter and MT mRNA was conducted using TaqMan real-time PCR (StepOnePlus Real-Time PCR System, Applied Biosystems, Life Technologies, Carlsbad, CA), as per the manufacturer's instructions. Inventoried TaqMan gene expression assays were obtained for *ZnT1*, *ZnT5*, *ZnT6*, *ZnT7*, *ZIP1*, *ZIP3*, *ZIP7*, *ZIP8*, *ZIP10*, *ZIP14*, *MT-1A*, *MT-2A*, and *18S*. The efficiency in amplification of the primers was determined using the standard curve method, with all primers generating efficiencies in the range of 85–95 %. The chosen zinc transporter and MT transcripts represent key mediators of cellular zinc homeostasis and transport in PBMC. All mRNA expression levels were normalised to *18S* rRNA expression as an endogenous reference and quantified using the ΔC_P method; fold change relative to baseline was quantified using the $\Delta\Delta C_P$ method.

Dietary intake analysis

Participants maintained a 3-day estimated food record (two weekdays and one weekend day) during the second week of the study. Participants were instructed to record all food and drinks consumed during the day with portions estimated using the food record templates provided. A research dietitian checked each completed food record with the participant to clarify any missing items or unusual portions. Food records were analysed (FoodWorks 7 Professional Edition 2012, Xyris Software, QLD, Australia) for the determination of energy, macro- and micronutrient intakes.

Statistical methods

Descriptive data were expressed as mean ± SD, and outcome measures were described as mean ± SEM. Differences between groups at baseline were analysed using independent *t*-tests. The primary outcomes, changes in gene expression and fold change (relative to baseline) between groups, were assessed by repeated-measures ANOVA for complete sets of data. Age, BMI, and physical activity were explored as covariates of the models used. Normality, sphericity, and residuals were checked for compliance with model assumptions. Greenhouse-Geisser corrections were used when Mauchly's sphericity test returned $P < 0.05$.

Factor analysis using principal components was used to identify 'clusters' of zinc transporter gene expression at

baseline. Multiple regression models were used to investigate whether the difference in *MT-2A* gene expression over the trial was determined by changes in the expression of zinc transporter genes, grouped either as 'all genes measured' or within the identified 'clusters'. Similarly, changes in measured zinc transporters and MT were used to predict the change in plasma zinc concentration. Multivariate ANOVA models were used to analyse whether dietary zinc intake and different components of physical activity levels (vigorous, moderate, and walking physical activity) determined the expression of zinc transporter and MT genes at baseline. Bivariate correlations were used to explore the possible relationships between physical activity levels, plasma zinc concentration, and zinc transporter and MT gene expressions at baseline. In primary analyses, i.e. comparisons between groups, $P < 0.05$ was used to denote statistical significance. In 'secondary' analyses, $P < 0.01$ was used to denote statistical significance with $P < 0.05$ being marginally significant due to the large number of statistical tests performed. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 21.0 (Armonk, NY: IBM Corp).

Results

Of the 153 enquiries received, 66 volunteers returned screening questionnaires and were assessed for eligibility to participate in the study. Twelve volunteers did not meet the inclusion criteria, 13 declined to participate, and two others withdrew their interest due to work commitments. Thirty-nine healthy participants (21 males and 18 females) were enrolled in the study and randomised into either the NT group ($n = 20$) or the ZT group ($n = 19$). Supplemental Figure 1 shows the trial profile.

Thirty-four participants completed the study, with five participants lost to follow-up at either Day 2 or 14. Two participants who completed the trial each had one missing time point (Day 0 and 7, respectively) due to unexpected circumstances. Given that the reasons for missing data and withdrawal were unrelated to the trial, only complete data were included for the analysis of primary outcomes. A high apparent compliance rate of 97 % was found in the ZT group. All participants reported that no nutritional supplements (other than the provided supplements) were consumed during the trial. The age and BMI of the participants who completed the trial were 26.5 ± 11.7 years (mean ± SD) and 22.5 ± 2.6 kg/m^2, respectively. There were no significant differences in the baseline levels of physical activity, biochemical measurements, or nutrient intakes between the two groups (Table 1).

Plasma Zinc and Serum CRP

At baseline, the mean plasma zinc concentration for both groups were within the reference range (10–18 μmol/L) (Brown et al. 2004) and were not significantly different between groups. Using repeated-measures ANOVA to determine the change in plasma zinc concentration over the course of the trial, no significant differences were found between the two groups ($P = 0.21$, Supplemental Figure 2). In the ZT group, plasma zinc concentration increased by 0.98 ± 0.67 μmol/L (mean ± SEM) at Day 21; however, this was not significantly higher than the baseline value ($P = 0.32$).

No differences were observed in serum CRP between the two groups at baseline ($P = 0.21$) and over the course of the trial ($P = 0.21$). Serum CRP concentrations for all participants were below 10 mg/L, suggesting the absence of acute inflammation (Myers et al. 2004).

Table 1 Selected baseline characteristics and dietary intakes (data shown as mean ± SD) of participants who completed the trial

	NT group ($n = 18$)	ZT group ($n = 16$)
Age (year)	25.8 ± 12.5	27.3 ± 10.9
BMI (kg/m^2)	22.8 ± 2.7	22.2 ± 2.6
Plasma zinc (μmol/L)	17.0 ± 3.1	16.0 ± 3.5
Serum CRP (mg/L)	1.2 ± 0.8	1.8 ± 1.8
Total physical activity (MET-minutes/week)	4159 ± 2982	2403 ± 1583
Weekday sitting time (min/day)	472 ± 148	420 ± 210
Energy (kJ/day)	10,904 ± 2934	10,700 ± 4095
Carbohydrate (% of energy)	46.1 ± 9.3	38.2 ± 10.7
Protein (% of energy)	20.3 ± 5.9	17.9 ± 4.1
Total fat (% of energy)	28.8 ± 5.8	36.6 ± 9.3
Dietary fibre (g/day)	30.2 ± 11.5	27.4 ± 12.1
Dietary zinc (mg/day)	15.6 ± 7.3	13.9 ± 7.4

NT no treatment, *ZT* zinc treatment

Zinc transporter and metallothionein gene expressions

Gene expressions of *MT-2A* were higher in the ZT group compared to the NT group ($P = 0.025$), as determined by repeated-measures ANOVA. When the data were expressed as fold change relative to baseline, fold changes of *MT-2A* were higher in the ZT group ($P = 0.016$; Fig. 1), specifically at Day 2 ($P = 0.011$). In the ZT group, the fold change of *MT-2A* was 1.40 ± 0.18 at Day 2, indicating an increase of 40 ± 18 % in the relative copies of *MT-2A* mRNA from baseline. The addition of age, BMI, and physical activity as covariates within the repeated-measures ANOVA models did not alter the overall results.

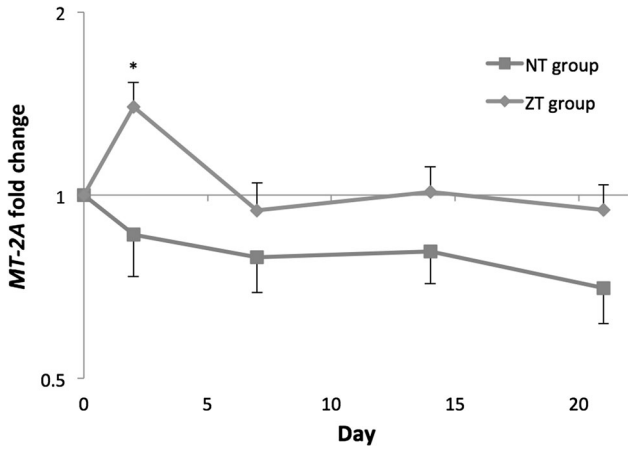

Fig. 1 *MT-2A* fold change in NT (no treatment, $n = 17$) and ZT (zinc treatment, $n = 15$) groups. Data expressed as estimated marginal mean ± SEM from repeated-measures ANOVA. $P = 0.016$ between groups, $P = 0.011$ at Day 2

Zinc supplementation did not change the gene expression (Supplemental Table 1) or fold change of zinc transporters or *MT-1A*. Of the measured ZnTs in this study, *ZnT7* was the most highly expressed zinc transporter at baseline as judged by relative C_p values, expressing at more than twofold higher than the other cytoplasmic zinc exporters. *ZIP1* and *ZIP3* had the highest expression of the measured ZIPs, while *ZIP14* had the lowest expression (Supplemental Figure 3). No significant differences were found in the gene expression of zinc transporters and MT at baseline between the two groups.

Relationships between zinc transporters and MT

Relationships between zinc transporter and MT gene expressions at baseline are shown in Table 2. Significant relationships were observed among all transporters in the ZnT family ($P < 0.01$). *ZIP7* and *ZIP10* had the largest number of significant associations, each with five significant positive relationships with other zinc transporters and MT ($P < 0.01$). Conversely, *ZIP8* gene expression was not related to any of the other measured zinc transporters or MT.

Factor analysis using principal components revealed two 'clusters' of zinc transporters in which the expression of zinc transporters within each cluster was related to each other at baseline. The clusters identified were: Cluster 1—*ZIP1, ZIP3, ZIP7, ZIP10*, and *ZIP14*; Cluster 2—*ZnT1, ZnT5, ZnT6, ZnT7*, and *ZIP8*. When multiple regression models were used to explore the factors determining the change in *MT-2A* gene expression between Days 0 and 21, changes in all measured zinc transporters were related to the change in *MT-2A* expression ($r^2 = 0.54$, $P = 0.029$; Table 3). Similarly, changes in the gene expression of zinc transporters within Cluster 1 determined the change in the

Table 2 Pearson correlation coefficients (r) between zinc transporters and metallothionein for all participants at baseline ($n = 38$)

	ZnT5	*ZnT6*	*ZnT7*	*ZIP1*	*ZIP3*	*ZIP7*	*ZIP8*	*ZIP10*	*ZIP14*	*MT-1A*	*MT-2A*
ZnT1	0.64***	0.60***	0.46**	0.32	0.33*	0.30	0.10	0.27	0.25	0.07	0.37*
ZnT5		0.64***	0.62***	0.17	0.33*	0.20	0.22	0.36*	0.30	0.09	0.11
ZnT6			0.65***	−0.03	0.24	−0.03	0.26	0.19	0.04	0.09	0.05
ZnT7				0.03	0.33*	0.08	0.26	0.27	0.15	0.23	0.12
ZIP1					0.64***	0.76***	0.09	0.41*	0.64***	−0.01	0.55***
ZIP3						0.52**	0.22	0.46**	0.50**	−0.07	0.21
ZIP7							−0.04	0.54***	0.52**	0.10	0.46**
ZIP8								0.15	0.20	0.20	0.14
ZIP10									0.68***	0.48**	0.44**
ZIP14										0.11	0.47**
MT-1A											0.35*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3 Multiple regression models using changes in all or 'clusters' of zinc transporters identified by principal component analysis to predict the change in *MT-2A* gene expression over the study period

Dependent variable	Factors	r^2	Overall *P* value	Univariate component	Standardised coefficient (β) ± standard error	Univariate *P* value
Δ *MT-2A*	Δ All ZnTs and ZIPs[a]	0.542	0.029	*ZIP1*	0.89 ± 0.26	0.003
	Δ Cluster 1[b]	0.477	0.003	*ZIP1*	0.77 ± 0.22	0.002
	Δ Cluster 2[c]	0.073	NS	–	–	–

Based on complete data available for analysis ($n = 33$)

[a] *ZnT1, ZnT5, ZnT6, ZnT7, ZIP1, ZIP3, ZIP7, ZIP8, ZIP10*, and *ZIP14*

[b] *ZIP1, ZIP3, ZIP7, ZIP10*, and *ZIP14*

[c] *ZnT1, ZnT5, ZnT6, ZnT7*, and *ZIP8*

expression of *MT-2A* gene ($r^2 = 0.48$, $P = 0.003$). In both models, *ZIP1* gene expression emerged as a significant univariate predictor of the change in *MT-2A* expression ($P < 0.01$).

Relationships between plasma zinc concentration, dietary zinc intake, and gene expression of zinc transporters and MT

In multivariate analysis, dietary zinc intake was a significant predictor of all measured zinc transporter and MT gene expressions at baseline ($P = 0.03$, Supplemental Table 2), explaining 61 % of variance in gene expressions; *MT-1A* expression emerged as a significant univariate factor within the multivariate ANOVA model ($P = 0.002$), while *ZIP10* reached marginal statistical significance ($P = 0.045$). At baseline, plasma zinc concentration was not correlated with any of the measured zinc transporter or MT gene expressions. Changes in the expression of zinc transporter and MT genes did not predict the change in plasma zinc concentration over the trial period.

Relationships between physical activity levels and zinc transporter and MT gene expressions

Weekday sitting time was not correlated with any of the measured zinc transporters or MT. When components of physical activity (vigorous, moderate, or walking) were used to predict the gene expression of all measured zinc transporters and MT, no significant relationships were found (data not shown). Total physical activity level was correlated with *ZIP7* gene expression at baseline (Fig. 2, $r = 0.36$, $P = 0.029$). One participant reported a total physical activity level of 13,914 MET-minutes/week, which was >3 SD above the group mean and therefore was considered to be an outlier. The positive relationship between total physical activity and *ZIP7* was strengthened after removal of the outlier within the data set ($r = 0.43$, $P = 0.009$).

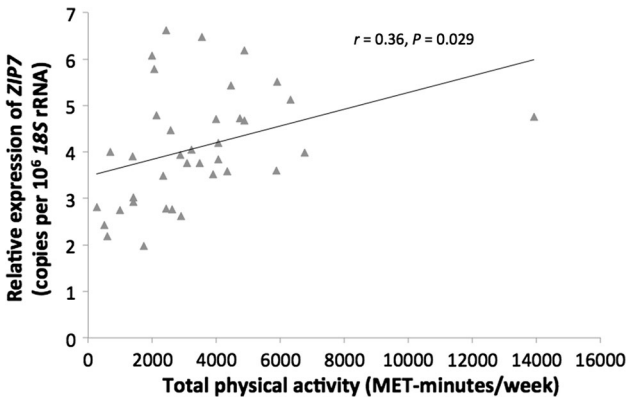

Fig. 2 Bivariate correlation between total physical activity (MET-minutes/week) and *ZIP7* gene expression at baseline for all participants ($n = 38$). The positive relationship remained after removal of the outlier within the data set ($r = 0.43$; $P = 0.009$)

Discussion

The present study explored the effects of zinc supplementation on the expression of zinc transporter and MT genes in PBMC of healthy adults. The primary finding is a significant upregulation of *MT-2A* gene expression within two days of zinc supplementation, in the absence of significant change in plasma zinc concentration. Our previous report suggests coordination among components of cellular zinc homeostasis (Foster et al. 2011), and the current findings extend these observations by demonstrating numerous relationships between the expression of zinc transporter and MT genes at baseline and during zinc supplementation. Specifically, the use of multiple regression models showed that the difference in *MT-2A* gene expression over the trial period was determined by changes in the expression of zinc transporter genes. In addition, to the best of our knowledge, this is the first study to report a relationship between habitual physical activity level and zinc transporter gene expression in humans.

MT represents a large group of metal-binding proteins, with at least 10 functional MT isoforms identified in humans (Blindauer 2014). While MT-2A responds to many physiological stresses, such as hormones and thiol oxidation, the principal role of MT-2A relates to the maintenance of cellular zinc homeostasis. The primary observation of an increase in *MT-2A* gene expression during zinc supplementation is supported by previous studies of similar duration as the current report. In a study that utilised a high dose of zinc supplementation (50 mg/day elemental zinc), a significant increase in MT mRNA was observed in monocytes throughout the intervention period, with a return to baseline level within 4 days of supplement cessation (Sullivan et al. 1998). A lower dose of elemental zinc (15 mg/day) with a shorter duration (10 days) elicited similar responses in MT gene expression within PBMC and subsets of leucocytes (Cao and Cousins 2000; Aydemir et al. 2006). Moreover, *MT-2A* gene expression is responsive to suboptimal dietary zinc intake. After 10 weeks of 4.6 mg of dietary zinc intake/day, *MT-2A* mRNA in T lymphocytes decreased significantly, before returning to the baseline level after 5 weeks of zinc repletion (Allan et al. 2000). While some of the earlier studies did not distinguish between the numerous isoforms of MT that are present in humans which present methodological differences in the present literature, the results are consistent in showing the responsiveness of MT transcription to changes in total zinc intake.

The mechanisms that regulate MT transcription in cells have been explored extensively using in vitro models (Vandeghinste et al. 2000; Jackson et al. 2009). Upregulation of MT transcription can be induced by the binding of activated metal-regulatory transcription factor-1 (MTF-1) to the metal-responsive region of the MT gene (Günther et al. 2012). Recently, a novel transcription factor, CCAAT/enhancer binding protein-α (C/EBPα), was found to be important in the induction of MT expression, both independently and synergistically with MTF-1 (Datta et al. 2007). Activation of C/EBPα by phosphorylation can be mediated through the phosphoinositide 3-kinase/Akt pathway, one of the many signalling pathways in which zinc plays a regulatory role as a second messenger (Foster and Samman 2010). In the current study, using multiple regression models that explored changes over the study period, the difference in *MT-2A* gene expression was determined by changes in the expression of zinc transporter genes, both when measured collectively and when grouped according to the 'clusters' identified from factor analysis using principal components. In particular, the present study shows that the change in *ZIP1* gene expression was a strong independent predictor of the change in *MT-2A* expression. *ZIP1* mRNA was abundantly expressed and is a major zinc importer at the plasma membrane. The relationship between *ZIP1* and *MT-2A* mRNA affirms the role of MT in maintaining cellular zinc homeostasis in response to changes in the levels of exogenous zinc; the potential cellular mechanisms that link ZIP1 with MT gene expression are shown in Fig. 3.

The lack of response in plasma zinc concentration during zinc supplementation is consistent with the modest effect of dietary zinc intake on systemic zinc concentration reported in a recent meta-analysis of randomised controlled trials (Lowe et al. 2012). The duration and dose of zinc supplement provided in the current study may be insufficient to elicit an increase in plasma zinc concentration as identified in other studies, particularly with the added limitation of a small sample size. In contrast to plasma zinc concentrations, *MT-2A* gene expressions in PBMC are more reflective of immediate changes in dietary zinc intake. The transient increase in *MT-2A* gene expression during zinc supplementation is intriguing and can be explained by the rigid regulation of zinc metabolism. The maintenance of whole-body zinc homeostasis is mediated primarily through the balance of endogenous zinc secretion and absorption within the gastrointestinal tract, which reflects immediate changes in dietary zinc intake (Chung et al. 2008). In a study which utilised zinc stable isotopes, six consecutive days of 20 mg/day zinc supplementation reduced fractional zinc absorption in the gut by 50 % (Tran et al. 2004), suggesting rapid physiological adaptation to increased dietary zinc intake. We propose that the *MT-2A* gene expression profile seen in the current study, comprising a rapid increase and subsequent return to baseline level by Day 7, is reflective of a reduction in systemic uptake of zinc in the gut in response to consecutive days of zinc supplementation.

The gene expression analysis in the present study is limited to 10 of the 24 zinc transporters identified in humans to date. Data on the expression of other zinc transporters may provide further details on the fluctuations in intracellular zinc concentration during zinc supplementation. For instance, in HEK-293 and MCF-7 cells, *ZnT2* gene expression has been shown to be modulated by numerous suppressive and active regulators, including zinc-induced activation of MTF-1 (Lu et al. 2015). In fibroblast cells, the upregulation of ZnT2 expression and its subsequent function of sequestering zinc within lysosomes are proposed to be one of the mechanisms in which cells maintain zinc homeostasis during excess zinc exposure (Falcón-Pérez and Dell'Angelica 2007). Moreover, gene transcription represents one of the many points of regulation in the ultimate protein function. It is plausible that zinc supplementation may modulate post-transcriptional modifications of zinc transporters and MT, impacting the localisation and function of these proteins. Further investigations into the role of zinc transporters and MT in maintaining cellular zinc homeostasis would benefit from proteomic and intracellular zinc analyses.

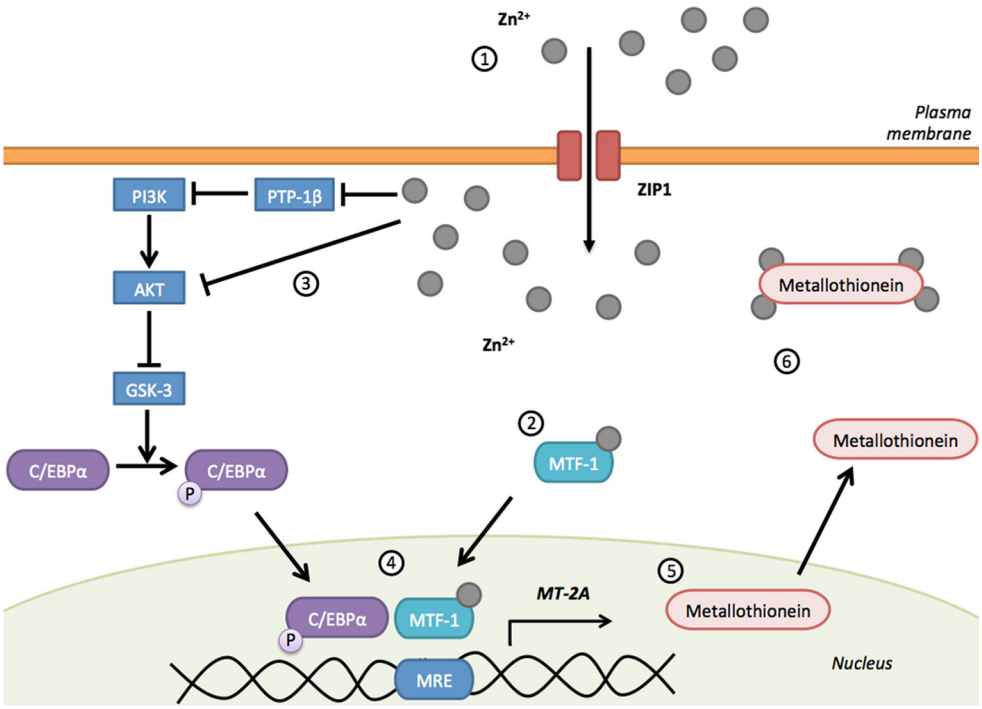

Fig. 3 Schematic showing hypothesised regulation of *MT-2A* gene expression by zinc.(*1*) Increased extracellular zinc is transported into the cell by the cellular zinc importer, ZIP1. (*2*) Higher concentration of intracellular zinc can activate transcription factor, MTF-1. (*3*) Zinc ions also can indirectly modulate the coactivator, C/EBPα, through the PI3K–Akt signalling pathway. (*4*) Activated MTF-1 and C/EBPα can translocate into the nucleus, binding to the promoter region of *MT-2A* gene, upregulating gene transcription. (*5*) Upregulation of gene expression can eventuate in increased MT-2A protein. (*6*) Higher concentration of MT serves to sequester intracellular zinc, thereby creating a negative feedback signal within the cell. *PTP-1* protein tyrosine phosphatase-1β, *PI3K* phosphoinositide 3-kinase, *GSK-3* glycogen synthase kinase-3, *C/EBPα* CCAAT/enhancer binding protein-α, *MTF-1* metal-regulatory transcription factor-1, *MRE* metal-responsive element

Although the inclusion of age as a covariate in the statistical models did not alter the overall results of the present study, age is a significant factor in maintaining immune and antioxidative functions of zinc (Mocchegiani et al. 2014). A recent trial in an elderly population with low zinc status (plasma zinc concentration <11.77 μmol/L) reported that zinc supplementation upregulates the gene expressions of *ZIP1* and *MT-1A* (Sharif et al. 2015). Discrepancies between the results reported by Sharif et al. (2015) and those of the present study may be related to differences in age, zinc status, and other baseline characteristics of the sampled populations. In addition, cellular uptake of zinc in response to zinc supplementation is influenced by polymorphisms in zinc transporter and MT genes. A study in an elderly population reported that participants with the G+ allele in the +1245 A/G MT1A gene polymorphism exhibited higher levels of intracellular labile zinc after zinc supplementation than those with the G− allele (Giacconi et al. 2014). Thus, the potential moderating effects of age, zinc status, and genetic polymorphisms on the gene expression of zinc transporters and MT in response to zinc supplementation and implications for oxidative stress warrant further research.

Recent studies have shown relationships between zinc transporters and skeletal muscles in the context of exercise. Variants of single nucleotide polymorphism in *ZnT8* gene have been associated with differences in muscle phenotype and function in men (Sprouse et al. 2014). The positive relationship between *ZIP7* gene expression and total physical activity level found in the current study may be reflective of the function of this particular zinc transporter in skeletal muscles. ZIP7 transporter resides on the endoplasmic reticulum of the cell and is responsible for transient influx of zinc into the cytosol in response to cellular signals (Taylor et al. 2012). The function of ZIP7 to elicit a rapid increase in cytosolic zinc is related to the propagation of cellular signalling pathways, specifically those reliant on the phosphorylation of Akt and extracellular signal-regulated kinases 1/2 (ERK-1/2). A recent study, which used siRNA to silence ZIP7 expression in a skeletal muscle cell line, found that both basal and insulin-stimulated glucose metabolism are compromised as a result of reduced ZIP7 expression (Myers et al. 2013). Due to the crucial role of ZIP7 in glycolysis, the authors suggest that this zinc transporter may present as a molecular target in the treatment of insulin resistance within skeletal muscles. This

supposition is supported by the action of zinc ions in the insulin signalling pathway (Jansen et al. 2009) and the role of mitochondrial MT–Zn complex in regulating cellular energy metabolism (Ye et al. 2001).

The current study presents the influence of physical activity and zinc supplementation on the expression of *ZIP7* and *MT-2A* genes, respectively. The modulations in the mediators of cellular zinc homeostasis by external stimuli, such as exercise and dietary zinc intake, may have implications for zinc-mediated intracellular signalling pathways and related cellular functions. MT-2A participates in 'zinc-muffling' reactions (Maret 2011), which involve the binding of excess zinc ions and delivering them to different subcellular compartments. Zinc-induced upregulation of MT protein may attenuate the spatial and temporal characteristics of transient increases in intracellular zinc concentration (Colvin et al. 2010), which impact zinc-mediated cellular signalling pathways, such as those activated by insulin. We have shown previously in PBMC that cytokine production, an important function of immune cells, is determined by zinc transporter and MT expressions (Chu et al. 2015), possibly via the modulation of ERK-1/2 and JAK/STAT pathways. The interactions between zinc homeostasis, intracellular signalling pathways, and cellular function warrant further investigation.

A number of limitations should be considered in the interpretation of this study, in particular the small sample size and the use of EDTA plasma tubes and CPT with sodium citrate for the analysis of plasma zinc and PBMC extraction, respectively. However, all precautions were taken to avoid zinc contamination in the collection and subsequent processing of the blood samples. Furthermore, the current study lacked the analysis of PBMC subtype populations which may influence the gene expression of zinc transporters and MT. PBMC represent a cohort of immune cells that can be modified by lifestyle factors, for example exercise (Deuster et al. 1988) and zinc status (Beck et al. 1997; Uciechowski et al. 2008). Whether changes in the PBMC transcriptome as a result of zinc supplementation derive from differences in the composition of the PBMC subtypes or transcriptional changes in a stable cohort of PBMC is currently unclear.

The current study demonstrates a transient increase in *MT-2A* gene expression during zinc supplementation in healthy adults. The difference in *MT-2A* gene expression over the trial period was correlated with the change in *ZIP1* expression, suggesting coordination in the maintenance of cellular zinc homeostasis. The positive correlation between physical activity level and *ZIP7* gene expression suggests an influence of lifestyle and biological factors on the mediators of cellular zinc homeostasis. Given the complex biological functions in which zinc is involved, the understanding of multiple variables, including expression of zinc transporter and MT genes, may be required to predict zinc status at the individual level.

Acknowledgments Financial support was obtained from Meat and Livestock Australia. The funding body did not play any role in designing the study; in the collection, analysis, and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication.

Compliance with ethical standards

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Human Research Ethics Committee of the University of Sydney) and with the Declaration of Helsinki 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Conflict of interest Anna Chu, Meika Foster, Sarah Ward, Kamrul Zaman, Dale Hancock, Peter Petocz, and Samir Samman declare that they have no conflict of interest.

References

Allan AK, Hawksworth GM, Woodhouse LR et al (2000) Lymphocyte metallothionein mRNA responds to marginal zinc intake in human volunteers. Br J Nutr 84:747–756

Aydemir TB, Blanchard RK, Cousins RJ (2006) Zinc supplementation of young men alters metallothionein, zinc transporter, and cytokine gene expression in leukocyte populations. Proc Natl Acad Sci USA 103:1699–1704. doi:10.1073/pnas.0510407103

Beck FW, Prasad AS, Kaplan J et al (1997) Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. Am J Physiol 272:E1002–E1007

Blindauer CA (2014) Metallothioneins. In: Maret W, Wedd A (eds) Binding, transport and storage of metal ions in biological cells. Royal Society of Chemistry, Cambridge, pp 606–665

Brown KH, Rivera JA, Bhutta Z et al (2004) International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. Food Nutr Bull 25:S94–S203

Cao J, Cousins R (2000) Metallothionein mRNA in monocytes and peripheral blood mononuclear cells and in cells from dried blood spots increases after zinc supplementation of men. J Nutr 130:2180–2187

Chu A, Foster M, Hancock D et al (2015) TNF-α gene expression is increased following zinc supplementation in type 2 diabetes mellitus. Genes Nutr 10:440. doi:10.1007/s12263-014-0440-4

Chung C, Stookey J, Dare D (2008) Current dietary zinc intake has a greater effect on fractional zinc absorption than does longer term zinc consumption in healthy adult men. Am J Clin Nutr 87:1224–1229

Colvin RA, Holmes WR, Fontaine CP, Maret W (2010) Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis. Metallomics 2:306–317. doi:10.1039/b926662c

Craig CL, Marshall AL, Sjöström M et al (2003) International physical activity questionnaire: 12-country reliability and validity. Med Sci Sports Exerc 35:1381–1395. doi:10.1249/01.MSS.0000078924.61453.FB

Datta J, Majumder S, Kutay H et al (2007) Metallothionein expression is suppressed in primary human hepatocellular carcinomas and is mediated through inactivation of CCAAT/

enhancer binding protein alpha by phosphatidylinositol 3-kinase signaling cascade. Cancer Res 67:2736–2746. doi:10.1158/0008-5472.CAN-06-4433

Deuster PA, Curiale AM, Cowan ML, Finkelman FD (1988) Exercise-induced changes in populations of peripheral blood mononuclear cells. Med Sci Sports Exerc 20:276–280

Falcón-Pérez JM, Dell'Angelica EC (2007) Zinc transporter 2 (SLC30A2) can suppress the vesicular zinc defect of adaptor protein 3-depleted fibroblasts by promoting zinc accumulation in lysosomes. Exp Cell Res 313:1473–1483. doi:10.1016/j.yexcr.2007.02.006

Fischer Walker CL, Ezzati M, Black RE (2009) Global and regional child mortality and burden of disease attributable to zinc deficiency. Eur J Clin Nutr 63:591–597. doi:10.1038/ejcn.2008.9

Foster M, Samman S (2010) Zinc and redox signaling: perturbations associated with cardiovascular disease and diabetes mellitus. Antioxid Redox Signal 13:1549–1573

Foster M, Samman S (2012) Zinc and regulation of inflammatory cytokines: implications for cardiometabolic disease. Nutrients 4:676–694. doi:10.3390/nu4070676

Foster M, Hancock D, Petocz P, Samman S (2011) Zinc transporter genes are coordinately expressed in men and women independently of dietary or plasma zinc. J Nutr 141:1195–1201. doi:10.3945/jn.111.140053

Fukada T, Yamasaki S, Nishida K et al (2011) Zinc homeostasis and signaling in health and diseases: zinc signaling. J Biol Inorg Chem 16:1123–1134. doi:10.1007/s00775-011-0797-4

Giacconi R, Simm A, Santos AN et al (2014) Influence of +1245 A/G MT1A polymorphism on advanced glycation end-products (AGEs) in elderly: effect of zinc supplementation. Genes Nutr 9:426. doi:10.1007/s12263-014-0426-2

Günther V, Lindert U, Schaffner W (2012) The taste of heavy metals: gene regulation by MTF-1. Biochim Biophys Acta 1823:1416–1425. doi:10.1016/j.bbamcr.2012.01.005

Hennigar SR, Kelleher SL (2012) Zinc networks: the cell-specific compartmentalization of zinc for specialized functions. Biol Chem 393:565–578. doi:10.1515/hsz-2012-0128

IPAQ Research Committee (2005) Guidelines for data processing and analysis of the international physical activity questionnaire (IPAQ)—Short and Long Forms

Jackson KA, Valentine RA, McKay JA et al (2009) Analysis of differential gene-regulatory responses to zinc in human intestinal and placental cell lines. Br J Nutr 101:1474–1483. doi:10.1017/S0007114508094634

Jansen J, Karges W, Rink L (2009) Zinc and diabetes—clinical links and molecular mechanisms. J Nutr Biochem 20:399–417. doi:10.1016/j.jnutbio.2009.01.009

King J (2011) Zinc: an essential but elusive nutrient. Am J Clin Nutr 94:679–684. doi:10.3945/ajcn.110.005744.2

Lehrach H, Diamond D, Wozney JM, Boedtker H (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry 16:4743–4751. doi:10.1021/bi00640a033

Lichten LA, Cousins RJ (2009) Mammalian zinc transporters: nutritional and physiologic regulation. Annu Rev Nutr 29:153–176. doi:10.1146/annurev-nutr-033009-083312

Lowe NM, Medina MW, Stammers A-L et al (2012) The relationship between zinc intake and serum/plasma zinc concentration in adults: a systematic review and dose-response meta-analysis by the EURRECA network. Br J Nutr 108:1962–1971. doi:10.1017/S0007114512004382

Lu Y, Liu Y, Lin M, et al (2015) Coordinative modulation of human zinc transporter 2 gene expression through active and suppressive regulators. J Nutr Biochem 26:351–359

Maret W (2003) Cellular zinc and redox states converge in the metallothionein/thionein pair. J Nutr 133:1460S–1462S

Maret W (2011) Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. Biometals 24:411–418. doi:10.1007/s10534-010-9406-1

Mocchegiani E, Costarelli L, Giacconi R et al (2014) Micronutrient-gene interactions related to inflammatory/immune response and antioxidant activity in ageing and inflammation. A systematic review. Mech Ageing Dev 136–137:29–49. doi:10.1016/j.mad.2013.12.007

Myers GL, Rifai N, Tracy RP et al (2004) CDC/AHA workshop on markers of inflammation and cardiovascular disease: application to clinical and public health practice: report from the laboratory science discussion group. Circulation 110:e545–e549. doi:10.1161/01.CIR.0000148980.87579.5E

Myers SA, Nield A, Chew G-S, Myers MA (2013) The zinc transporter, slc39a7 (Zip7) is implicated in glycaemic control in skeletal muscle cells. PLoS ONE 8:e79316. doi:10.1371/journal.pone.0079316

National Health and Medical Research Council (2006) Nutrient Reference Values for Australia and New Zealand including recommended dietary intakes

Oteiza PI (2012) Zinc and the modulation of redox homeostasis. Free Radic Biol Med 53:1748–1759. doi:10.1016/j.freeradbiomed.2012.08.568

Prasad A, Halsted J, Nadimi M (1961) Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism and geophagia. Am J Med 31:532–546

Ryu M, Langkamp-Henken B, Chang S-M et al (2011) Genomic analysis, cytokine expression, and microRNA profiling reveal biomarkers of human dietary zinc depletion and homeostasis. Proc Natl Acad Sci USA 108:20970–20975. doi:10.1073/pnas.1117207108

Samman S (2007) Zinc Nutr Diet 64:S131–S134. doi:10.1111/j.1747-0080.2007.00200.x

Sharif R, Thomas P, Zalewski P, Fenech M (2015) Zinc supplementation influences genomic stability biomarkers, antioxidant activity, and zinc transporter genes in an elderly Australian population with low zinc status. Mol Nutr Food Res 59:1200–1212. doi:10.1002/mnfr.201400784

Sprouse C, Gordish-Dressman H, Orkunoglu-Suer EF et al (2014) SLC30A8 nonsynonymous variant is associated with recovery following exercise and skeletal muscle size and strength. Diabetes 63:363–368. doi:10.2337/db13-1150

Sullivan VK, Burnett FR, Cousins RJ (1998) Metallothionein expression is increased in monocytes and erythrocytes of young men during zinc supplementation. J Nutr 128:707–713

Taylor KM, Hiscox S, Nicholson RI, et al (2012) Protein kinase CK2 triggers cytosolic zinc signaling pathways by phosphorylation of zinc channel ZIP7. Sci Signal 5:ra11. doi: 10.1126/scisignal.2002585

Tran C, Miller L, Krebs N (2004) Zinc absorption as a function of the dose of zinc sulfate in aqueous solution. Am J Clin Nutr 80:1570–1573

Uciechowski P, Kahmann L, Plümäkers B et al (2008) TH1 and TH2 cell polarization increases with aging and is modulated by zinc supplementation. Exp Gerontol 43:493–498. doi:10.1016/j.exger.2007.11.006

Vandeghinste N, Proost P, De Ley M (2000) Metallothionein isoform gene expression in zinc-treated human peripheral blood lymphocytes. Cell Mol Biol 46:419–433

Ye B, Maret W, Vallee BL (2001) Zinc metallothionein imported into liver mitochondria modulates respiration. Proc Natl Acad Sci USA 98:2317–2322. doi:10.1073/pnas.041619198