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Sensitivity and reliability of zinc transporter and metallothionein gene expression in peripheral blood mononuclear cells as indicators of zinc status: responses to *ex vivo* zinc exposure and habitual zinc intake in humans

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Abstract

Zn is an essential nutrient for humans; however, a sensitive biomarker to assess Zn status has not been identified. The objective of this study was to determine the reliability and sensitivity of Zn transporter and metallothionein (MT) genes in peripheral blood mononuclear cells (PBMCs) to Zn exposure *ex vivo* and to habitual Zn intake in human subjects. In study 1, human PBMCs were cultured for 24 h with 0–50 μM $ZnSO_4$ with or without 5 μM N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), and mRNA expression of *SLC30A1-10*, *SLC39A1-14*, *MT1* subtypes (*A*, *B*, *E*, *F*, *G*, *H*, *L*, *M* and *X*), *MT2A*, *MT3* and *MT4* mRNA was determined. In study 2, fifty-four healthy male and female volunteers (31·9 (SD 13·8) years, BMI 25·7 (SD 2·9) kg/m^2) completed a FFQ, blood was collected, PBMCs were isolated and mRNA expression of selected Zn transporters and MT isoforms was determined. Study 1: *MT1E*, *MT1F*, *MT1G*, *MT1H*, *MT1L*, *MT1M*, *MT1X*, *MT2A* and *SLC30A1* increased with increasing concentrations of Zn and declined with the addition of TPEN. Study 2: Average daily Zn intake was 16·0 (SD 5·3) mg/d (range: 9–31 mg/d), and plasma Zn concentrations were 15·5 (SD 2·8) μmol/l (range 11–23 μmol/l). PBMC *MT2A* was positively correlated with dietary Zn intake (r 0·306, $P = 0·03$) and total Zn intake (r 0·382, $P < 0·01$), whereas plasma Zn was not ($P > 0·05$ for both). Findings suggest that *MT2A* mRNA in PBMCs reflects dietary Zn intake in healthy adults and may be a component in determining Zn status.

Key words: **Biomarkers: Zinc transporter: Metallothionein: Leucocytes**

Zn status is currently assessed by measuring the concentration of Zn in plasma or serum. Despite frequent use for the assessment of Zn status at the population level, circulating concentrations of Zn are considered a poor indicator of Zn status[1–3]. The lack of a specific, responsive and reliable indicator of Zn status is a substantial barrier to the development of targeted nutritional interventions and human health, as an estimated 20 % of the world's population (about 1·1 billion people) is a risk for inadequate Zn intake[4–6]. Thus, various alternative biomarkers have been considered (reviewed in Lowe *et al.*[7]). One potential alternative is assessing expression of Zn-binding and Zn-transporting proteins. These proteins control Zn homoeostasis in response to fluctuations in Zn intake and thus their measurement in circulating blood cells may be an effective means to assess Zn status.

Metallothioneins (MTs) are small, cysteine-rich, cytosolic Zn-binding proteins. In mammals, four tandemly clustered MT genes (*MT1*, *MT2A*, *MT3* and *MT4*) have been identified. Expansion of the *MT1* gene in humans has led to the identification of a total of thirteen paralogs (*MT1A-J*, *MT1L*, *MT1M* and *MT1X*) five of which are predicted to be no longer active forms (*MT1C*, *MT1D*, *MT1I*, *MT1J* and *MT1L*). *MT1* and *MT2* are the major isoforms and are expressed ubiquitously, while *MT3* and *MT4* are minor isoforms found in specialised cells such as neurons and stratified squamous epithelium, respectively. MTs increase in response to extracellular Zn and are degraded when MT-bound Zn is released in low Zn conditions[8]. MT expression is induced by Zn through binding of Zn to the Zn-finger transcription factor, metal-regulatory transcription factor 1, which

Abbreviations: MT, metallothionein; PBMCs, peripheral blood mononuclear cells; TPEN, N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine.

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binds to metal-responsive elements in the promoter of the MT gene[9].

Twenty-four Zn transporters have been described in mammals and can be divided into two distinct families: the Zrt-, Irt-like protein (ZIP) (solute carrier family (*SLC*) *39A1-14*) family of Zn importers, which function to import Zn into the cell or transport Zn from within a subcellular compartment into the cytoplasm, and the Zn Transporter (ZnT, *SLC30A1-10*) family of Zn exporters, which function to export Zn out of the cell or transport Zn from the cytoplasm into subcellular compartments. Expression and localisation of Zn transporters are tissue- and cell-type specific[10]. Similar to MTs, some Zn transporters, such as ZnT1(*SLC30A1*)[8], ZnT2 (*SLC30A2*)[11] and ZIP10 (*SLC39A10*)[12], also contain metal-responsive elements that respond to Zn. In addition, other Zn transporters, such as ZnT5 (*SLC30A5*)[13], contain Zn-responsive elements in their promoters[14], suggesting additional mechanisms by which Zn may regulate Zn transporter expression.

Previous studies have examined the utility of MT and/or Zn transporter expression in various blood cell types as biomarkers of Zn status[15–30]. A recent systematic review of these studies determined that MT mRNA in leucocyte subtypes may be a more reliable and sensitive indicator of Zn exposure in healthy individuals than plasma Zn or MT mRNA in cells from the erythroblast lineage[31]. However, limitations of these studies include few having distinguished between MT isoforms and subtypes and few having determined Zn transporter expression. Whether specific MT isoforms or subtypes, or expression of Zn transporters, could provide more reliable and sensitive measures of Zn status remains unknown. Thus, the objective of this study was to determine the reliability and sensitivity of MT isoforms and subtypes and Zn transporter genes in peripheral blood mononuclear cells (PBMCs) to changes in Zn exposure.

Methods

Cell culture

Normal, human PBMCs (PCS-800-011) were purchased from American Type Culture Collection. Cells were cultured in RPMI-1640 (American Type Culture Collection) alone (control, 0 μM $ZnSO_4$) or RPMI-1640 with 10 μM $ZnSO_4$ (0·37 mg elemental Zn), 50 μM $ZnSO_4$ (1·83 mg elemental Zn) or 50 μM $ZnSO_4$ and 5 μM N,N,N′,N′-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN, Sigma-Aldrich). TPEN is a membrane permeable metal chelator with a higher metal-binding constant ($K_{Zn} = 10^{15.58}/M$) than MT ($K_{Zn} = 3{\cdot}2 \times 10^{13}/M$). The Zn concentrations were chosen to represent a modest and large fluctuation in circulating concentrations of Zn[32]. At 24 h later, cells were collected in TRIzol® (Life Technologies) and stored at –80°C.

Human subjects

Volunteers were part of a larger study determining the effects of consuming a diet of military food rations on gut microbiota composition[33]. The study conformed to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board at the U.S. Army Research Institute of Environmental Medicine (USARIEM) and registered at www.clinicaltrials.gov as NCT02423551. Healthy men and women aged 18–61 years with a BMI ≤ 30 kg/m^2 were recruited. Exclusion criteria included antibiotic use, not willing to cease dietary supplements, vegetarian diet, history of gastrointestinal disease, use of non-steroidal anti-inflammatory drugs, actively trying to lose weight, pregnant/lactating, not willing to avoid strenuous activity or alcohol consumption 24 h prior to testing and prior blood donation (<8 weeks).

Subjects (n 54) completed the 2014 Block FFQ (NutritionQuest) at baseline to assess habitual Zn intake[34]. The Block FFQ is an extensive 127-item questionnaire developed from analysis of two waves of National Health and Nutrition Examination Survey (NHANES) dietary recall data. The nutrient and food group analysis database was developed from the United States Department of Agriculture's Food and Nutrient Database for Dietary Studies (FNDDS 5.0), the Food Pyramid Equivalents Database and the Nutrient Database for Standard Reference (SR27). Fasted blood samples were collected at baseline in the early morning in BD Vacutainer Cell Preparation Tubes. Plasma and PBMC were isolated by centrifugation according to the manufacturer's instructions. PBMC pellets were collected in TRIzol® and PBMC, and plasma was stored at –80°C. Plasma Zn concentrations were determined from plasma isolated from BD Vacutainer Cell Preparation Tubes and determined by inductively coupled plasma MS (ICP-MS) (Thermo Fisher Scientific X Series 2)[35].

Gene expression

RNA was isolated, and cDNA was synthesised as described previously[16]. Real-time relative RT-PCR was performed with TaqMan Gene Expression Assays (Life Technologies) and run on a StepOne Plus real-time PCR instrument (Applied Biosystems). The programme was run for a 10 min activation step at 95°C followed by forty cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 60 s. Primers and probes for TaqMan Gene Expression Assays are included in online Supplementary Table S1. Each sample was analysed in duplicate. For study 1, target mRNA expression was calculated using the ΔΔCt method. Fold change relative to controls was quantified using $2^{(-\Delta\Delta Ct)}$ where $\Delta Ct = Ct_{Target\ gene} - Ct_{Reference\ gene}$ and $\Delta\Delta Ct = \Delta Ct_{Treatment} - \Delta Ct_{Controls}$. The average of *18S* and *ACTB* was used as the reference gene, and the average ΔCt of the untreated controls was used as the calibrator. In study 2, target mRNA expression was normalised to *18S* ($\Delta Ct = Ct_{Gene} - Ct_{18S}$), and gene expression was quantified using the $2^{(-\Delta Ct)}$ comparative Ct method[36].

Data analysis

All participants in the larger study[33] with PBMCs were used for data analysis; a formal power calculation was not calculated. In study 1, the mRNA expression of all MT isoforms and subtypes and all Zn transporters determined in PBMCs cultured with Zn or TPEN *ex vivo* for 24 h were compared by one-way ANOVA. *Post hoc* significant differences were made using Tukey's adjustment for multiple comparisons. In study 2, Pearson correlations were used to correlate Zn responsive genes isolated from PBMCs with

habitual dietary Zn intake determined by FFQ. Dietary Zn, total Zn, phytate and phytate:Zn were adjusted for energy intake using the residual method[37]. Data that were not normally distributed were log transformed. The α level for statistical significance was $P < 0{\cdot}05$. Data were analysed using SPSS version 24 (IBM Corp) and are presented as mean values and standard deviations.

Results

Study 1

A heat map depicting relative gene expression and a table with fold changes in MT and Zn transporter expression in PBMCs exposed to Zn and TPEN *ex vivo* are shown in Fig. 1 and Table 1, respectively. Data used to derive the heat map can be found in online Supplementary Table S2. *MT1E*, *MT1F*, *MT1G*, *MT1H*, *MT1L*, *MT1M*, *MT1X*, *MT2A* and *SLC30A1* increased with increasing concentrations of Zn and decreased with the addition of TPEN. Genes that increased with the addition of 10 μM $ZnSO_4$ compared with control ($P < 0{\cdot}05$) were *MT1M* (106·2-fold increase), *MT1L* (49·8-fold), *MT1H* (22·0-fold), *MT1F* (15·8-fold), *MT1X* (13·7-fold), *MT1G* (13·3-fold), *MT1E* (7·1-fold), *MT2A* (6·6-fold) and *SLC30A1* (2·3-fold). Genes that increased with the addition of 50 μM $ZnSO_4$ compared with 10 μM $ZnSO_4$ ($P < 0{\cdot}05$) were *MT1L* (4270·5-fold increase), *MT1M* (3958·5-fold), *MT1H* (858·9-fold), *MT1A* (598·6-fold), *MT1G* (388·3-fold), *MT1X* (219·6-fold), *MT1F* (192·1-fold), *MT1E* (143·8-fold), *MT2A* (69·7-fold), *SLC30A1* (7·2-fold), *SLC39A8* (5·8-fold) and *MT1B* (2·5-fold). All of the transporters that responded to Zn returned to control (*MT1A*, *MT1B*, *SLC30A1* and *SLC39A8*) or 10 μM $ZnSO_4$ levels (*MT1E*, *MT1F*, *MT1G*, *MT1H*, *MT1L*, *MT1M*, *MT1X* and *MT2A*) with the addition of 50 μM $ZnSO_4$ and TPEN ($P > 0{\cdot}05$). *MT3*, *SLC30A3*, *SLC30A4*, *SLC30A5*, *SLC30A6*, *SLC30A7*, *SLC30A9*, *SLC39A2*, *SLC39A3*, *SLC39A4*, *SLC39A5*, *SLC39A6*, *SLC39A7*, *SLC39A9*, *SLC39A10*, *SLC39A12*, *SLC39A13* and *SLC39A14* were detected, but were not responsive to Zn ($P > 0{\cdot}05$). *SLC30A8* was not detected in control cells but was detected in at least some of the replicates in all other treatment conditions (online Supplementary Table S2). *SLC30A2* was detected in response to 50 μM $ZnSO_4$ but no other treatment condition and *SLC30A10* was only detected in one of the replicates in the control condition. *MT1E*, *MT1F*, *MT1G*, *MT1H*, *MT1L*, *MT1M*, *MT1X*, *MT2A* and *SLC30A1* increased with increasing concentrations of Zn and decreased with the addition of TPEN. *MT1A*, *MT1B*, *SLC39A8* and *SLC39A11* increased with 50 μM $ZnSO_4$, but not 10 μM $ZnSO_4$. There was an overall treatment effect for *SLC39A1* ($P = 0{\cdot}0349$) but no *post hoc* differences. *MT4* was not detected in any of the treatment conditions.

Study 2

Expression of MT (*MT1E*, *MT1F*, *MT1G*, *MT1H*, *MT1L*, *MT1X* and *MT2A*) and Zn transporter (*SLC30A1*, *SLC30A9*, *SLC39A8*, *SLC39A10* and *SLC39A11*) genes that were found to be sensitive to Zn in study 1 were examined in PBMCs isolated from human subjects. Subject demographics and dietary data are shown in

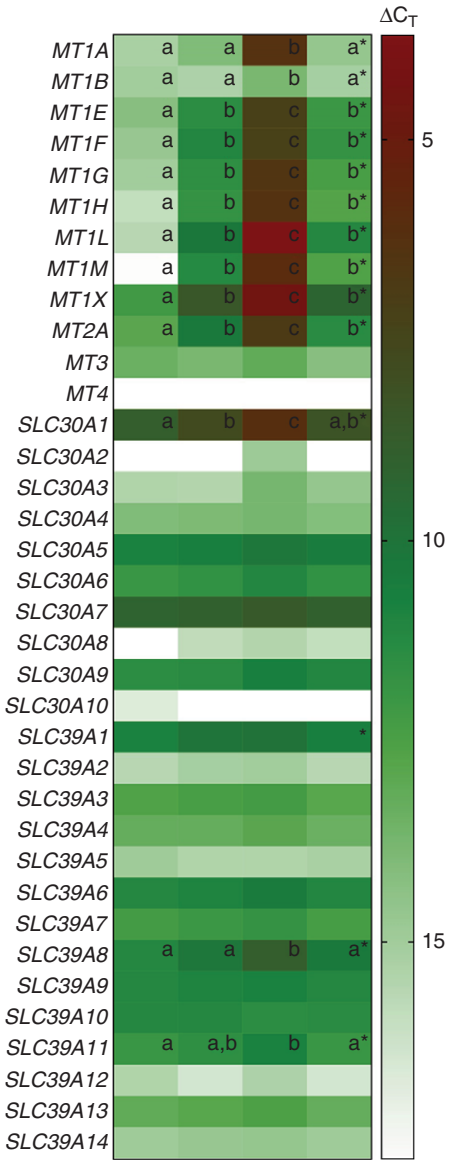

Fig. 1. Heat map of metallothionein and zinc transporter expression in peripheral blood mononuclear cells (PBMCs) exposed to zinc and N,N,N′, N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) *ex vivo*. Columns represent ΔC_T values of metallothionein isoforms and subtypes and zinc transporters in control cells and cells cultured with 10 μM $ZnSO_4$ or 50 μM $ZnSO_4$ or with 50 μM $ZnSO_4$ plus TPEN for 24 h. Lower ΔC_T values represent greater gene expression (red) and genes not expressed are shown in white. * Significant overall *P* value ($P < 0{\cdot}05$). [a,b,c] Unlike letters within a row indicate a significant difference ($P < 0{\cdot}05$). Data used to derive this figure are shown in online Supplementary Table S2. CT, cycle threshold.

Table 2. Adjusted dietary Zn intakes were above the Estimated Average Requirement (EAR) for all participants (range 9–20 mg/d)[38]. Of the subjects, 22 % (12/54) reported taking a dietary supplement containing Zn. Dietary phytate varied considerably between subjects (183–1371 mg/d), and phytate:Zn molar ratios indicated that diets were of good-to-medium Zn bioavailability[39]. Plasma Zn concentrations were within the normal range with one female and two males having concentrations less than established cut-offs (<70 or 74 μg/dl or <10·7 or 11·3 μmol/l, respectively[40]).

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Table 1. Metallothionein and zinc transporter expression in peripheral blood mononuclear cells exposed to zinc and N,N,N′,N′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) *ex vivo**
(Mean values and standard deviations)

	Fold change								
	Control		10 μM $ZnSO_4$		50 μM $ZnSO_4$		50 μM $ZnSO_4$ + 5 μM TPEN		
Gene	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*
MT1A	1·1[a]	0·7	2·4[a]	1·0	598·6[b]	376·9	1·9[a]	1·4	<0·0001
MT1B	1·0[a]	0·3	0·8[a]	0·2	2·5[b]	1·6	1·0[a]	0·2	0·0013
MT1E	1·1[a]	0·5	7·1[b]	2·7	143·8[c]	51·0	4·9[b]	2·2	<0·0001
MT1F	1·0[a]	0·3	15·8[b]	10·3	192·1[c]	97·5	8·9[b]	4·2	<0·0001
MT1G	1·0[a]	0·3	13·3[b]	7·6	388·3[c]	153·0	6·1[b]	1·7	<0·0001
MT1H	1·1[a]	0·4	22·0[b]	12·2	858·9[c]	398·1	10·3[b]	4·7	<0·0001
MT1L	1·0[a]	0·3	49·8[b]	31·5	4270·5[c]	2356·4	29·5[b]	20·2	<0·0001
MT1M	1·5[a]	1·5	106·2[b]	69·0	3958·5[c]	1766·1	34·2[b]	11·1	<0·0001
n	2								
MT1X	1·0[a]	0·3	13·7[b]	7·3	219·6[c]	90·4	7·5[b]	2·5	<0·0001
MT2A	1·2[a]	0·8	6·6[b]	2·9	69·7[c]	20·5	3·1[b]	0·8	<0·0001
MT3	1·0	0·3	0·9	0·4	1·3	0·6	0·6	0·3	0·1638
MT4	ND		–		–		–		
SLC30A1	1·1[a]	0·6	2·3[b]	0·9	7·2[c]	2·4	1·6[a,b]	0·3	<0·0001
SLC30A2	ND		–		–		–		
SLC30A3	1·2	0·9	1·0	0·5	4·6	4·7	1·9	1·0	0·1044
n	2		4				4		
SLC30A4	1·0	0·3	1·1	0·4	1·5	0·9	1·0	0·4	0·6760
SLC30A5	1·0	0·3	1·2	0·4	1·8	0·8	1·3	0·6	0·2276
SLC30A6	1·1	0·5	1·3	0·5	1·9	1·0	1·3	0·7	0·2856
SLC30A7	1·0	0·3	1·2	0·4	1·6	0·7	1·3	0·7	0·4295
SLC30A8	ND		–		–		–		
SLC30A9	1·0	0·3	1·2	0·5	1·9	1·0	1·5	0·9	0·4223
SLC30A10	ND		–		–		–		
SLC39A1	1·1[a]	0·7	1·9[a]	0·6	1·9[a]	0·6	1·1[a]	0·3	0·0348
SLC39A2	1·2	0·7	1·6	0·9	2·0	1·6	1·1	0·4	0·6747
n							4		
SLC39A3	1·0	0·3	1·2	0·3	1·4	0·6	1·0	0·4	0·2384
SLC39A4	1·0	0·1	1·0	0·3	1·3	0·3	0·9	0·2	0·1021
SLC39A5	1·2	1·0	0·8	0·4	0·8	0·5	1·0	0·7	0·8127
n	4								
SLC39A6	1·0	0·3	1·1	0·3	1·7	0·8	1·1	0·5	0·1915
SLC39A7	1·1	0·4	1·2	0·3	1·5	0·6	0·9	0·3	0·1605
SLC39A8	1·1[a]	0·4	2·1[a]	0·8	5·8[b]	3·4	1·9[a]	0·9	0·0002
SLC39A9	1·0	0·3	1·2	0·4	1·3	0·5	1·1	0·5	0·7178
SLC39A10	1·0	0·3	1·0	0·3	0·9	0·4	0·9	0·4	0·8582
SLC39A11	1·1[a]	0·4	1·3[a,b]	0·3	2·1[b]	0·7	1·0[a]	0·3	0·0032
SLC39A12	1·0		0·5	0·1	1·1	0·4	0·5	0·3	0·0606
n	1		3				3		
SLC39A13	1·0	0·3	1·3	0·2	1·7	0·7	1·0	0·5	0·1498
SLC39A14	1·0	0·2	1·3	0·5	1·5	0·9	1·1	0·5	0·7179

ND, not detected.
[a,b,c] Unlike superscript letters within a row indicate a significant difference ($P < 0·05$).
* Fold change of metallothionein isoforms and subtypes and Zn transporters in control cells and cells cultured with 10 μM $ZnSO_4$ or 50 μM $ZnSO_4$ or with 50 μM $ZnSO_4$ plus TPEN for 24 h relative to control. *n* 5/treatment unless replicates within a treatment were not detected. Data are corrected for the average of *18S* and *ACTB*. *P* values and superscript letters are for log-transformed data.

With the exception of *MT1H*, *MT1G* and *MT1M*, which were only detected in 26, 15 and 35 % of subjects, respectively, and are not reported, the majority of subjects expressed *MT1A* (93 %), *MT1E* (72 %), *MT1F* (81 %), *MT1L* (72 %), *MT1X* (83 %), *MT2A* (93 %), *SLC30A1* (94 %), *SLC39A8* (98 %) and *SLC39A11* (96 %). Adjusted dietary Zn (R 0·306, $P = 0·031$) and total Zn (R 0·382, $P = 0·006$), which included the sum of Zn from diet and supplements, were positively correlated with *MT2A* (Table 3). Adjusted dietary phytate was positively correlated with MT1A (R 0·287, $P = 0·044$), and plasma Zn concentrations were negatively correlated with *MT1A* (R −0·279, $P = 0·050$) and *MT2A* (R −0·289, $P = 0·042$).

Discussion

To our knowledge, this is the first study to comprehensively examine the reliability and sensitivity of Zn exposure and depletion on all Zn transporters and MTs in PBMCs. PBMC isolated from human subjects were exposed to physiological concentrations of Zn or TPEN *ex vivo* to identify MT isoforms and subtypes and Zn transporter genes that responded to Zn. Candidate genes were subsequently measured in PBMCs isolated from human subjects and correlated to their habitual Zn intake. The major finding of this study was that although the majority of MT isoforms and subtypes were detected in

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Table 2. Volunteer demographics and dietary data*
(Mean values, standard deviations and ranges; percentages; *n* 54)

Variable	Mean	SD	Range
Male (%)	94		
Ethnicity (%)			
White	72		
Black	9		
Other	19		
Age (years)	31·9	13·8	18–61
Weight (kg)	78·4	12·4	52–114
BMI (kg/m^2)	25·7	2·9	18–31
Energy (kcal/d)†	2380·7	928·5	797–4985
Plasma Zn (μmol/l)	15·5	2·8	11–23
Dietary Zn (mg/d)	14·0	5·2	5–26
Adjusted dietary Zn‡ (mg/d)	14·3	2·5	9–20
Adjusted total Zn‡ (mg/d)	16·0	5·3	9–31
Adjusted dietary phytate‡ (mg/d)	693·6	235·5	183–1371
Adjusted phytate:Zn molar ratio‡	4·8	1·5	2–10

* Total Zn includes dietary Zn and Zn from supplements.
† To convert values from kcal to kJ, multiply by 4·184.
‡ Values adjusted for energy intake using the residual method.

Table 3. Correlations between habitual zinc intake, plasma zinc and metallothionein and zinc transporter mRNA†
(Pearson correlation coefficients; *n* 54)

	Dietary Zn	Total Zn	Phytate	Phytate:Zn	Plasma Zn
Plasma Zn	0·134	0·018	0·003	–0·050	1
PBMC *MT1A*	0·099	0·113	0·287*	0·274	–0·279*
PBMC *MT1E*	0·269	0·299	0·188	0·035	–0·112
PBMC *MT1F*	0·209	0·123	0·164	0·053	–0·125
PBMC *MT1L*	–0·020	–0·009	0·067	0·089	0·023
PBMC *MT1X*	0·040	0·010	0·205	0·208	–0·028
PBMC *MT2A*	0·306*	0·382*	0·237	0·118	–0·289*
PBMC *SLC30A1*	0·232	0·209	0·168	0·046	–0·081
PBMC *SLC39A8*	0·195	0·156	0·127	0·029	–0·256
PBMC *SLC39A11*	0·138	0·210	0·094	0·003	–0·197

* Significant correlation ($P < 0·05$).
† *MT1A* was detected in fifty of fifty-four participants, *MT1E* (39/54), *MT1F* (44/54), *MT1L* (39/54), *MT1X* (45/54), *MT2A* (50/54), *SLC30A1* (51/54), *SLC39A8* (53/54), *SLC39A11* (52/54). Gene data are log-transformed. Values for dietary Zn, total Zn, phytate and phytate:Zn adjust for energy intake using the residual method. Total Zn includes dietary Zn and Zn from supplements.

PBMCs and were sensitive to changes in Zn, only *MT2A* mRNA in PBMCs reflected habitual dietary Zn intake in healthy adults. These findings further suggest that *MT2A* mRNA in PBMCs may be a component in determining Zn status.

Of those transporters studied, most MT isoforms and subtypes, with the exception of *MT3* and *MT4*, were expressed in PBMCs at an appreciable level and responded to Zn. This finding agrees with previous work that demonstrated that MTs increase in response to extracellular Zn and are degraded when MT-bound Zn is released when Zn is low[13]. In contrast, while the majority of Zn transporters were expressed in PBMCs, few responded to Zn. Mammalian MTs are characterised by their twenty invariant cysteines. These cysteine residues are responsible for binding and sequestering Zn through their thiol (-SH) moieties and bind up to seven Zn atoms (three Zn atoms in the β domain and four Zn atoms in the α domain). Excess Zn is thought to displace Zn from MT[41]. Free Zn can then fill Zn fingers within the DNA-binding domain of cytosolic metal-regulatory transcription factor 1[42,43]. Holo-metal-regulatory transcription factor 1 can then translocate to the nucleus where it binds to metal-responsive elements in the promoter of the MT gene[9]. A consensus sequence for the central core of the metal-responsive element (TGCRCNC, were R is an A or G and N is any nucleotide) has been identified by examining a host of known metal-responsive genes[44]. Although MTs are capable of binding other essential (e.g. Cu) and non-essential (e.g. Cd) metals, MT-bound Zn is thought to be the predominant form of MT in human tissue. For example, MT is not present in the livers of Zn-deficient rats, even when liver Cu concentrations are high[45]. Importantly, metal-regulatory transcription factor 1 DNA-binding activity is reversibly activated in response to changes in free Zn concentration[46].

In the present study, *MT2A* was highly expressed in PBMCs, was sensitive to changes in Zn exposure and depletion and was positively correlated to habitual Zn intake in human subjects. These findings are consistent with that of Cao & Cousins[19]

and Cao *et al.*[20] who supplemented male subjects with placebo or 15 mg Zn/d (as $ZnSO_4$) for 10 d. PBMC *MT2A* mRNA increased with Zn supplementation at the earliest time point measured (day 2) and remained elevated until supplementation ceased when levels returned to baseline. Chu *et al.*[21] provided female and male subjects with no treatment or 22 mg Zn/d for 21 d and determined mRNA expression of *MT1A*, *MT2A*, *SLC30A1*, *SLC30A5*, *SLC30A6*, *SLC30A7*, *SLC39A1*, *SLC39A3*, *SLC39A7*, *SLC39A8*, *SLC39A10* and *SLC39A14* in PBMCs. *MT2A* was the only gene affected by Zn supplementation and increased with Zn supplementation on day 2 but returned to baseline on days 7–21. Conversely, Ryu *et al.*[25] determined the expression of *MT1/2*, *SLC30A1*, *SLC30A4*, *SLC30A5*, *SLC30A6*, *SLC30A7*, *SLC39A1*, *SLC39A3*, *SLC39A5*, *SLC39A6*, *SLC39A8*, *SLC39A10* and *SLC39A14* mRNA in PBMCs isolated from male subjects fed a Zn-depleted diet for 10 d. In this study, a common amplicon region was identified and a combination of two forward primers, two reverse primers and two TaqMan probes was used to amplify *MT1H*, *MT1H*-like, *MT1G*, *MT1L*, *MT1E*, *MT1A* and *MT2*. Expression of *MT1/2*, *SLC30A1*, *SLC30A4* and *SLC30A5* decreased with Zn depletion by day 10. These findings indicate that *MT2A* mRNA in PBMCs is sensitive to Zn supplementation and depletion and that detection of *MT2A* mRNA in PBMCs may be a reliable marker to assist in the detection of Zn status in a clinical or field setting.

In contrast to MTs, there was a lack of consistency in the Zn transporter genes. While the majority of Zn transporters were expressed in at least one of the treatment conditions, few responded to Zn supplementation and/or depletion. This may be expected as MTs, *SLC30A1* and *SLC30A2* contain metal response elements in their promoters that respond to Zn through metal response element-binding transcription factor-1[25]. While *SLC30A2* was only expressed in response to higher concentrations of Zn, *SLC30A1* was highly expressed in PBMCs and increased with increasing concentrations of Zn and declined with Zn depletion. This is consistent with findings from Overbeck *et al.*[47] who found that *SLC30A1* expression increased incrementally in cells cultured with 15 and 30 μM Zn and decreased slightly with TPEN. Despite being Zn responsive, *SLC30A1* mRNA was not correlated with habitual Zn intake.

The finding that plasma Zn was not correlated with habitual dietary Zn intake in the current study is consistent with most studies that show that plasma Zn does not respond consistently to changes in dietary Zn[31]. Zn is required for multiple aspects of general metabolism and thus complex systems tightly regulate Zn homoeostasis. When Zn intake is reduced, there is a reduction in endogenous losses to conserve Zn[48] and Zn is mobilised from small, rapidly exchangeable pools in the plasma, liver and possibly bone[49,50]. Thus, changes in plasma and tissue Zn are relatively insensitive to changes in dietary Zn because of mechanisms that tightly control Zn homoeostasis. Interestingly, *MT2A* and *MT1A* mRNA in PBMCs were negatively correlated with plasma Zn in study 2. Kwon *et al.*[51] found a similar negative correlation between mononuclear cell *MT2A* mRNA and plasma Zn in a larger cohort of 50–80-year-old males and females from South Korea (*n* 110). One possible explanation that the authors suggest is that over time the presence of adequate MT2A protein levels may feedback to down-regulate gene expression[51].

The strengths of this study are that it is the first to comprehensively examine MT and Zn transporter expression in PBMCs. Limitations include the homogenous study population and measurement of dietary intake by FFQ at one timepoint. Plasma Zn concentrations should be interpreted with caution as plasma was isolated from BD Vacutainer Cell Preparation Tubes. These tubes are not trace element-free and may be a source of Zn contamination; however, any variability due to contamination would likely be equally distributed across participants. Further, this study assessed the mRNA expression of MT and Zn transporters, which precludes comparison of the relative concentrations clinically. Reference material for MT concentrations needs to be developed in order to compare MT reliably across different immunoassays. Other questions such as whether the different proportion of blood cell subtypes in peripheral blood, which may vary across individuals[19], affects the sensitivity to Zn supplementation and depletion should be assessed. Future studies should examine the expression of MT and Zn transporters in PBMCs in diverse populations, adolescents, children and individuals with disease. Further, controlled feeding studies in humans are required to determine whether the identified genes respond to short- and long-term changes in dietary Zn intake and can be used to predict Zn status.

Collectively, these data may help inform the development of a multivariable model to provide a comprehensive assessment of Zn status while minimising the influence of confounders on any single variable. Other nutrients use multiple indicators and a multivariable model. Thus, the combination of MT (perhaps in conjunction with plasma Zn) may provide a more comprehensive view of Zn status while minimising the influence of any single variable. This is important as MT and Zn transporter expressions are affected by various conditions (e.g. inflammatory agents, free radicals, glucocorticoids and pharmacological agents[45,46]), which would affect the interpretation.

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Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114520002810

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