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Zinc-Induced Metallothionein in Centenarian Offspring From a Large European Population: The MARK-AGE Project

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Abstract

Metallothionein (MT) family are cysteine-rich proteins that regulate zinc (Zn) homeostasis and protect against oxidative damage. Studies in transgenic mice have shown that MT favorably influence longevity, although their role in human aging is not completely understood. Within the European multicenter study MARK-AGE, we analyzed MT induction after Zn treatment in peripheral blood mononuclear cells (PBMCs) and its relation with redox biomarkers in 2,936 age-stratified subjects (35–75 years) including the general population (RASIG), centenarian offspring (GO), and their spouses (SGO). We found that the lymphocyte capability to induce MT in response to Zn is not affected by aging. However, GO participants showed lower Zn-induced MT and increased basal expression of MT1A, MT1X, and ZnT-1 genes than RASIG subjects. Moreover, Zn-induced MT levels were found to be inversely related with oxidative stress markers (plasma protein carbonyls,

3-nitrotyrosine, and malondialdehyde) in the whole population, but not in GO subjects. In conclusion, our results support the hypothesis that the response to Zn is attenuated in PBMCs of centenarian offspring compared to the general population as a consequence of a tighter control of Zn homeostasis which is likely to provide them constant protection against stress stimuli over the whole lifespan.
Keywords: Metallothionein, Longevity, Zinc, Aging, Senescence

Metallothioneins (MT) are zinc-responsive small cysteine-rich proteins that are involved in metal homeostasis and detoxification, as well as in protection against reactive oxygen species (ROS) related damage. Other properties attributed to MT are the modulation of mitochondrial respiratory function (1) and the transduction of nitric oxide (NO) into zinc (Zn) signals (2). Hence, it is not surprising that these proteins are claimed to regulate immune response (3), cellular senescence (4), and tissue regeneration (5).

The relevance of MT in the aging process has been documented both in animal models and humans as these proteins are responsive to anti-aging interventions including caloric restriction (CR) and inhibition of the insulin/insulin-like signaling (IIS) pathway (5–7).

An increased expression of MT has been associated with a higher lifespan in daf-2 mutant worms (8). Consistently, increased survival has been observed both in cardiac-specific MT transgenic mice (9) and in MT-1 transgenic mice (10). By contrast, MT-1 and MT-2 gene knockout mice display shorter lifespan than wild type mice (11).

A large body of evidence also indicates that MT prevent diabetic complications in mice models (12,13), attenuate aging and diabetes induced cardiac dysfunction (14), protect against coagulatory and fibrinolytic disturbance (15) and against lipid metabolic or inflammatory damages (15,16) which are common age-related disorders. The implication of MT in human longevity is suggested by genetic evidence. Italian centenarian females show higher frequency of the +647 MT1A Asp/Asp genotype (3), while the +647 MT1A Thr allele has been associated with increased susceptibility to diabetes mellitus and cardiovascular complications (17).

Other findings in humans show increased MT protein levels in peripheral blood mononuclear cells (PBMCs) from elderly subjects as compared to young ones at basal condition (18), while reduced MT protein concentrations have been found in healthy nonagenarians/centenarians with respect to septua-octagenarians (19). However, it is still unknown if these age-related changes are due to modifications in systemic or intrinsic intracellular signals that alter the capability of the cell to induce MT. Mechanisms underlying transcriptional induction of MT genes are not completely understood, but MT expression can be induced by a variety of physiological agents and environmental stressors such as transition metals, glucocorticoids, cAMP, phorbol esters, alkylating agents, oxidizing agents, ultraviolet and ionizing radiation, and hypoxia (20). However, as a consequence of various signal transduction pathways, the mobilization of intracellular Zn ions has been frequently reported as common mediator of these stimuli (21). Moreover, while the Zn binding function is exerted by MT proteins, MT protein levels often bear no clear relationship with mRNA levels (22). Hence, the direct measurement of MT protein levels in response to stimulation with Zn appears as a likely unambiguous way to study the overall capability to induce MT and investigate if intrinsic processes associated with aging affect this function.

Therefore, in this study we will assay Zn-induced MT production in PBMCs from a large cohort of subjects recruited in MARK-AGE project including participants recruited in an age-stratified manner from the "general population" (RASIG), offspring of centenarians previously studied in the GEHA study, termed "GEHA offspring" (GO) and their spouses (SGO), as a control for environmental factors and lifestyle (23). Association among Zn-induced MT and biochemical laboratory parameters will be also assessed. The data will be complemented and related to baseline mRNA expression of individual MT isoforms and Zn transporters.

Methods

Study Population, Recruitment, Data and Blood Sample Collection

MARK-AGE is an European-wide cross-sectional population study aimed at the identification of biomarkers of aging (23).

In the present work, we measured MT production in PBMCs after 24 hours of culture with 50 µM of Zn from 2,936 participants in the age range of 35–75 years recruited in eight different European countries. The studied population consisted of 2,150 RASIG, 520 GO, and 266 SGO. Basal MT and Zn transporters mRNA levels were measured in a subgroup of 120 participants.

Details of the recruitment procedures and of the collection of anthropometric, clinical and demographic data, as well as of laboratory parameters assays have been previously reported (24–26).

PBMCs isolation procedure has been described (24). Briefly, PBMCs were isolated from lithium-heparin whole blood, obtained by phlebotomy after overnight fasting, by discontinuous density gradient centrifugation in Percoll and subsequently cryopreserved and stored in liquid nitrogen. Samples were then shipped from the various recruitment centers to the MARK-AGE Biobank located at the University of Hohenheim, Stuttgart, Germany. From the Biobank, coded samples were subsequently sent to the Scientific and technological Pole of INRCA of Ancona on dry ice, where they were stored in liquid nitrogen until use.

PBMC Thawing, Viability Assay, and Culture

One aliquot of 1 million of PBMCs for each subject was thawed in a 37°C water bath, and the cells were diluted in prewarmed complete medium (RPMI-1640 + 10% FCS) and centrifuged at 250 g for 10 minutes and immediately resuspended in fresh complete medium. Then the cell viability was measured using Trypan blue dye exclusion technique.

PBMCs were cultured in 24 well plates with 1 mL of RPMI-1640 medium (GIBCO) supplemented with 100 units/mL penicillin and streptomycin, 1% glutamine and 10% heat-inactivated fetal calf serum (FCS) (37°C; 5% CO_2) for 24 h. The day after, Zn sulfate (50 µM) was added in the medium and PBMCs were incubated for further 24 hours (37°C; pH 7.4; 5% CO_2).

PBMCs were recovered and washed in phosphate-buffered saline (PBS, pH 7.4) buffer at 250 g for 10 minutes and used for MT and propidium iodide assays. One aliquot up to 1×10^5 was resuspended in 300 µL of PBS and stained with 5 µL of propidium iodide (1 mg/mL of stock solution). After 1 minute of incubation, the cells were immediately analyzed by flow cytometry (FC).

Flow Cytometry Assay of Zn-Induced Metallothioneins

An aliquot of PBMCs (0.5×10^6) was treated with 3% paraformaldehyde in PBS and incubated for 15 minutes at 4°C. Samples were

washed with 2 mL of PBS and centrifuged (800 g, 10 minutes), the resulting supernatants aspirated, and the cells resuspended in a permeabilization buffer (1 mL) containing 3% FCS, 0.1% sodium azide, and 0.1% saponin in PBS for 30 minutes at room temperature.

Cells were then washed with 3 mL of staining buffer (3% FCS, 0.1% sodium azide in PBS, pH 7.4) and centrifuged (300*g*, 5 minutes). Cells were resuspended in 800 µL of staining buffer and subdivided in two aliquots. The first aliquot was incubated with 1.6 µg of anti-human Metallothionein rabbit polyclonal antibody (FL-61; sc-11377, Santa Cruz Biotechnology, Inc.) for 30 minutes at room temperature, then washed with 3 mL of staining buffer and centrifuged (300*g*, 5 minutes). Then, both aliquots of PBMCs were incubated with a secondary antibody (goat anti-rabbit IgG-FITC; Santa Cruz Biotechnology, Inc.) for 30 minutes at room temperature in the dark. PBMCs were then washed with 3 mL of staining buffer (300*g*, 5 minutes), resuspended in 500 µL of PBS, and the relative cellular fluorescence which reflects cellular MT levels, was determined using flow cytometry.

Non-viable cells and debris were excluded, based on forward and side light-scatter properties and cells were analyzed selecting the most appropriate lymphocyte and monocytes gates, based on the combination of forward- and side-scatter. For each sample, the MT-specific mean fluorescence intensity (MFI) was calculated by subtracting the background MFI (only secondary antibody) from the MFI obtained after staining with both primary and secondary antibodies.

Standardization was performed by the use of fluorescent beads (Quantum FITC-5 MESF, Bangs Laboratories, Inc.) made up of five calibrated populations with a known number of molecular equivalent of soluble fluorochrome (MESF) and one non-fluorescent bead population (blank). The MT MFI value, expressed as average fluorescence channel (arbitrary units scaled from 0 to 1024), was transformed into a MESF value by means of linear regression analysis and normalized by a reference sample, previously cryopreserved, used as an internal control.

Determination of Total Free Cysteine in Whole Blood, Plasma Protein Carbonyls 3-Nitrotyrosine, and Malondialdehyde

The measurements of the redox biomarkers were performed as previously reported (27).

Extraction of Total RNA and Real-Time PCR Assay

Total RNA from PBMCs was extracted using the RNeasy kit (Qiagen, Italy) according to the manufacturer's instruction and quantified by NanoDrop spectrophotometer.

cDNA synthesis from total RNA was performed using i-Script reverse transcriptase (Bio-Rad, Hercules, CA) according to the manufacturer's guidelines. The resulting cDNA were used for real-time PCR assay to detect the expression levels of β-actin housekeeping gene as well as MT1A, MT1E, MT1F, MT1X, MT1H, MT2A, SLC39A2 (ZIP2), SLC39A3 (ZIP3), and SLC30A1 (Znt1).

Primers were designed using the software Beacon Designer 3.0 (Bio-Rad) and reported in Supplementary Table 4S.

One microgram of cDNA was amplified in a total volume of 25 µL containing iQ SYBR GREEN SUPERMIX (Bio-Rad) on a BioRad iQ5 optical realtime PCR (Bio-Rad), employing primer concentrations of 200 nM for MT1H, Mt1X, Mt2A, Znt1, and ZIP3 genes; 150 nM for β-ACTIN, and 300 nM for MT1A, MT1E, MT1F, and ZIP-2 genes. Assays for each transcript were carried out as duplicates. Any inefficiencies in RNA input or reverse transcription were corrected by normalization to the housekeeping gene.

Relative amounts of gene target mRNAs were calculated based on standard curves prepared by a serial dilution of control cDNA. The fold changes were calculated using the comparative CT method [$\Delta\Delta C_t$ (Cycle Threshold)].

Statistical Analysis

Subject's characteristics were reported as mean ± standard deviation (SD) or percentages for continuous and categorical variables, respectively. For continuous variables, normal distribution was verified by the one-sample Kolmogorov–Smirnov test. Differences among groups were checked by one-way analysis of variance for continuous variables and Pearson's χ^2 test for categorical variables. All the variables not normally distributed were log-transformed.

The one-way nonparametric ANOVA (Kruskal–Wallis test), analysis and (after correction for several confounding factors) were used to determine if there was a difference in Zn-induced MT in relation to age groups GO, SGO and RASIG and other categorical variables. When a significant *p*-value was found ($p < .05$), a multiple comparison test by generalized linear models of Zn-induced MT in lymphocytes and monocytes (linear model with log-transformed values and identity link-function) and generalized mixed linear models with random effects were used to determine differences among groups. We used Spearman correlations to assess associations between Zn-induced MT and other variables. All the analyses were performed using the SPSS/Win program (version 22.0; SPSS, Inc., Chicago, IL).

Results

Participant's Characteristics

The characteristics of the MARK-AGE study participants are presented in Table 1 and Supplementary Table 1S. Two thousand nine hundred and thirty-six subjects with mean age of 58.2 ± 10.8 have been subdivided in four age classes (35–44; 45–54; 55–64; 65–75). The percentage of males was similar in all the age groups.

The prevalence of smoking habits was higher in younger subjects as compared to older ones ($p < .001$), while BMI was increased in subjects with age >55 years (Supplementary Table 1S, $p < .001$) according to a previous report (28).

The significant differences in the mean age among the recruiting centers (Supplementary Table 1S, $p < .001$) were due to a different subject group distribution by centers and to the presence of young individuals only in RASIG group (Table 1). C-reactive protein (CRP), homocysteine levels, serum glucose, and glycosylated hemoglobin A1C levels and free fatty acids were increased in older donors (Supplementary Table 1S, $p < .001$). Lipid profile differed in relation to age as also previously reported in the same cohort (28). In particular, we observed that total cholesterol and LDL cholesterol were higher in subjects with age >45 years than in subjects with an age range of 35–44 years. The same concerned triglycerides levels in the 55–64 age class compared to younger ages (Supplementary Table 1S, $p < .001$). Fibrinogen levels increased significantly with age (Supplementary Table 1S, $p < .001$), while HDL levels together with white blood cell, monocyte and lymphocyte counts were similar throughout all age classes.

Table 1 reports the characteristic of subject groups.

GO and SGO groups consist of individuals of higher age and show lower prevalence of current smokers but similar percentage of males as compared to the RASIG group.

Table 1. Characteristics of RASIG, GO, and SGO Subjects

	RASIG (a)	GO (b)	SGO (c)	*p*-Value
Age (y)	56.2 ± 11.3	64.6 ± 5.8[a]	63.8 ± 6.2[a]	**0.001**
Male % (*n*)	47.1 (1011)	42.4 (220)	50.4 (134)	**0.067**
Smoker				
Never	51.7 (1111)	48.7 (253)	48.5 (129)	**0.001**
Former	30.1 (646)	39.3 (204)	42.5 (113)	
Current	18.2 (390)	11.9 (62)	9.0 (24)	
BMI (kg/m^2)	26.3 ± 5.9	27.04 ± 9.3	27.3 ± 4.5	0.42
Country				
Finland	4.2 (90)	27.1 (141)	20.3 (54)	**0.001**
Italy	17.4 (373)	25.0 (130)	25.6 (68)	
Austria	17.4 (374)	0 (0)	0 (0)	
Greece	14.7 (316)	3.1 (16)	1.9 (5)	
Poland	18.4 (397)	11.8 (61)	13.2 (35)	
The Netherlands	0 (0)	17.0 (88)	24.4 (65)	
Belgium	11.2 (241)	16.0 (83)	14.7 (39)	
Germany	16.7 (359)	0 (0)	0 (0)	
CRP (mg/L)	2.1 ± 3.1	2.6 ± 4.2	2.6 ± 3.8	0.092
Homo (µmol/L)	15.05 ± 5.99	15.84 ± 7.00	15.38 ± 6.01	0.058
FSG (mmol/L)	5.20 ± 1.17	5.34 ± 1.15[a]	5.38 ± 1.19	**0.001**
HbA1c (%)	5.99 ± 0.63	6.10 ± 0.59	6.09 ± 0.56	0.45
TG (mmol/L)	1.25 ± 0.82	1.24 ± 0.77	1.31 ± 0.96	0.60
T-CHOL (mmol/L)	5.54 ± 1.03	5.67 ± 0.93	5.45 ± 1.04	0.19
HDL (mmol/L)	1.53 ± 0.43	1.58 ± 0.44	1.46 ± 0.44	0.084
LDL (mmol/L)	3.31 ± 0.87	3.37 ± 0.80	3.26 ± 0.87	0.53
FFA (mg/dL)	0.66 ± 0.30	0.67 ± 0.30	0.70 ± 0.47	0.62
Fibrinogen (mg/mL)	3.66 ± 1.47	4.29 ± 1.47[a]	4.26 ± 1.46[a]	**0.001**
WBC*	21.7 ± 6.8	5.9 ± 0.1	11.2 ± 3.7	0.71
Mono (*n* × 1000/µL)*	1.48 ± 0.44	0.45 ± 0.072	0.78 ± 0.25	0.58
Lympho (*n* × 1000/µL)*	54.56 ± 16.07	19.67 ± 0.26	32.04 ± 9.26	0.59

Note: Values are mean ± SD or (*) standard error and percentage (number), all such variables; *p*-value: one-way-ANCOVA (continuous variables) including the effects of gender, recruitment center and age as covariates and chi-square test (prevalence). CRP = C-reactive protein; FSG = fasting serum glucose; Homo = homocysteine; HbA1c = glycosylated hemoglobin; TG = triglycerides; T-CHOL = total cholesterol; FFA = free fatty acids; WBC = white blood cell count; Mono = monocytes; Lympho = lymphocytes.

Subject group distribution varied across recruiting centers; no enrolment of SGO and GO individuals occurred in Germany and Austria (Table 1, $p < .001$), while RASIG subjects were not provided by The Netherlands.

Lower serum fasting glucose and fibrinogen levels were found in RASIG subjects as compared to GO only or to both GO and SGO subjects, respectively (Table 1, $p < .001$). No differences in BMI and in any other laboratory parameters were observed (Table 1, $p > .05$).

Zn-Induced MT Association With Age Years and Subject Groups

A representative FC analysis (histograms and dot-plot) of human PBMCs stained with MT polyclonal antibody (FL-61) and gated in lymphocytes and monocytes is reported in Supplementary Figure 1S. The comparison between FC determination by the use of this antibody and of the monoclonal one (clone E9) reported in our previous studies (3) demonstrated a strong correlation coefficient ($R^2 = .98$; Supplementary Figure 2S). Moreover, the basal MT levels measured in a group of 54 healthy Italian elderly (mean of age 70.7 ± 7.5 years; 28 females and 26 males) by the present FC assay (MT median value = 72 pmol/10^7 cells) were comparable (Spearman rho correlation; $R = .43$; $p < .001$; Supplementary Figure 2SB) with those we previously obtained by HPLC-ICP-MS analysis (Malavolta et al. given in Supplementary Materials).

Nonparametric comparison by Kruskal–Wallis test shows that Zn-induced MT is unaffected by age both in lymphocytes and monocytes from the whole population (Supplementary Table 2S, $p > .05$).

Median values of Zn-induced MT in lymphocytes were significantly higher in RASIG as compared to GO also after correction for age, gender, and country by using a generalized linear model (GLM) with linear distribution (Supplementary Table 2S, $p < .05$). A similar trend concerned monocytes of RASIG versus GO. Differences were found also across countries with the highest production in Austria and the lowest one in Poland (Supplementary Table 2S, $p < .001$). No significant differences were found in relation to gender, BMI, smoking habits, and quartiles of Zn plasma levels (Supplementary Table 2S, $p < .05$).

With regard to laboratory measurements, Zn-induced MT levels were not associated with lipid profile (total level of HDL and LDL cholesterol, triglycerides and free fatty acids in serum), serum homocysteine, C-reactive protein, glucose levels, monocyte, and lymphocyte counts (Supplementary Table 3S). On the other hand, an association was observed with glycosylated Haemoglobin A1C and Fibrinogen serum levels. In particular, the Zn-induced MT levels was lower in the second quartile of glycosylated Haemoglobin A1C as compared to the 95th percentile in lymphocytes, while in monocytes the values of 25th percentile was decreased with respect to all the other percentiles (Supplementary

Table 3S, $p < .01$). An opposite trend was observed in relation to fibrinogen's quartiles, particularly in lymphocytes (Supplementary Table 3S, $p < .01$).

Basal Levels of MT and Zn Transporters Gene Expression in PBMCs From a Subgroup of GO, SGO, and RASIG Subjects

We randomly selected 120 subjects from the GO, SGO, and RASIG groups (each group consisted of 40 subjects equally spitted into two subgroups of 20 based on the high or low MT protein induction upon Zn treatment of PBMCs) matched for age (age range 61–75 years), gender and country to measure the basal MT and Zn transporters gene expression. In particular, we assessed MT1A, MT1E, MT1F, MT1X, MT1H, MT2A, SLC39A2 (ZIP2), SLC39A3 (ZIP3), and SLC30A1 (Znt1) genes transcripts. We found an increase of MT1A and ZnT-1 in GO as compared to RASIG and SGO and of MT1X in GO compared to RASIG (Figure 1, $p < .05$). No differences were observed for MT1H, MT2A, ZIP2, and ZIP3, while MT1E and MT1F isoforms were undetectable.

Bivariate Correlation Among Zn-induced MT and Gene Expression of MT and Zn Transporters

Spearman's correlation demonstrated that Zn-induced MT of both lymphocytes and monocytes were inversely correlated with mRNA levels of MT and Zn transporters genes. However, while this correlation among MT2A, MT1X, and Zn-induced MT levels is independent of the cell type (Table 2), the negative correlation among Zip3, Znt1 genes and Zn-induced MT levels was observed only in monocytes (Table 2).

By contrast, no significant correlation was found among Zn-induced MT and MT1A, MT1H, and Zip2 mRNA levels.

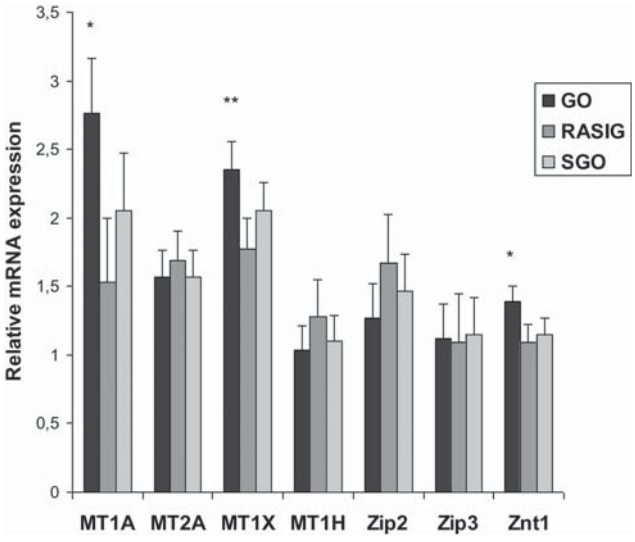

Figure 1. Gene expression level of MT isoforms and Zn transporters in PBMCs from GO, SGO, and RASIG participants. Expression levels of MT1A, MT2A, MT1X, MT1H, Zip2, Zip3, and Znt1 mRNA in PBMCs from 150 GO, SGO, and RASIG elderly subjects (age range 61–75 years). Significant differences in the mRNA expression levels of MT1A, MT1X, and ZnT1 across GO, SGO, and RASIG subjects. Data are expressed as relative to the delta CT mean of GO samples. Values shown are means ± SEM. *$p < .05$ as compared to RASIG and SGO subjects; **$p < .05$ as compared to RASIG subjects.

Zn-Induced MT in GO, SGO, and RASIG Subjects

The possible modulation of Zn-induced MT among GO, SGO, and RASIG subjects was tested using ANCOVA analyses after adjusting for effects of age, gender, and country.

As shown in Figure 2, GO subjects showed lower Zn-induced MT in lymphocytes (Figure 2A, $p < .05$) as compared to RASIG, while only a trend was found for monocytes (Figure 2B). No significant differences were observed between GO and SGO participants, although these last ones had values similar to RASIG. Notably, differences in lymphocytes Zn-induced MT between GO and RASIG participants remained significant even when our analysis was restricted to individuals aged more than 54 years to uniform the age range across all subject groups (Figure 2C and D, $p < .001$).

To further confirm our results, we performed a generalized mixed linear models analysis that included the identification number of each MT measurement session as random effects in order take into account possible batch effects. As shown in Supplementary Figure 3S, this analysis confirmed the decrement of Zn-induced MT in lymphocytes from GO participants compared to RASIG.

Relationship Among Zn-Induced MT and Redox Biomarkers

We performed an ANCOVA analysis after correction for age, gender, country to evaluate the relationship among Zn-induced MT and redox biomarkers (Figure 3).

Zn-induced MT levels in lymphocytes were higher at the lower percentile of 3-nitrotyrosine, malondialdehyde, protein carbonyls in plasma samples and of total cysteine in the whole blood considering both RASIG and whole population ($p < .001$; $p < .01$; $p < .05$). A negative correlation among Zn-induced MT in lymphocytes with redox biomarkers was also observed (Supplementary Figure 4S and Supplementary Table 5S).

Discussion

Metallothioneins are stress-response factors that mediate potent cytoprotective responses, most notably against oxidative stress damage (2). Their action against environmental stress or toxins favors organism survival and might improve the lifespan. Overexpression of MT has been associated with extended longevity in animal models (8–10), while findings in human peripheral blood mononuclear cells show an inverted U-shaped relationship with aging, which is consistent with an example of antagonistic pleiotropy. In other words, the defensive response offered by MT against various types of stress associated with aging, may have the risk of becoming potentially deleterious, thereby contributing to the progression of aging and age-related diseases. This is a concept that recurs for many hallmarks of aging related to stress response and might explain the late life decreased expression of MT as a selection of low MT producers (19). However, a reduction of MT levels has been also reported in aged skin (29), in senescent $CD4^+$ T-cell clones (19) and in peripheral blood cells with critically shortened telomeres (30), thus, suggesting that the age-related accumulation of senescent cells and their reduced proliferation rate may be responsible for the decreased expression of MT in very old age. As a consequence of various signal transduction pathways originated by oxidative stress as well as nutritional, toxicological and inflammatory stimuli, the uptake or mobilization of intracellular Zn ions mediates the induction of MT (2,21).

Hence, the assessment of Zn-induced MT protein levels may represent an indirect measurement of the cell capability to respond to

Table 2. Bivariate Correlation Between Zn-Induced MT and MT and Zn Transporters Transcript Levels

	MT1A	MT2A	MT1X	MT1H	Znt1	Zip2	Zip3
	Correlation Coefficient, *p Value*						
Zn-i MT in lymphocytes	−0.023	−0.168	−0.21	−0.024	−0.157	0.057	−0.152
	0.803	***0.048***	***0.021***	*0.848*	*0.085*	*0.554*	*0.096*
Zn-i MT in monocytes	−0.048	−0.203	−0.276	−0.075	−0.248	−0.024	−0.232
	0.599	***0.026***	***0.002***	*0.545*	***0.006***	*0.807*	***0.011***

Note: Zn-i MT = Zn-induced MT. Spearman's correlation among Zn-induced MT in lymphocytes and monocytes with mRNA levels of MT and Zn transporters genes. The bold and italic values are attributed to the significant *p* values <.05.

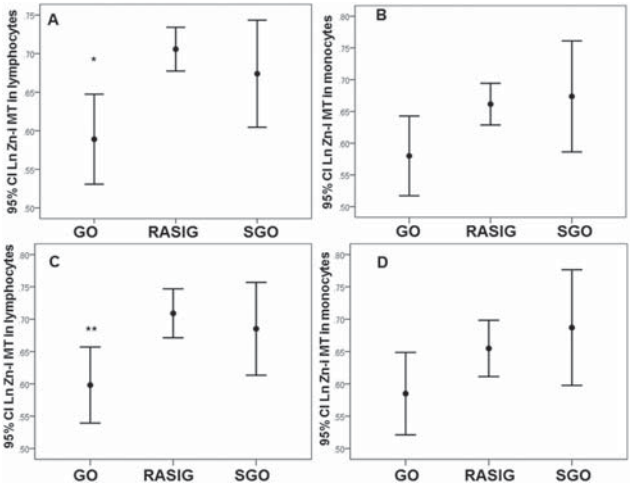

Figure 2. Zn-induced MT protein levels of lymphocytes and monocytes from GO, SGO, and RASIG either in the whole age range or in individuals aged > 54 years. Graphical representation (box-plot) of Zn-induced MT protein levels in lymphocytes (**A**) and monocytes (**B**) from GO (*N* = 520), SGO (*N* = 266), and RASIG (*N* = 2,150) of all ages. Zn-induced MT in lymphocytes (**C**) and monocytes (**D**) from GO (*N* = 499), SGO (*N* = 248), and RASIG (*N* = 1,212) with age >54 years. Analysis was performed in individuals aged above 54 years due to nonrepresentative numbers of GO and SGO below this age. **p* < .05; ***p* < .001; as compared to RASIG by ANCOVA analysis after correction for age, gender, and country.

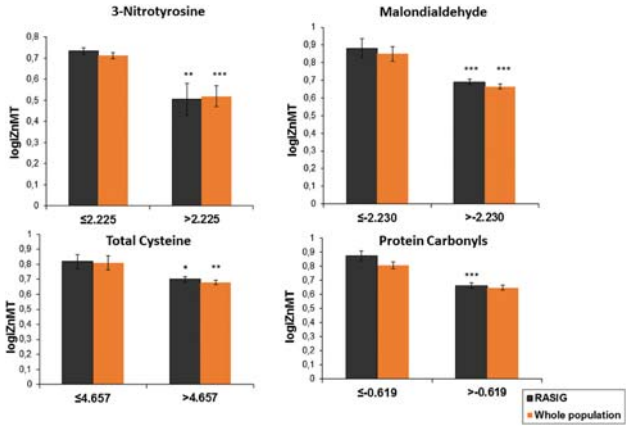

Figure 3. Zn-induced MT protein levels in lymphocytes according to median categories of oxidative stress biomarkers. iZnMT log-transformed values in relation to the median categories of 3-nitrotyrosine, malondialdehyde, and protein carbonyls in plasma samples as well as of total cysteine in the whole blood measured in RASIG and whole population ****p* < .001; ***p* < .01; **p* < .05 as compared to the 50th percentile of the log transformed oxidative stress variables by ANCOVA analysis after correction for age, gender, and country.

stress stimuli, but whether intrinsic processes associated with aging affect this function it is not at present explored.

In this study, we provide evidence that Zn-induced MT in lymphocytes and monocytes does not change with aging, at least in the age range considered here (35–75 years). However, Zn-induced MT levels in lymphocytes are surprisingly lower in centenarian offspring (GO) than in RASIG. A decreasing trend also concerns monocytes, albeit statistically not significant. The lower MT induction by Zn, observed in this leukocyte population with respect to lymphocytes, may depend on the higher basal MT gene expression in monocytes than lymphocytes (31). No significant differences were found between centenarian offspring and their spouses probably due to the relatively small number of subjects in this last group.

We have also screened 120 MARK-AGE elderly subjects subdivided in GO, SGO, and RASIG for the basal expression of MT genes in PBMCs. Results show that certain MT gene transcripts (ie, MT1A and MT1X) are upregulated in GO donors as compared to RASIG, which suggests that stress response pathways are activated in centenarian offspring. We have not collected data on MT basal protein levels, as cells were kept in the same culture conditions for 24 hours before the treatment with Zn. In these conditions, basal MT levels tend to normalize (data not shown) and, most importantly, these levels are negligible compared to the five- to sixfold increment, obtained after treatment with 50 µM of Zn sulfate (32).

MT and ZnT1 are described as the main responders to acute changes in cellular Zn (33) and the higher mRNA levels in GO participants with respect to RASIG, suggest that centenarians' offspring can keep labile Zn levels under better control than individuals undergoing normal aging. Hence, efficient Zn homeostatic machinery can be part of the longevity phenotype. It is interesting to note that, although we did not detect any age-related change of Zn-induced MT, the absolute values of the oldest group were the lowest in our population.

An efficient Zn homeostatic machinery is relevant for cell survival and function, as well as for efficient energy metabolism (2,34), while an alteration of this machinery has been correlated with age-related diseases (35,36).

The low Zn-MT induction in centenarian offspring may also be consistent with our knowledge about the deregulation of nutrient-sensing pathways in aging. This possibility is also supported by the negative correlation between Zn-induced MT and the basal expression of some MT isoforms and Zn transporters herein found.

The sensitivity of nutrient-sensing pathways has shown to decline with age in several organisms (37). A decreased insulin and insulin-like growth factor-1 (IIS) pathway is a common characteristic of

both physiological and accelerated aging but, paradoxically, a constitutively decreased IIS extends longevity (38). Impaired insulin or TOR signaling extend lifespan in multiple organisms and delays the age-dependent functional decline and the onset of age-related diseases such as cancer, diabetes, and cardiovascular diseases (39,40). Moreover, both insulin and TOR signaling have been shown to be tightly regulated by Zn signals, so much so that insulin-like or insulin-promoting effects of Zn have been reported in cellular (41,42) and animal models (43). Recent data obtained in worms have shown an impact of Zn on lifespan mediated in part by the insulin/IGF-1 pathway. Increasing Zn levels reduced the mean and maximum lifespan, whereas lowering Zn levels by means of a Zn-selective chelator increased the mean and maximum lifespan in Caenorhabditis *elegans*. Hence, regulation of Zn homeostasis in the worm may be an example of antagonistic pleiotropy (44).

A "reduced sensitivity" of Zn-dependent pathways with age is supported by the evidence of impaired intracellular Zn and Zn uptake in PBMC from elderly as compared to young subjects (35). In addition, plasma Zn is known to decline with aging independently of Zn dietary intake (45). In aged mice, an age-related epigenetic deregulation of Zn transporter expression has been associated with reduced intracellular Zn in immune cells (5), while a recent study demonstrated that fibroblasts from long-lived rodent species had reduced rates of metal uptake (cadmium and Zn) as compared to those from short-lived species (47), suggesting a more effective defense mechanism against metal toxicity with longevity. All these observations support the idea that the phenomenon called age-related Zn deficiency, when not caused by a proven dietary deficit or reduced absorption, may indeed reflect a deregulation of Zn sensing pathways. Interestingly, we found that a reduced capability of MT induction is associated with higher oxidative stress parameters such as plasma protein carbonyls, 3-nitrotyrosine and malondialdehyde in the whole population. These findings confirm the role of MT in counteracting oxidative stress. However, in centenarian offspring the lower oxidative stress status than in RASIG subjects (27) may result from other antioxidant defense mechanisms as supported by higher levels of total cysteine and other antioxidants (27). Epigenetic mechanisms may also be involved in the different regulation of Zn induced MT production between GO and RASIG. In fact, a reduced DNA methyltransferase DNMT1 expression has been found in GO with respect to RASIG, that is associated with a global hypomethylation and hypermethylation of specific gene promoters (48). This point suggest that epigenetic factors may be involved in our findings concerning lower Zn-induced MT protein levels in GO individuals than RASIG, as also supported by the association between low DNMT1 mRNA levels and low MT induction (Supplementary Figure 5S).

Reduced induction of MT in centenarian offspring might also depend on increased degradation pathways related to autophagy. In fact, it has been demonstrated that autophagy and proteasome activity are enhanced in longer-lived species (49) and it has been suggested that Zn-induced MT may be autophagocitated to improve lysosome stability by reducing both intralysosome oxidation and lysosomes membrane destabilization (50). Moreover, autophagy appears to be better maintained in members of families with extended longevity and positively correlates with improved T-cell function (51). These highly suggestive indications deserve to be investigated and confirmed in the centenarian offspring.

We have also found a positive association between glycosylated hemoglobin A1C and Zn-induced MT. Interestingly, this association appears to confirm in a large population setting previous evidence linking Zn and MT functions with energy metabolism, ATP

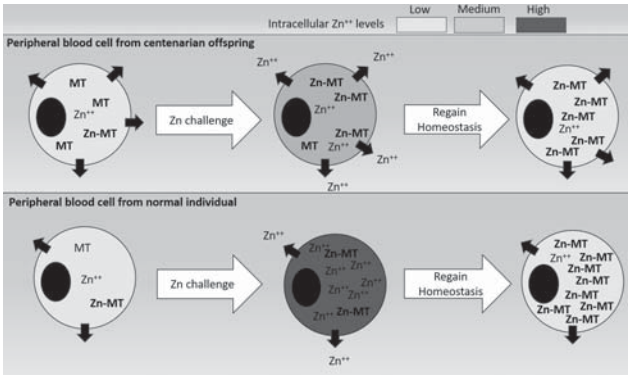

Figure 4. Control of Zn homeostasis and MT induction in peripheral blood cells from centenarian offspring compared to normal individuals. Induction of MT in response to Zn or other stressors exerts in general a protective function. However, cells from centenarian offspring display higher basal levels of MT and Zn exporters (black arrows in cell membrane). This condition allows a tight control of Zn homeostasis that limit intracellular Zn ions levels during a Zn challenge. Once the cell regains the homeostasis this is reflected by a lower induction of MT in cells from centenarian offspring as compared to normal aged subjects (RASIG).

regulation, and endocrine signaling (5). Of particular relevance are findings showing that MT may have a protective role against insulin resistance. In fact, glucose treatment increases the cytosolic concentration of free Zn via the upregulation of MT gene expression in pancreatic islet β-cells (52). In addition, overexpression of MT prevents diabetes-linked pathological changes of organs (13). Although all these evidence may reasonably support a role for MT in longevity, further research is needed to clarify the exact mechanisms involved.

In summary, Zn-induced MT in lymphocytes and monocytes are not associated with age, but are decreased in lymphocytes from centenarian offspring. Our results confirm the role of MT in counteracting oxidative stress and reinforce the hypothesis around the existence of a distinctive intrinsic (eg, epigenetic) regulation of Zn homeostasis in centenarian offspring. This is suggestive of a hypothesis whereby an increased resistance to Zn treatment is a consequence of a tighter control of Zn homeostasis in blood cells of potential long living people compared to the general population (the hypothesis is schematically illustrated in Figure 4).

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Conflict of interest.

None declared.

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