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RESEARCH PAPER

Influence of +1245 A/G MT1A polymorphism on advanced glycation end-products (AGEs) in elderly: effect of zinc supplementation

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Abstract Advanced glycation end-products (AGEs) stimulate reactive oxygen species (ROS) generation and represent a risk factor for atherosclerosis, while their formation seems to be prevented by zinc. Metallothioneins (MT), zinc-binding proteins exert an antioxidant function by regulating intracellular zinc availability and protecting cells from ROS damages. +1245 A/G MT1A polymorphism was implicated in type 2 diabetes and in cardiovascular disease development as well as in the modulation of antioxidant response. The purpose of this study was to investigate the influence of +1245 A/G MT1A polymorphism on AGEs and ROS production and to verify the effect of zinc supplementation on plasma AGEs, zinc status parameters and antioxidant enzyme activity in relation to this SNP. One hundred and ten healthy subjects (72 ± 6 years) from the ZincAge study were supplied with zinc aspartate (10 mg/day for 7 weeks) and screened for +1245 MT1A polymorphism. +1245 MT1A G+ (Arginine) genotype showed higher plasma AGEs and ROS production in peripheral blood mononuclear cells (PBMCs) than G− (Lysine) one at the baseline. No significant changes after zinc supplementation were observed for AGEs, ROS and MT levels as well as for enzyme antioxidant activity in relation to the genotype. Among zinc status parameters, major increases were observed for the intracellular labile zinc (iZnL) and the NO-induced release of zinc in PBMCs, in G+ genotype as compared to G−

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one. In summary, +1245 G+ carriers showed increased plasma AGEs and ROS production in PBMCs at baseline and a higher improvement in iZnL after zinc intervention with respect to G− individuals.

Keywords MT1A polymorphism · Advanced glycation end-products (AGEs) · Zinc supplementation · Aging · Intracellular free zinc

Introduction

Non-enzymatic modification of proteins by reducing sugars leads to the formation of newly modified molecular species known as "advanced glycation end-products" (AGEs). These reactions take part during aging and substantially accelerate during diabetes, and atherosclerosis generating and accumulating AGEs at many sites of the body including the heart and large blood vessels as a result of chronic hyperglycemia and enhanced oxidative stress (Basta et al. 2004). AGEs trigger proinflammatory, profibrotic and procoagulant cellular responses that are capable of damaging tissues, leading to vascular dysfunction, promoting atherosclerosis and cardiovascular disease (Daroux et al. 2010; Jandeleit-Dahm and Cooper 2008).

Some authors demonstrated an inhibitory effect of zinc on in vitro albumin glycation (Tupe and Agte 2010; Seneviratne et al. 2011), suggesting this oligoelement as a potent agent in reducing AGE formation. A more recent study reports the effect of zinc treatment in restoring NO production through eNOS expression and reactivation, and in suppressing NF-κB activation in AGE-pretreated endothelial cells (Zhuang et al. 2012). Therefore, zinc protects from endothelial dysfunction as well as from oxidative stress and regulates the inflammatory response (Giacconi et al. 2012; Bao et al. 2010; Wong et al. 2013). A growing body of evidence suggests that an abnormal zinc homeostasis may be involved in the pathogenesis of atherosclerosis and type 2 diabetes (DM-2) (Miao et al. 2013; Jansen et al. 2012; Mocchegiani et al. 2008a).

The intracellular zinc buffering and the modulation of transient changes in cytosolic zinc ion concentration is tightly controlled by metallothioneins (MT) (Colvin et al. 2010), which are low molecular antioxidant zinc-binding proteins (Mocchegiani et al. 2008a). During aging, the increased reactive oxygen species (ROS) production and the chronic inflammation may lead to MT dysfunction with consequent zinc dyshomeostasis and cardiovascular disease (CVD) appearance (Giacconi et al. 2010; Barbato et al. 2007).

Polymorphisms of these genes have been associated with reduced intracellular zinc ion availability and with DM-2 or CVD development (Giacconi et al. 2008, 2010; Yang et al. 2008).

+1245 A/G MT1A polymorphism (rs 8052394) is implicated in DM-2 onset, and it influences serum SOD activity in diabetic Chinese patients (Yang et al. 2008), while our evidence show an increased susceptibility of the MT1 ACG haplotype to CVD in the Greek population (Giacconi et al. 2010).

Zinc supplementation trials have demonstrated beneficial effects of zinc in restoring metal cation homeostasis, the enzyme antioxidant activity and the immune-inflammatory response (Mocchegiani et al. 2008b; Mariani et al. 2008a; Bao et al. 2010) and suggested an important role for the gene nutrient interaction (Mariani et al. 2008b; Mocchegiani et al. 2012).

On this basis, the aim of the present study was to investigate the influence of +1245 A/G MT1A polymorphism on AGEs and ROS production and to verify the benefit of zinc supplementation in vivo on plasma AGEs, zinc status, antioxidant enzyme activity [superoxide dismutase (pSOD), glutathione peroxidase (GPx), catalase (CAT)] and lipid profile in relation to this SNP.

Materials and methods

Subject population

The study included 110 healthy randomly selected subjects (57 men and 53 women; mean age 72 ± 6 years, range 65–85) enrolled in the ZincAge project. Subjects were recruited by five European centers located in Italy, Greece, Poland, France and Germany (Mocchegiani et al. 2008b).

All subjects were in good health condition and without functional impairment, according to the inclusion criteria of the ZincAge protocol. In particular, healthy elderly non-institutionalized men and women were selected on the basis of the SENIEUR protocol for immuno-gerontological studies. The participants of the study had to be free of medication such as steroids, diuretics, anticonvulsants, anti-depressive drugs, antibiotics, antimetabolites, nonsteroid anti-inflammatory drugs and micronutrient supplementation. Subjects were excluded if they had autoimmune, neurodegenerative, cardiovascular, kidney or liver diseases, diabetes, infections, cancer, chronic inflammatory bowel disease or acrodermatitis enteropathica, sickle cell anemia, chronic skin ulcerations and endocrine disorders.

Healthy status was evaluated by a specific questionnaire on health and morbidity planned for the study. Ethical approval was obtained by all centers performing the recruitment and all subjects signed an informed consent form.

Zinc supplementation was performed with 10 mg/day of zinc aspartate (Unizink 50, KÖ HLER PHARMA Corp.,

Alsbach-Hähnlein, Germany) in Italy, Germany, France and Greece. In Poland, zinc supplementation was carried out with 10 mg/day of an identical form of zinc aspartate (Zincas, Zakład Chemiczno-Farmaceutyczny FARM-APOL, Poznan, Poland) approved by the respective local University Health Authorities. The supplementation period was 48 ± 2 days.

Laboratory measurements and biological sample separation

Venous peripheral blood samples, collected after an overnight fast, underwent basal biochemical laboratory determinations. Leucocytes and hemoglobin counts were performed by standard automated procedures (Sysmex XE-2100). The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by an UV test according to a standardized method on Roche automated clinical chemistry analyzers; the rate of the photometrically determined coenzyme nicotinamide adenine dinucleotide (NADH) decrease is directly proportional to the rate of formation of pyruvate or oxaloacetate and thus, respectively, the ALT or AST activity. The total cholesterol, HDL-cholesterol, triglycerides, fasting glucose and albumin were measured by an enzymatic colorimetric test on automated clinical chemistry analyzers (Roche-Hitachi). Serum concentration of high sensitive C-reactive protein (hs-CRP) was determined by amplified immunonephelometry assay (CardioPhase hsCRP-Dade Behring Inc Deerfield, IL). Serum, plasma, granulocytes, PBMCs and buffy coats were separated, aliquoted and stored frozen at −80 °C in the Biological Bank of INRCA until analysis.

Assessment of dietary zinc intake

A qualitative food frequency questionnaire, designed for the needs of ZincAge project, was used for the assessment of dietary zinc intake in healthy elderly subjects. The consumption of 53 food items was recorded and, based upon these data, a "zinc score" for each volunteer was determined. To provide a continuous variable, representative of zinc dietary habits, frequency, quantity estimation and zinc content of foods consumed were all considered for the "zinc score" calculation (zinc score = frequency × quantity × zinc content). A validation study of the "zinc score" has been previously reported (Kanoni et al. 2010).

AGEs measurements

For the measurement of the AGE autofluorescence in the plasma samples, plasma was diluted 1:150 in PBS. 200 μL of the diluted plasma samples were in triplicate brought on a 96-well microtiterplate. The AGE intrinsic fluorescence (360 nm excitation and 440 nm emission) was measured with a plate reader (FluoStarOptima). For calculation of the concentration of the AGE modification, in vitro-modified human plasma was used as standard (0.001, 0.003, 0.01, 0.03, 0.11, 0.33, 1, 3, 10 μg modified plasma protein/mL). The measured fluorescence was normalized to the plasma total protein concentration (Simm et al. 2007).

Determination of total reactive oxygen species production

Total cellular reactive oxygen species (ROS) production in thawed PBMCs were analyzed by flow cytometry after loading of cells with a highly sensitive fluorescent probe [5 and 6 chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA)].

About 250,000 cells were incubated at 37 °C for 30 min in the dark with 2 μM of probe in PBS buffer. CM-H_2-DCFDA is cleaved by intracellular esterases and transformed into a fluorescent dye when oxidized. Cells were then washed with PBS and analyzed by flow cytometry (Coulter Epics XL).

The mean fluorescence intensity (MFI) of 5,000 cells (corrected for autofluorescence) for each subject before and after zinc supplementation was taken as a measure for the total ROS load.

Genotyping of +1245 A/G MT1A polymorphism

Genomic DNA of PBMC was extracted by the phenol chloroform method, according to the standard procedure.

We screened the +1245 A/G SNP in the coding region of MT1A, corresponding to a Lys51Arg amino acid change. PCR restriction fragment length analysis (PCR–RFLP) was performed, as previously described (Giacconi et al. 2008).

Multiple alignment of MT proteins in Vertebrata Subphylum

Sequence alignments were performed using protein BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PAGE=Proteins) to verify whether the mutated allele (Arg51) is located in a conserved region of the MT protein (Electronic Supplementary Material, Table 5).

Analysis of tridimensional structure of MT1A Lys51Arg variants

We used I-TASSER online Structure & Function Prediction server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) for protein 3D structure prediction (Electronic

Supplementary Material, I-TASSER results for MT1A Lys51 and MT1A Arg51 variants).

Zinc, concentrations in plasma, and granulocytes

Zinc concentrations in plasma and granulocyte samples were determined with Thermo XII Series ICP-MS (Thermo Electron Corporation, Waltham, MA, USA), following the manufacturer's instructions (AN_EO604) with slight modifications (Malavolta et al. 2010). Plasma samples were diluted 1:10 and granulocytes 1:26 with a diluent, containing 0.1 % triton, to maintain a stable emulsion with the diluted sample and 0.15 % HNO_3, to ensure solubility of the trace elements, in order to achieve washout of these elements between samples. External calibration solutions containing Zn (blank to 2,000 ppb) were prepared by serial dilution of a parent multi-element solution (1,000 ppm for Zn) (VHG Labs, Manchester, USA), using the same diluent as for the samples. Rhodium (Rh) at 200 ng/mL was used as internal standard. Data were acquired for 66Zn. Quality of the analysis was assured by assessment of "quality standard samples" (SERONORM^{TM} TRACE ELEMENT SERUM, Sero AS, Billingstad, Norway). Zinc levels of the quality standard samples were within 10 % of the certified levels, as previously reported (Malavolta et al. 2010).

Limits of detection estimated with the post-column calibration were 5 ppb for 66Zn. The instrument was operated with a Peltier cooled impact bead spray chamber, single piece quartz torch (1.5 mm i.d. injector) together with Xi interface cones and a Cetac-ASX 100 autosampler (CETAC Technologies, Omaha, NE, USA). A Burgener Trace nebulizer was used as this device does not block during aspiration of clinical samples. The instrument was operated in standard mode (non-CCT), using 1,400 W RF power, 1.10 L/min nebulizer gas flow, 0.70 L/min auxiliary gas flow, 13.0 L/min cool gas flow, 70 ms dwell time, 30 s sample uptake 35 s wash time (2 repeats per sample).

Flow cytometric analysis of zinc ion availability and NO-induced release of zinc

Zinc Ion Availability and NO-induced release of zinc (iZnR) were tested as previously reported (Malavolta et al. 2006).

Briefly, thawed PBMC were divided into three equal aliquots of 2×10^5 cells, incubated with 20 μM Zinpyr-1 (ZP-1) (Neurobiotex, Galveston, TX, USA) for 30 min at 37 °C, 5 % CO_2 in HEPES-buffered zinc-free RPMI medium containing 1 mM EDTA, as an extracellular chelator, of free zinc, which remained in the medium and/or was adsorbed to the cell membrane. In the second aliquot, was added 50 μM *N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (Sigma-Aldrich), to detect the autofluorescence of the zinc-free ZP-1 probe, which represented the minimum of mean fluorescence intensity (MFI_{min}) for ZP-1 fluorescence. The last aliquot was incubated in the same condition plus 500 μM diethylamine NOnoate acetoxymethylated (AcOM-DEA/NO) (Calbiochem, VWR International s.r.l., MI, Italy) to detect the release of intracellular free zinc from MT (iZnR).

Table 1 Laboratory and biochemical parameters of study subjects

	Males $n = 57$	Females $n = 53$	p value
Age (years)	72.2 ± 6.5	71.9 ± 6.9	NS
WBC (10^3/μL)	6.3 ± 1.7	6.4 ± 1.3	0.02
Lymphocytes (%)	29.6 ± 7.6	32.3 ± 6.7	0.044
Neutrophils (%)	58.4 ± 8.1	53.7 ± 5.8	0.002
Monocytes (%)	7.2 ± 1.5	7.0 ± 1.8	NS
Erythrocytes (10^6/μL)	4.9 ± 0.4	4.5 ± 0.4	0.001
Hemoglobin (g/dL)	15.2 ± 1.2	14.1 ± 1.2	0.001
AST (U/L)	25.0 ± 6.8	21.8 ± 8.4	0.036
ALT (U/L)	33.0 ± 11.9	30.3 ± 13.1	NS
Creatinine (mg/dL)	1.02 ± 0.18	0.88 ± 0.2	0.001
CRP (pg/mL)	0.48 ± 0.69	0.36 ± 0.31	NS
Albumin (g/dL)	4.1 ± 0.24	4.0 ± 0.22	0.047
Glycemia (mg/dL)	96.8 ± 11.0	96.4 ± 14.6	NS
TG (mg/dL)	119.4 ± 59.6	131.9 ± 55.2	NS
TC (mg/dL)	211.4 ± 28.2	233.5 ± 45.6	0.001
HDL-C (mg/dL)	48.5 ± 28.2	55.5 ± 16.4	0.008
AGEs (ng/mL)	1.51 ± 0.63	1.34 ± 0.56	0.166
ROS (MFI)	15.3 ± 12.6	12.0 ± 11.8	0.201
pSOD (U/mL)	21.74 ± 3.34	21.13 ± 2.60	0.367
GPx (nmol NADPH/min/mL)	0.099 ± 0.004	0.100 ± 0.007	0.930
CAT (μmol/min/mg prot)	21.47 ± 2.61	21.07 ± 2.97	0.528
Zn plasma levels (μM)	10.67 ± 2.39	10.85 ± 2.45	0.734
Zn granulocytes (nmol/mg protein)	0.21 ± 0.13	0.20 ± 0.11	0.718
MT (MFI)	98.2 ± 40.8	82.5 ± 31.2	0.161
iZnL	1.24 ± 0.10	1.29 ± 0.10	0.035; 0.070[a]
iZnR	0.17 ± 0.04	0.18 ± 0.09	0.410
Zinc score	130 ± 57	121 ± 51	0.020; 0.030[a]

Data are mean ± SD

WBC white blood cells, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *CRP* C-reactive protein, *TG* triglycerides, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *AGEs* advanced glycation end-products, *ROS* reactive oxygen species, *pSOD* plasma superoxide dismutase, *GPx* glutathione peroxidase, *CAT* catalase, *MT* metallothioneins, *iZnL* intracellular labile zinc, *NO-iZnR*-induced release of zinc

[a] Comparisons between males and females were performed by ANCOVA analysis adjusting for age and country and lipid profile

Data of zinc ion availability (iZnL) were reported as normalized fluorescence intensity (MFI/MFImin), while iZnR was calculated as the difference between MFI (+NO)/MFImin and MFI/MFImin.

Metallothionein determination

Thawed PBMCs (2×10^5) were treated with 0.3 % paraformaldehyde and stored at 4 °C for 2 days before processing. MT determination was performed using the monoclonal mouse anti-horse Metallothionein clone E9 antibody (Dakocytomation, Denmark). Results are expressed as MFI (Giacconi et al. 2014).

Antioxidant enzyme activity determinations

SOD3 (pSOD) (U/mL), CAT (μmol/min/mg protein) and GPx (nmol NADPH/min/mL) activities in plasma were measured, according to the methods previously reported (Mariani et al. 2006).

Statistical analysis

The differences in allele distribution of +1245 A/G MT1A polymorphism from Hardy–Weinberg's equilibrium elderly subjects were compared by Pearson's Chi-squared test. The effect of polymorphism on continuous variables was investigated by analysis of covariance (ANCOVA) adjusting for confounding factors, such as age, gender and country. Statistical significance was defined as $p < 0.05$. All the analyses were performed using the SPSS/Win program (version 15.0; SPSS Inc., Chicago, IL, USA).

Results

Clinical and biochemical parameters of the subjects

The study population characteristics are reported in Table 1. Comparisons were performed in relation to gender considering the higher dietary zinc intake in males than females ($p < 0.05$). No differences were observed for the age of the subjects.

No significant differences between men and women were found in the percentage of monocytes ALT, CRP, fasting glucose and triglyceride levels.

Conversely neutrophils, erythrocytes, hemoglobin, AST, creatinine and albumin levels were higher in men than women ($p < 0.05$, $p < 0.01$). An increment in the white blood cell count, the percentage of lymphocytes, HDL and total cholesterol serum levels were observed in women as compared to men ($p < 0.05$, $p < 0.01$). Among biochemical variables, no significant differences were found in AGEs, ROS and MT levels, as well as in the antioxidant enzyme activity. With regard to zinc status parameters (zinc plasma levels, zinc concentrations in granulocytes, iZnL, iZnR), higher levels of intracellular labile zinc were observed in women than men, but this difference became not significant after lipid profile correction in the ANCOVA analysis, evidencing an association between low

Table 2 MT1A +1245 A/G genotypic and allelic frequencies in elderly study subjects according to gender

	Males $n = 57$	Females $n = 53$	All subjects $n = 110$
Genotypes			
1245 AA (Lys/Lys)	70.2 % (40)	83 % (44)	76.4 % (84)
1267 AG (Lys/Arg)	28 % (16)	17 % (9)	22.7 % (25)
1267 GG (Arg/Arg)	1.8 % (1)	0 % (0)	0.9 % (1)
Alleles			
A allele	0.84	0.92	0.88
G allele	0.16	0.08	0.12

Pearson $\chi^2 = 3.009$, $df = 2$, $p = 0.22$ (genotypic frequency)

Pearson $\chi^2 = 2.718$, $df = 1$, $p = 0.09$ (allelic frequency)

Lys Lysine, *Arg* Arginine

Table 3 Influence of +1245 MT1A A/G SNP on clinical and biochemical parameters

	G− genotype Baseline	G+ genotype Baseline	*p* value
CRP (mg/dL)	0.55 ± 1.46	0.42 ± 0.58	0.91
Albumin (g/dL)	4.1 ± 0.2	4.0 ± 0.2	0.41
Glycemia (mg/dL)	96.7 ± 13.8	96.1 ± 9.1	0.53
Total cholesterol (mg/dL)	223.2 ± 41.8	219.2 ± 34.3	0.70
HDL-cholesterol (mg/dL)	53.1 ± 14.2	49.2 ± 16.2	0.28
Triglycerides (mg/dL)	122.1 ± 52.8	133.7 ± 72.5	0.24
AGEs (ng/mL)	1.31 ± 0.50	1.66 ± 0.62	**0.036**
ROS (MFI)	4.1 ± 2.1	24.0 ± 18.3	**0.01**
pSOD (U/mL)	21.53 ± 2.85	21.18 ± 3.26	0.65
GPx (nmol NADPH/min/mL)	0.100 ± 0.006	0.099 ± 0.006	0.37
CAT (μmol/min/mg prot)	20.80 ± 2.67	21.40 ± 2.92	0.35
Zn plasma levels (μM)	10.58 ± 2.21	11.12 ± 2.24	0.22
Zn granulocytes (nmol/mg protein)	0.21 ± 0.12	0.20 ± 0.12	0.92
MT (MFI)	87.52 ± 32.13	89.21 ± 38.78	0.88
iZnL	1.27 ± 0.12	1.24 ± 0.09	0.44
iZnR	0.18 ± 0.07	0.17 ± 0.06	0.47

Data are mean ± SD

Comparisons between G+ (AG+GG) and G− (AA) genotype were performed by ANCOVA analysis correcting for age, gender and country

Bold values indicate a significant difference

Table 4 Changes on clinical and biochemical parameters after zinc supplementation according to +1245 MT1A A/G genotypes

	G− genotype	G+ genotype	*p* value
CRP (mg/dL)	62.8 ± 25.9	−1.4 ± 11.1	0.35
Albumin (g/dL)	2.5 ± 0.95	5.2 ± 2.2	0.28
Glycemia (mg/dL)	−1.75 ± 1.9	−1.0 ± 2.8	0.95
Total cholesterol (mg/dL)	−0.1 ± 1.4	1.3 ± 3.0	0.82
HDL-cholesterol (mg/dL)	6.4 ± 1.7	4.3 ± 2.1	0.81
AGEs (ng/mL)	8.3 ± 7.0	−19.8 ± 15.6	0.12
ROS (MFI)	8.2 ± 6.9	−22.4 ± 19.3	0.14
pSOD (U/mL)	14.9 ± 1.2	18.4 ± 2.6	0.28
GPx (nmol NADPH/min/mL)	−0.76 ± 0.22	0.11 ± 0.45	0.086
CAT (μmol/min/mg prot)	−8.9 ± 1.1	−9.4 ± 2.3	0.64
Zn plasma levels (μM)	9.3 ± 2.1	4.5 ± 2.9	0.50
Zn granulocytes (nmol/mg protein)	83.3 ± 21.7	163.9 ± 43.3	0.14
MT (MFI)	64.5 ± 28.4	31.9 ± 67.4	0.58
iZnL	7.0 ± 2.3	19.8 ± 5.6	**0.020**
iZnR	14.5 ± 7.5	60.3 ± 14.6	**0.031**

Data are mean of % changes ± SE

Comparisons between G+ (AG+GG) and G− (AA) genotype were performed by ANCOVA analysis correcting for age, gender and country

Bold values indicate a significant difference

iZnL levels and higher total cholesterol as previously reported in obese subjects (Costarelli et al. 2010).

+1245 A/G MT1A genotype and allele frequency distribution

Allele and genotype distribution of +1245 A/G MT1A polymorphism in the studied population is summarized in Table 2. The observed frequencies of this SNP were compared with the expected frequencies and did not deviate from the Hardy–Weinberg equilibrium ($p > 0.05$). No significant differences were observed in the genotype and allele frequency distribution between males and females.

Association analysis of +1245 MT1A A/G polymorphism with laboratory and biochemical parameters at baseline

+1245 MT1A A/G polymorphism did not affect baseline clinical parameters such as CRP, albumin, fasting glucose levels and lipid profile (Table 3).

+1245 MT1A A/G polymorphism has a significant influence on baseline AGEs and ROS production. In particular, G+ carriers showed higher plasma AGEs associated with increased ROS production in PBMCs than G− carriers (Table 3, $p < 0.05$, $p < 0.01$).

pSOD, GPx and CAT activity were not influenced by MT1A genotypes (Table 3). No significant differences were observed for zinc status parameters (zinc plasma levels, zinc concentrations in granulocytes, iZnL, iZnR) and MT levels (Table 3).

Changes on clinical and biochemical parameters after zinc supplementation according to +1245 MT1A A/G genotypes

The changes in clinical and biochemical parameters after 7 weeks of zinc supplementation were compared between genotypes with adjustment for age, gender and country.

After correction for multiple testing, the effect of zinc intervention on clinical parameters was not different between G− and G+ genotypes (Table 4).

A slight trend for a down-regulation of AGEs and ROS levels was observed in G+ carriers after zinc supplementation (Table 4). No changes on pSOD, GPx and CAT activity and MT were observed between genotypes after zinc supplementation (Table 4).

Among zinc status parameters (zinc plasma levels, zinc concentrations in granulocytes, iZnL, iZnR), significant differences were observed for the intracellular labile zinc and the iZnR in PBMCs, which increased in G+ genotype as compared to G− one (Table 4, $p < 0.05$).

Multiple alignment of MT proteins in Vertebrata Subphylum and analysis of the three-dimensional of MT1A Lys51Arg variants

+1245 MT1A polymorphism yields a Lys51Arg amino acid ex-change. Results from multiple alignment of MT proteins in Vertebrata Subphylum show that Lysine is phylogenetically conserved at position 51, in several classes and orders of vertebrates (Electronic Supplementary Material, Table 5), with the exception of Amphibia, where Lysine is replaced with Glutamic acid.

Therefore, Lys 51 is located in a conserved region of the MT alpha domain, between two cysteine residues, suggesting a critical role for protein stability and folding.

Both Arginine and Lysine are positively charged basic amino acids and are mostly exposed to protein surface (see SA: Predicted solvent accessibility in Tables 7 and 8 of Electronic Supplementary Material), playing important roles in protein stability by forming electrostatic interactions. However, Arginine forms a higher number of electrostatic interactions compared with Lysine by means the guanidinium group. Due to different steric and electrostatic effects, these two amino acids may also influence the stability of cysteine clusters and consequently the zinc-binding affinity (Sokalingam et al. 2012; Munoz et al. 2000). We have also calculated predicted 3D structure of MT1A

variants from amino acid sequence using I-TASSER online Structure & Function Prediction (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy et al. 2010).

The results demonstrate some changes in the predicted secondary structure and in the predicted solvent accessibility of the cysteine (Cys) residues (Electronic Supplementary Material, Tables 7, 8).

In particular, Cys21 pass from a random coil (in MT1A Lys51) to a beta-strand (in MT1A Arg51), while Cys26 and Cys57 are buried in Lys51 variant and exposed in Arg51 variant.

These data further suggest that Lys51Arg variation in MT1A protein can influence the stability of cysteine clusters and consequently the zinc-binding affinity.

I-TASSER server develops four models of predicted 3D structures in MT1A Lys51 variant and three models of predicted 3D structures in MT1A Arg51 variant. These models are reported in the supplementary material (Electronic Supplementary Material, I-TASSER results1; I-TASSER results1; Figs. 1A B, C, D, 2A, B, 3C). However, the model 1 for both MT1A isoforms shows higher accuracy than other models [on the basis of the lower value of residue-specific quality (RSQ1) of the model, which is defined as the estimated deviation of the residue on the model from the native structure of the protein] (Electronic Supplementary Material, Figs. 1A, 2A, Tables 7, 8).

Discussion

Oxidative stress triggers the development and progression of atherosclerosis. MT are ROS scavengers that regulating zinc homeostasis, protect cells from the stress condition (Maret and Krezel 2007), including apoptosis induced by AGEs (Lim et al. 2008). MT genetic variants have been associated with a modulation of intracellular zinc in PBMCs and with the onset of diabetes and cardiovascular complications (Giacconi et al. 2008, 2010). +1245 A/G MT1A polymorphism influences the antioxidant enzyme activity in diabetic Chinese patients and Greek CVD patients (Yang et al. 2008; Giacconi et al. 2010).

In elderly has been reported an increased incidence of a moderate zinc deficiency (Prasad et al. 2007; Mocchegiani et al. 2008b), and this condition may predispose to atherosclerosis development (Beattie et al. 2012; Giacconi et al. 2007).

Zinc supplementation exerts anti-atherogenic effects reducing inflammatory markers such as C-reactive protein (CRP), interleukin-6 (IL-6), macrophage chemoattractant protein 1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1) in elderly (Bao et al. 2010). Zinc-supplemented subjects show also improvements in oxidative stress parameters (lipid peroxidation products, DNA oxidation products) (Bao et al. 2008). However, some intervention studies found no effect of zinc on oxidative stress markers in elderly or diabetic patients (Andriollo-Sanchez et al. 2008; Seet et al. 2011). It is possible that the heterogeneity between studies on the effect of zinc supplementation may be due to confounding factors, such as environmental factors or the genetic background. By discovering key genes influencing zinc homeostasis, we may identify people who most likely would benefit from a nutritional intervention. Previous researches have evidenced different immune-inflammatory response to zinc supply both in vitro than in vivo according to MT genetic variants (Mazzatti et al. 2008; Mariani et al. 2008b), which are in strong linkage disequilibrium with +1245 MT locus (Giacconi et al. 2008).

In the present investigation, we have evaluated the antioxidant effect of zinc supplementation in relation to +1245 A/G MT1A polymorphism.

+1245 G+ carriers displayed enhanced baseline AGE production and oxidative stress with respect to G− carriers suggesting a major susceptibility to atherogenesis.

In fact, AGEs have been shown to play an important role in atherosclerosis, contributing to endothelial dysfunction (Ando et al. 2013), even in non-diabetic subjects (Yamagishi et al. 2007a), and they are positively correlated with cardiovascular mortality (Kilhovd et al. 2005; de Vos et al. 2014). Besides, dietary AGE intake has been associated with systemic inflammation, such as C-reactive protein, in a large group of healthy subjects (Uribarri et al. 2005). These compounds are also implicated in lipid dysmetabolism. Glycation of apoA-I, the major protein component of HDL, is strongly associated to produce dysfunctional HDL, which can accelerate cellular senescence and atherosclerosis (Park et al. 2010). All these findings suggest that the increased AGEs levels in G+ carriers could be associated with dysfunctional HDL and may predispose to atherosclerosis development as previously demonstrated in Greek CVD patients (Giacconi et al. 2010).

Some recent evidence report an inhibitory effect of zinc on in vitro AGE formation (Tupe and Agte 2010; Seneviratne et al. 2011). Our study shows that zinc supplementation tended to decrease AGE plasma concentrations and ROS levels in G+ carriers with; however, no significant improvement also when the changes were analyzed in all 110 subjects (data not shown), according to a previous report (Andriollo-Sanchez et al. 2008).

The significant beneficial effects of zinc intervention on antioxidant enzyme activity, MT and intracellular zinc homeostasis (iZnL, iZnR in PBMCs, Zinc content in granulocytes) have been previously demonstrated in the same group of subjects, independently of the genetic background (Mocchegiani et al. 2008b; Mariani et al. 2008a).

Here, we found no changes on antioxidant response and MT production after zinc supplementation between G− (Lys 51) or G+ (Arg51) carriers. Among intracellular and extracellular zinc parameters only iZnL and iZnR showed significant higher increments in G+ subjects than G− ones. These results might depend on a major zinc uptake or cytosolic zinc buffering in PBMCs in relation to the +1245 MT1A genotype. Indeed, here, we have demonstrated that Lys 51 is located in a conserved region of the MT alpha domain, between two cysteine residues, and Arg51 substitution determines some changes in the secondary and tertiary protein structure in correspondence of Cys21, Cys26 and Cys57 residues.

These findings demonstrate that Lys51Arg variation in MT1A protein can influence the stability of cysteine clusters and consequently the zinc-binding affinity.

On the other hand, the higher increment of zinc ion availability in G+ carriers after zinc supplementation, suggests a prompt response of cells exposed to increased oxidative stress condition at the baseline, in incorporating zinc which will bind to zinc-dependent transcription factors involved in the expression of antioxidant genes (Maret 2011). In fact, it has been demonstrated that increments of intracellular zinc, released by NO donors, induce Glutathione (GSH) synthesis, protecting endothelial cells from oxidative stress (Cortese-Krott et al. 2009). Although we found only a trend for ROS decrement in G+ supplemented subjects, zinc intervention may be useful for the improvement of enzyme antioxidant activity (Mariani et al. 2008a) and other oxidative stress parameters (Bao et al. 2008).

Moreover, considering that lowering of dietary, AGEs suppresses the neointimal formation in apolipoprotein E-deficient mice, and reduces inflammation in non-diabetic population (Lin et al. 2002; Yamagishi et al. 2007a; b; Uribarri et al. 2005) a decreased intake of food-derived AGEs could represent a novel target for therapeutic intervention in AT or DM-2 patients, especially those who are genetically more predisposed to increased AGE production (such as G+ carriers). Limitations of this investigation could be the unbalanced number of G+ and G− participants to zinc supplementation trial and the presence of possible environmental differences in our multi-centric study, therefore may be useful to confirm our findings in a larger and homogeneous population study. We have not included a control group, since it is reasonable to assume that the "placebo effect" is similar in G+ and G− carriers; in this case, the presence of a placebo group could be not necessary as reported also by other authors in trials evaluating the effect of gene nutrient interaction (Meplan et al. 2008).

In conclusion, +1245 MT1A polymorphism modulates plasma AGEs and ROS production in PBMCs. Zinc supplementation tended to decrease AGE plasma levels in G+ carriers with higher improvement in intracellular zinc ion availability, in G+ zinc-supplemented individuals, than G− ones.

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Conflict of interest All the authors declare that they have no conflict of interest.

Ethical standard The local Research Ethics Committees approved the study protocol and all the participants have given their written informed consent.

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