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ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 1008–1014
Journal of Nutritional Biochemistry

Association of MT1A haplotype with cardiovascular disease and antioxidant enzyme defense in elderly Greek population: comparison with an Italian cohort

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Received 20 February 2009; received in revised form 9 June 2009; accepted 20 August 2009

Abstract

Metallothioneins (MT), the antioxidant zinc-binding proteins, seem to mediate cardioprotection. It has been postulated that zinc homeostasis and MT function may be altered, as a consequence of oxidative stress, in cardiovascular disease (CVD), with a potential implication of MT genetic polymorphisms. The present study explores the role of +647A/C and +1245A/G MT1A polymorphisms on the susceptibility to CVD, zinc status and enzyme antioxidant activity, in the Greek and Italian populations. The country selection was based on the lower zinc status and the reduced zinc dietary intake in Greece than in Italy despite the similar Mediterranean dietary pattern. A total of 464 old, healthy control subjects and 369 old CVD patients more than 70 years of age were studied. Logistic regression model indicated that +1245 MT1A G+ genotype significantly increased the risk of CVD in Greece (34.4% vs. 23.2%; odds ratio=1.88, 95% confidence interval=1.14–3.08; P=.013) but not in Italy. Haplotype analysis showed an increment of CG haplotype frequency in CVD Greek patients (17.4% vs. 10.6%, P<.05). Differential country-related frequency distribution was also recorded. Applying a multivariate regression model, +647/+1245 MT1A haplotype was associated with a modulation of enzyme antioxidant activities in both countries. Decreased plasma zinc and reduced intracellular Zn release, as well as increased enzyme antioxidant activity, were more apparent in Greek healthy donors than in Italy. In conclusion, +1245 MT1A polymorphism and +647/+1245 MT1A haplotype are implicated in CVD in Greece but not in Italy, suggesting a role of gene–diet interaction in the disease predisposition.
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Keywords: Metallothionein polymorphisms; Zinc; Cardiovascular disease; Enzyme antioxidant activity

1. Introduction

Experimental and clinical studies have suggested an implication of increased oxidative stress in the pathophysiology of atherosclerosis and cardiovascular disease (CVD) [1–4], and oxidative stress may also result from an imbalance between oxidant production and antioxidant defenses [5]. Metallothioneins (MTs), low molecular mass zinc-binding proteins, exert an antioxidant function by regulating intracellular zinc availability and protecting heart tissue from damages induced by reactive oxygen and nitrogen species [6,7]. Several investigators report hypozincemia in atherosclerosis and CVD [8,9] or a zinc dyshomeostasis during myocardial ischemia–reperfusion model [10]. In addition, longitudinal or nested case–control studies, within a prospective population, describe the general involvement of zinc deficiency in increased CVD mortality [11], especially in the presence of concomitant dysmetabolism of other trace elements [12]. Zinc is also an important cofactor of two isoforms of Cu/Zn superoxide dismutase (SOD1 and SOD3 with cytoplasmatic and extracellular localization, respectively). Alterations in antioxidant defense mechanisms are correlated with CVD and atherosclerosis severity [13,14]. Furthermore, a dyshomeostasis of other micronutrients that are essential cofactors of SOD and glutathione peroxidase (GPx), such as copper and selenium [15], induces oxidative stress and modulates endogenous defenses that combat it [16]. Interestingly, zinc supplementation at physiological doses improves both zinc status [17] and plasma and erythrocyte superoxide dismutase (pSOD

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doi:10.1016/j.jnutbio.2009.08.008

and eSOD, respectively) levels, in a trial of healthy elderly participants from ZINCAGE study [18]. Supplementation with a high dose of zinc has also been suggested as a therapeutic strategy in terminating angina pectoris [19]. Moreover, the use of zinc ionophore pyrithione in ischemia–reperfusion model improves myocardial recovery, promoting the increment of intracellular labile zinc in response to oxidative stress [10]. Recent investigations suggest a strong association among +647A/C MT1A polymorphism (rs11640851) and zinc homeostasis in DM2 patients, affected by CVD complications [20], while +1245A/G MT1A polymorphism (rs 8052394) appears to influence serum SOD activity in diabetic Chinese patients [21]. The analysis of these two polymorphisms, characterized by an amino acid transition, may be useful to better elucidate the implication of MT1A isoform on CVD susceptibility. Hence, the aim of the present study is to investigate the potential impact of +647A/C and +1245A/G MT1A polymorphisms on CVD susceptibility, in a Greek and Italian population. The selection of these two countries was based upon the evidence of lower plasma zinc levels in Greek elderly donors than Italian ones (age range, 60–84 years) [22], despite the similar Mediterranean dietary pattern [23]. Antioxidant enzyme activity (pSOD, eSOD, CAT and GPx), zinc status and plasma levels of some trace elements were also assessed.

2. Materials and methods

2.1. Patients and controls

The study population consisted of four groups of subjects: 244 Italian and 220 Greek healthy control subjects (Groups A and B) and 215 Italian and 154 Greek CVD patients (Groups C and D).

Patients and controls recruited from Italy were born in Marche, a region in Central Italy. Patients (n=215) (mean age=77±10 years) were enrolled from the Department of Surgical Pathology, INRCA Geriatric Hospital during the 2005–2008 period, upon diagnosis of ischemic heart disease by clinical history and by resting electrocardiogram and/or carotid artery disease by Doppler ultrasonography period. The clinical features of the patients are summarized in Table 1. The control group consisted of 244 free-living individuals (mean age=75±10 years), characterized as healthy, on the basis of their clinical history and blood tests. In particular, they did not suffer from diabetes or any other clinical symptoms or history of cardiovascular and carotid artery disease.

Greek participants were randomly selected in the Athens region between February 2005 and December 2006 [24]. A complete medical and surgery record was obtained. All participants who reported a diagnosed history of angina, heart failure, coronary heart disease, stroke, myocardial infarction or surgeries such as heart bypass and angioplasty were characterized as cardiovascular patients (n=154, mean age=74±6 years). The control group consisted of 220 free-living healthy subjects (mean age=72 ±7 years), based on their clinical history and blood tests. The descriptive characteristics of the Greek subjects are summarized in Table 1.

The INRCA Hospital and Harokopio University Ethics Committees approved the project. Informed consent was obtained from each individual in compliance with Italian and Greek legislation.

2.2. Laboratory measurements

Venous peripheral blood samples, collected after an overnight fast, underwent basal biochemical laboratory determinations. Serum total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were measured by automated enzymatic methods with reagents from Boehringer-Mannheim (Germany). Fasting glucose and serum C-reactive protein (CRP) levels were determined using standard laboratory methods. The erythrocyte sedimentation rate (ESR) was determined by the classical Westergren method. Plasma was collected and frozen at −80°C until used. Peripheral blood mononuclear cells (PBMCs) were separated by conventional density gradient centrifugation, collected, washed and immediately used and/or in part cryopreserved in liquid nitrogen aliquots of 2×10^6 cells in 1 ml of RPMI (GIBCO) with 10% of fetal calf serum. Genomic DNA of PBMC was extracted by the phenol chloroform method, according to the standard procedure.

2.3. Genotyping of +647A/C MT1A and +1245C/G MT1A polymorphisms

We screened for two single-nucleotide polymorphisms (SNPs) found in dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) (PubMed Reference rs 8052394 and rs 11640851) corresponding to an A/C transition at +647 nt position and to an A/G transition at +1245 nt position, respectively, in the coding region of the human MT1A gene. The first SNP results in the Asp27Thr amino acid substitution, while the second one corresponds to Lys51Arg amino acid change. PCR-restriction fragment length analysis was performed, as previously described [25].

Table 1
Clinical characteristics of CVD patients and controls

	Controls		CVD patients	
	Italy (n=244) (A)	Greece (n=220) (B)	Italy (n=215) (C)	Greece (n=154) (D)
Age (years)	75±10	72±7 *	77±10	74±6 ‡‡
Male (%)	40.9	29.6	60 ***	46.1 †
Hypertension (%)	51	70 ***	72 ***	84 ‡‡†
Diabetes mellitus (%)	0	0	25 ***	20 ‡‡‡
Fasting glucose (mg/dl)	92.3±13.0	91.8±9.1	108.7±38.5 *	105.1±27.4 ‡‡
BMI (kg/m^2)	25.2±3.6	28.8±4.1 *	24.4±4.1	29.9±4.8 ‡‡†
ESR (mm/h)	12.2±10.0	17.9±11.7 *	16.7±13.6 *	29.4±22.9 ‡‡†
CRP plasma levels (mg/dl)	0.38×/÷0.48	0.32×/÷0.52	0.78×/÷2.19 *	0.93×/÷2.24 ‡‡†
LDH (UI/L)	168.8±26.3	175.0±25.3	164.4±40.2	232.9±104.4 ‡‡†
Total cholesterol (mg/dl)	211.4±38.3	220.3±30.8	200.1±41.3 *	229.5±42.6 ‡‡
HDL cholesterol (mg/dl)	56.1±14.8	58.4±12.5	59.7±21.5	53.5±12.8 ‡‡†
LDL cholesterol (mg/dl)	135.1±35.3	139.3±28.5	130.3±44.4	149.1±37.1 ‡‡
Triglycerides (mg/dl)	112.8±80.5	112.4±51.9	130.7±67.0 *	134.3±60.6 †

LDH, total serum lactic dehydrogenase.
Univariate analysis (using age and gender as covariates) or χ^2 test for categorical variables were reported.
* P<.05 versus Group A.
‡‡ P<.01 versus Group C.
*** P<.001 versus Group A.
† P<.05 versus Group B.
‡‡‡ P<.001 versus Group C.

2.4. Zinc, copper and selenium concentrations in plasma

Plasma zinc, copper and selenium concentrations from Greek and Italian samples were measured in the Laboratory of Nutrigenomic and Immunosenescence (Ancona, Italy) with Thermo XII Series ICP-MS (Thermo Electron Corporation, Waltham, MA, USA), following the manufacturer's instructions (AN_EO604) with slight modifications. Plasma samples were diluted 1:10 with a diluent, containing 0.1% Triton X-100, 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, Cu (blank to 2000 ppb) and Se (blank) were prepared by serial dilution of a parent multielement solution (1000 ppm for Zn, Cu and Se) (VHG Labs, Manchester, NH), using the same diluent as for the samples. Rhodium, at 200 ng/ml, was used as internal standard. Data were acquired for ^{66}Zn, ^{65}Cu and ^{82}Se. Quality of the analysis was assured by assessment of "quality standard samples" (SERONORM™ TRACE ELEMENT SERUM, Sero AS, Billingstad, Norway). Zinc levels of the quality standard samples were within 10% of the certified levels, as previously reported [17].

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). 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 1400 W RF power, 1.10 L min^{-1} nebulizer gas flow, 0.70 L min^{-1} auxiliary gas flow, 13.0 L min^{-1} cool gas flow, 70 ms dwell time, 30 s sample uptake and 35 s wash time (2 repeats per sample).

2.5. Flow cytometric analysis of intracellular zinc ion availability, zinc release by MT and MT determination

Flow cytometry assessments were carried out in the Laboratory of Nutrigenomic and Immunosenescence. "Zinc-free" RPMI medium (zinc concentration less than 1 ppb) was obtained by treatment of RPMI with 5% Chelex 100 (Sigma-Aldrich, Milan, Italy), adding EDTA and HEPES buffer at the final concentrations of

1 and 25 mM, respectively (pH 7.4). Thawed or fresh PBMCs were divided into two equal aliquots of 2×10^5 cells, at least. One aliquot was incubated with 20 μM Zinpyr-1 (ZP-1) (Neurobiotex, Galveston, TX) for 30 min at 37°C and 5% CO_2 in HEPES buffered "zinc-free" RPMI medium containing 1 mM EDTA, as extracellular chelator, of free zinc eventually still present in the medium and/or adsorbed to the cell membrane.

The second aliquot was always incubated in the same conditions plus 50 μM *N,N′,N′*-tetrakis (2-pyridylmethyl)ethylenediamine (Sigma-Aldrich), in order to detect the autofluorescence of the zinc-free ZP-1 probe.

After incubation, the aliquots were immediately analyzed by flow cytometry (Coulter Epics XL). After selecting lymphocyte population according to the forward light and side scatters, the mean fluorescence intensity for ZP-1 was detected (excitation wavelength, 488 nm; detection at 525±15) in the two aliquots. Data were recorded as the ratio of ZP-1 fluorescence/ZP-1 autofluorescence and represented the intracellular labile Zn (iZn_L) [26].

To investigate the NO-induced release of Zn, an additional aliquot was incubated with 20 μM ZP-1 plus 100 μM diethylamine NONOate acetoxymethylated (AcOM-DEA/NO) (Calbiochem, VWR International s.r.l., Milan, Italy). In fact, AcOM-DEA/NO is a cell-permeable acetoxymethylated diazeniumdiolate compound that donates NO "intracellularly" following the action of intracellular esterases. Once the incubation period was terminated, all aliquots were immediately read by the flow cytometer. The difference between iZn_L in the presence and absence of NO donor was used to estimate the intracellular release of Zn (iZn_R), as previously reported [20].

MT determination was performed as previously reported by Malavolta et al. [26], in thawed PBMC (2×10^5) treated with 0.3% paraformaldehyde and stored at 4°C for 2 days before processing using the monoclonal mouse anti-horse MT clone E9 antibody (Dakocytomation, Denmark). Results are expressed as mean fluorescence intensity.

2.6. Antioxidant enzyme activity determinations

The assessments of antioxidant enzyme activity in frozen samples from the Greek and Italian population were performed at the Institute of Gerontology and Geriatrics, University of Perugia (Italy). SOD3 (pSOD) (U/ml), CAT (μmol/min/mg protein) and GPx (nmol NADPH/min/ml) activities in plasma were measured, according to the methods of L'Abbé and Fisher [27], Beers and Sizer [28] and Flohé and Gunzler [29], respectively. To determine SOD1 activity in erythrocytes (eSOD), red blood cells were hemolyzed with cold distilled water. The enzymatic activity (U/g Hb) was measured in the supernatant, according to Winterbourn et al. [30], following the extraction with ethanol/chloroform mixture (1:1).

2.7. Assessment of dietary zinc intake

A qualitative food frequency questionnaire, designed for the needs of ZINCAGE project [31], 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 developed zinc score has been previously reported [32].

2.8. Statistical analysis

Data were analyzed with SPSS/Win program (version 15.0; SPSS Inc., Chicago, IL). Differences among groups were compared by univariate analysis using one-way analysis of variance for continuous variables and χ^2 test for categorical variables. Post hoc tests were performed when appropriate. The frequency distribution of +647A/C and +1245A/G MT1A polymorphisms and the differences in allele distribution from Hardy–Weinberg's equilibrium in control and patient groups were compared by χ^2 test.

Armitage's trend test and multiple logistic regression analysis were used to evaluate the association between +647A/C and +1245A/G MT1A polymorphisms and the study groups, after adjustment for potential cofounders, with the calculation of odds ratios (ORs) and 95% confidence intervals (CIs). Haplotype frequencies were estimated by using an expectation maximization algorithm, implemented with Arlequin package.

Pairwise linkage disequilibrium (LD) between the two SNPs was also assessed. Bonferroni correction was used to adjust for multiple testing, with the single test significance level established as $\alpha=.05$, divided by the number of tests. Multiple regression analysis for cardiovascular biochemical markers was performed, according to the CG haplotype of the MT1A gene after controlling for age, gender, body mass index (BMI) and hypertension. The calculation of partial correlation coefficient, corrected for age, was performed after the log transformation of variables with skewed distribution. A probability value less than .05 was considered statistically significant.

3. Results

3.1. Clinical characteristics and biochemical parameters

Table 1 presents the clinical profile of the participants. Hypertension was more prevalent in Greece than Italy, both in healthy subjects and in CVD patients ($P<.001$ and $P<.05$, respectively). Greek subjects had a higher mean BMI value than the Italian participants ($P<.05$). An increased inflammatory status (ESR) was observed in Greek healthy controls and CVD patients, when compared to the Italians ($P<.05$). With regard to CRP, a significant increment was observed exclusively in the CVD group for both countries, compared to healthy controls ($P<.05$). Total serum lactic dehydrogenase was enhanced in Greek CVD patients in relation to their Italian counterparts ($P<.01$). No country-dependent difference was recorded in the lipid profile of the healthy elders. Significant differences in total cholesterol, LDL and HDL cholesterol levels were observed between CVD patients from Italy and Greece ($P<.05$) (Table 1). CVD patients showed higher triglyceride and fasting glucose levels than healthy subjects in both countries ($P<.05$).

Table 2 reports the biochemical parameters of the subjects. A decrease in plasma zinc levels was observed in Greek healthy controls, as compared to the Italian ones ($P<.05$). A higher prevalence of subjects with a mild zinc deficiency (cutoff ≤11 μM) was observed in Greek healthy subjects when compared to Italian ones (47.3% vs. 32.4%; $P<.01$). Moreover, a zinc deficiency was found in Italian CVD patients compared to the Italian healthy controls ($P<.05$). The determination of zinc ion availability (iZn_L) and the *in vitro* NO-induced zinc release (iZn_R) was performed in a subpopulation, including 40 CVD patients and 40 healthy subjects, randomly selected from each country using cryopreserved PBMCs. In order to verify if the methodology [26] could be applied in frozen samples, we have previously evaluated the correlations between iZn_L and iZn_R in fresh versus frozen PBMCs in 28 Italian elderly controls (13 males and 15 females). A significant correlation between frozen and fresh samples (iZn_L fresh and frozen PBMCs: $r=.661$, $P<.001$; iZn_R fresh and frozen PBMCs: $r=.4$, $P<.03$) was found. iZn_L values were lower in Italian CVD

Table 2
Metal trace elements, zinc status and antioxidant enzymes

	Controls		CVD patients	
	Italy ($n=244$) (A)	Greece ($n=220$) (B)	Italy ($n=215$) (C)	Greece ($n=154$) (D)
Zn plasma levels (μM)	11.8±1.8	11.2±1.4*	10.7±2.5*	11.1±2.2
Se plasma levels (μM)	1.21±0.44	1.45±0.50*	1.22±0.53†	1.43±0.44‡
Cu plasma levels (μM)	17.5±3.4	18.0±3.8	18.6±5.0	18.8±5.0
iZn_L[a]	1.34±0.12	1.33±0.22	1.24±0.10*	1.35±0.15‡
iZn_R[a]	0.21±0.07	0.16±0.08*	0.18±0.06	0.14±0.1†‡
MT (pmol/mg prot)	21.7±9.5	21.3±8.1	23.7±12.1	26.5±8.9†
MT/iZn_R	368±202	365±165	468±160*	480±90†
pSOD (U/ml)	22.4±4.3	22.5±3.8	22.3±4.0	23.1±2.9‡
eSOD (U/g Hb)	2970.3±660.3	3365.6±760.3*	3094.3±716.3	3207.5±780.6
GPx (nmol NADPH/min/ml)	0.099±0.014	0.104±0.010*	0.100±0.008	0.101±0.011
CAT (μmol/min/mg prot)	19.2±3.2	22.9±2.9*	19.4±2.6	23.5±4.0‡

Univariate analysis (using age as covariate) was performed.

* $P<.05$ versus Group A.

† $P<.05$ versus Group B.

‡ $P<.05$ versus Group C.

[a] Tested in a subgroup of 40 CVD patients and 40 healthy controls randomly chosen.

Table 3
Correlations between antioxidant enzyme activity, zinc score and Zn status in Greek and Italian healthy controls

	LogMT/iZn$_R$		iZn$_R$		Plasma Zn	
	Coefficient[a]	Significance	Coefficient[a]	Significance	Coefficient[a]	Significance
Italy						
pSOD	0.027	0.8	-0.1	0.6	−0.16	0.4
eSOD	−0.24	0.1	0.4*	0.045	−0.07	0.6
GPx	0.12	0.5	−0.16	0.4	0.15	0.4
CAT	0.08	0.9	−0.1	0.3	−0.27	0.1
Zn score	−0.1	0.6	0.1	0.09	0.17**	0.002
Greece						
pSOD	−0.25	0.1	0.11	0.5	−0.11	0.6
eSOD	−0.38*	0.021	0.32*	0.009	0.21	0.3
GPx	0.02	0.6	−0.03	0.8	0.07	0.6
CAT	0.1	0.8	−0.12	0.5	−0.05	0.8
Zn score	−0.21	0.09	0.3*	0.015	0.1	0.1

[a] Partial correlation coefficients were computed after controlling for age.
* Correlation is significant at least at the .05 level.
** Correlation is significant at least at the .01 level.

than controls ($P<.05$), whereas no difference was observed in Greece, as well as between Greek and Italian healthy controls. iZn$_R$ values decreased in the presence of disease in Greece ($P<.05$), while a slight downward trend was observed in Italy. In addition, iZn$_R$ values were significantly lower in the Greek population than the Italian one ($P<.05$) (Table 2).

No country-related differences in MT levels were recorded. However, MT levels were significantly increased in CVD Greek patients, compared to their healthy counterparts ($P<.05$), while MT/iZn$_R$ increased concomitantly to the pathology, in both populations ($P<.05$) (Table 2).

With regard to other trace elements, no significant disease-related differences were recorded in copper and selenium levels, although Greek subjects presented higher selenium plasma concentrations, compared to the Italians ($P<.05$).

Antioxidant enzyme activity was not affected by the disease state, but differences were observed in relation to country. In particular, eSOD, GPx, and CAT activities were increased in Greek controls, as compared to the Italian ones ($P<.05$), while CAT and pSOD activities were higher in CVD Greek patients than Italians ($P<.05$).

Partial correlation coefficients, among enzyme antioxidant activity, zinc parameters and zinc score, are reported in Table 3. In Greek healthy controls, an inverse correlation between MT/iZn$_R$ and eSOD was found ($r=-0.38$, $P<.05$). Positive correlations were observed between iZn$_R$ and eSOD in Greece and in Italy ($r=.32$, $P<.01$ and $r=.4$, $P<.05$, respectively). Moreover, significant correlations were observed between zinc score and iZn$_R$ in Greece ($r=.3$, $P<.05$) or plasma zinc levels and zinc score in Italy ($r=.17$, $P<.01$).

3.2. +647A/C and +1245A/G MT1A genotype distribution

The genotype frequency distributions for +1245A/G polymorphism were in Hardy–Weinberg's equilibrium, in cases and controls in both countries. The +647A/C MT1A polymorphism genotype

Table 4
Genotype distribution of +647A/C and +1245A/G MT1A polymorphisms in CVD and healthy controls

(A) Italy

	Controls ($n=244$)	CVD patients ($n=215$)	P value (Pearson χ^2 test) [Armitage's trend test]	OR (95% CI) [P value] (logistic regression model)
+647A/C MT1A genotype				
AA	47.5%	55.3%	.21	0.83 (0.39–1.75) [.62[a]]
AC	45.5%	37.7%	[OR=0.851, $\chi^2=1.80$, $P=.17969$]	
CC	7.0%	7.0%		
+1245A/G MT1A genotype				
AA	75%	80%	.47	0.68 (0.31–1.48) [.34[b]]
AG	22.9%	18.7%	[OR=0.775, $\chi^2=1.67$, $P=.19683$]	
GG	2.1%	1.3%		

(B) Greece

	Controls ($n=220$)	CVD patients ($n=154$)	P value (Pearson χ^2 test) [Armitage's trend test]	OR (95% CI) [P value] (logistic regression model)
+647A/C MT1A genotype				
AA	44.5%	34.2%	.12	1.25 (0.75–2.13) [.38[a]]
AC	48.2%	58.6%	[OR=1.243, $\chi^2=2.48$, $P=.11567$]	
CC	7.3%	7.2%		
+1245A/G MT1A genotype				
AA	76.8%	65.6%	.021	1.88 (1.14–3.08) [.013[b]]
AG	23.2%	33.1%	[OR=2.103, $\chi^2=6.66$, $P=.00989$]	
GG	0%	1.3%		

Logistic regression analysis was performed with adjustment for age, gender, hypertension, BMI and diabetes.
ORs for genotypes were calculated, grouping [a]A/C+C/C (C+ genotype) versus A/A (C− genotype) and [b]A/G+G/G (G+ genotype) versus A/A (G− genotype).

Table 5
Haplotypic frequencies in CVD and healthy controls

(A) Italy

+647 MT1A allele	+1245 MT1A allele	Controls, frequency (%)	CVD patients, frequency (%)	*P* value
A	A	63.9	69.5	.085
C	A	22.7	20.3	.40
C	G	6.7	5.7	.51
A	G	6.7	4.5	.15

(B) Greece

+647 MT1A allele	+1245 MT1A allele	Controls, frequency (%)	CVD patients, frequency (%)	*P* value
A	A	67.4	63.2	.26
C	A	21.1	19.0	.43
C	G	10.6	17.4	.007**
A	G	0.9	0.4	.33

For (A): likelihood ratio=3.9, df=3, P=.27 by Pearson χ^2 when comparing all groups.
For (B): likelihood ratio=8.1, df=3, P=.04 by Pearson χ^2 when comparing all groups.
Each P value is referred to the comparison between one haplotype versus all other ones.
** P<.05 after adjusting for multiple comparisons, using Bonferroni correction.

frequency was consistent with Hardy–Weinberg's equilibrium in the various groups, except for the CVD Greek patients.

No significant different genotypic distribution of +647A/C MT1A and +1245 MT1A polymorphisms was observed between CVD patients and healthy old controls in Italy (Table 4A).

Similarly, no differences were observed in +647A/C MT1A genotype distributions between healthy controls and CVD patients in Greece. However, a significant association was found in Greece between +1245A/G MT1A polymorphism and CVD, with increased G+ frequency (AG and GG genotypes) in the patient group, with respect to healthy controls (P<.05 by Pearson χ^2 test) (Table 4B). Multiple logistic regression analysis, corrected for age, gender, hypertension, BMI and diabetes, indicated, however, that the +1245 G+MT1A genotype was an independent risk factor for CVD (P=.013; OR=1.88, 95% CI=1.14–3.08) (Table 4B).

3.3. Haplotype determination

Two single nucleotide polymorphisms of MT1A cluster were determined, and the frequency of haplotypes was analyzed using the Arlequin software package. The polymorphism of +647 MT1A was in strong LD with the +1245 MT1A one, as indicated by the D' value, which was estimated in all subjects for both countries (D'=0.34, P<.00001 and D'=0.92, P<.00001 for Italy and Greece, respectively). Moreover, a significant difference in haplotype frequency distribution between CVD patients and control subjects was evident in Greece (likelihood ratio=8.1, df=3, P=.04 by Pearson χ^2) (Table 5B), but not in Italy (likelihood ratio=3.9, df=3, P=.27 by Pearson χ^2) (Table 5A).

MT1A CG (Thr/Arg) haplotype was more prevalent in Greek CVD patients, as compared to old individuals (17.4% vs. 10.6%; P=.007), and these differences remained statistically significant after Bonferroni correction for four comparisons. Haplotype frequency distribution between Greek and Italian populations, within the same subject group, displayed significant differences in both controls and CVD patients (controls: likelihood ratio=13.4, P<.01; CVD patients: likelihood ratio=18.5, P<.001).

3.4. Phenotype assessment according to +1245A/G MT1A polymorphism and MT1A haplotypes in Greek and Italian participants

We evaluated the influence of MT1A haplotype on phenotype markers (antioxidant enzymes and zinc homeostasis) in Greek and Italian populations.

Multiple regression analysis of enzyme antioxidant activity and iZn_R according to the CG haplotype of the MT1A gene, including age, gender, BMI and hypertension, as covariates, was performed.

The Greek participants with CG haplotype (both CG+/CG− and CG+/CG+, labeled as CG+) had higher GPx activity in healthy donors (P<.001) (Fig. 1B) and lower CAT activity (Fig. 1A) and iZn_R in CVD patients (Fig. 1C) than subjects without CG haplotypes (labeled as CG−/CG−) (P<.05). No differences in relation to CG haplotype were observed for CAT activity and iZn_R in the control group and for GPx activity in CVD patients. In Italy, CG haplotype was associated with higher pSOD in the control subjects and increased eSOD in CVD patients (P<.01 and P<.05, respectively) (Fig. 2A and B). No significant differences were found for GPx, CAT activity and iZn_R. Using the same multiple regression model, we found that none of the other haplotypes was associated with

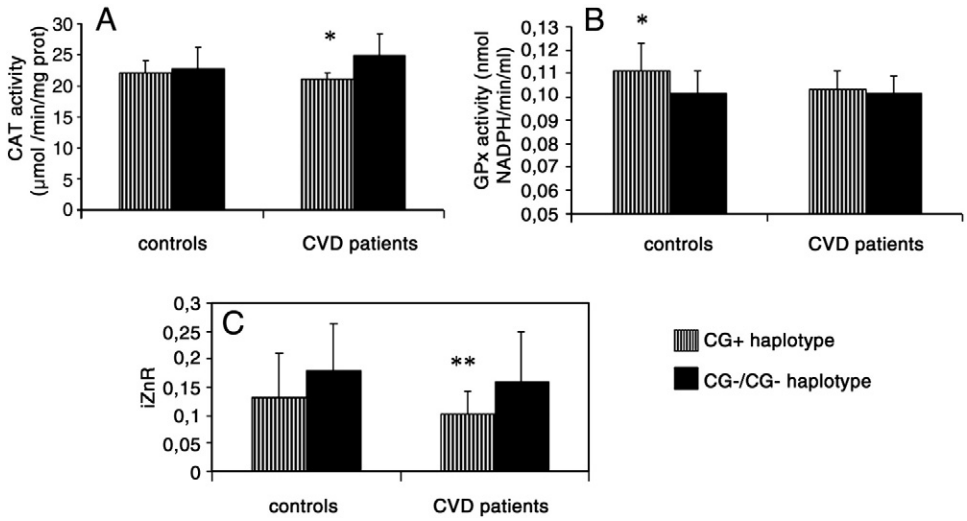

Fig. 1. Catalase, GPx activity and intracellular zinc release according to +647/+1245 MT1A haplotypes in the Greek cohort. **P<.001 as compared to CG−/CG− haplotype; *P<.05 as compared to CG−/CG− haplotype, using multivariate regression analysis correcting for age, gender, BMI and hypertension. CG+ haplotype identifies heterozygous and homozygous subjects with CG haplotypes, while CG−/CG− represents subjects without CG haplotype.

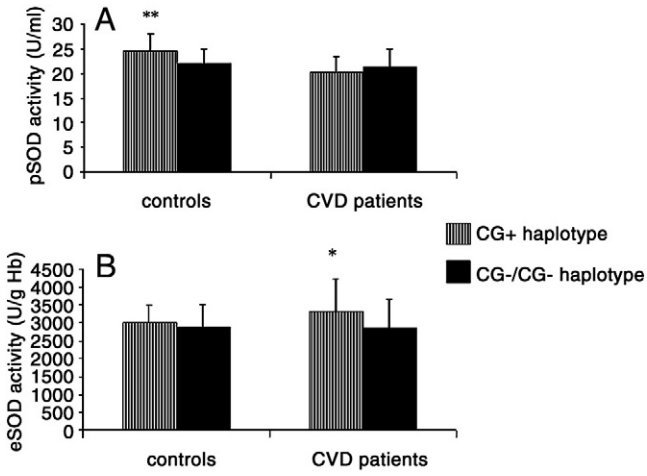

Fig. 2. **$P<.01$ as compared to CG−/CG− haplotype; *$P<.05$ as compared to CG−/CG− haplotype, using multivariate regression analysis correcting for age, gender, BMI and hypertension. CG+ haplotype identifies heterozygous and homozygous subjects with CG haplotypes, while CG−/CG− represents subjects without CG haplotype.

antioxidant enzyme activity or other clinical and biochemical markers (data not shown).

4. Discussion

Oxidative stress and zinc dyshomeostasis have been implicated in CVDs [33,34]. A cardioprotective role has been suggested for MT [6,35], zinc-binding proteins regulating zinc homeostasis and antioxidant response [36,37]. Recently, an involvement of +647A/C MT1A polymorphism in DM2 CVD complications coupled with altered modulation of intracellular zinc homeostasis has been demonstrated [20], whereas +1245A/G MT1A polymorphism seems to influence the antioxidant enzyme activity in diabetic Chinese patients [21]. In the present study, a logistic regression model revealed an association of +1245 MT1A polymorphism with CVD in Greece, but not in Italy. The variable LD among the Italian and the Greek population, as well as the different lifestyle and environmental factors, including diet, could explain the lack of association between +1245 MT1A polymorphism with CVD in the Italian cohort. Several studies demonstrated that gene–environment combinations may modify the disease risk with a particular relevance for dietary habits [38–42].

No association between the +647A/C MT1A polymorphism and CVD was observed. However, haplotype analysis showed a significant association of the MT1A CG haplotype with CVD in the Greek sample, as well as a different country-related haplotype frequency distribution, suggesting heterogeneity in the frequencies of MT variants in different European populations (unpublished data from ZINCAGE study).

CG haplotype modulated enzyme antioxidant activity in Greece (CAT and GPx) and in Italy (eSOD and pSOD), and it was associated with decreased iZn_R in CVD Greek patients (Figs. 1 and 2). All these findings are relevant because they show for the first time the implication of MT1A gene variants in antioxidant efficiency and CVD susceptibility. In the Greek cohort, we have also observed a higher prevalence of healthy subjects with mild zinc deficiency (cutoff ≤11 μM in the plasma) than Italian ones (47.3% vs. 32.4%; $P<.01$). The choice of this cutoff has been previously suggested to identify subjects with a "potential zinc deficiency", taking also into account a variability of 10% in plasma zinc concentrations [17,43]. The lower zinc status in Greece was also supported by impaired iZn_R, which, in turn, reflects a more reduced zinc intake in elderly Greek population than in Italy, as previously reported [32]. The presence of a positive correlation between iZn_R and the dietary zinc intake in Greece confirms this assumption. A moderate zinc deficiency may predispose to CVD development or mortality, as also suggested by epidemiological studies in other countries [11,44,45]. Zinc plays an antiatherogenic role through the inhibition of both oxidative stress and endothelial cells apoptosis during inflammation [46,47]. Moreover, zinc enhances the activity and expression of antioxidant proteins, such as MTs [48] and SOD [18]. Therefore, chronic zinc deficiency affects the antioxidant response determining susceptibility to oxidative stress and modulation of inflammatory parameters [49,50], increasing, as such, the risk of CVDs. In addition, since one of the protective effects of MT on CVD may be attributed to their capacity to release zinc [51], Greek subjects with impaired iZn_R may be more vulnerable to the pathology occurrence or progression. On the other hand, the Greek cohort presents high prevalence of CVD risk factors, such as hypercholesterolemia and hypertension [24], and increased BMI and ESR levels than Italian ones (present study). The increment of enzyme antioxidant activity (pSOD, eSOD and CAT) in Greece might be, in part, promoted by a reduced zinc intake [52] or by the presence of atherogenic stimuli [53,54]. Finally, the decreased zinc dietary intake in Greek healthy elderly cohort [32] may be the main cause of the different results between Greece and Italy, further supporting the relevance of the gene–dietary habit interaction for CVD predisposition [41]. Interestingly, the Mediterranean diet is widespread in both countries and is associated with lower incidence of CVDs [55]. However, the Mediterranean diet score is lower in Greece than in Italy and is associated with increased circulating levels of pro-inflammatory cytokines [56].

In conclusion, our data support +1245A/G MT1A polymorphism and +647/+1245 MT1A haplotype as susceptibility markers for CVD in the Greek population and suggest a possible influence of MT1A gene variants on antioxidant enzyme activity and intracellular zinc release.

However, a study with a large sample size is needed to validate or replicate our association results, especially from other ethnic populations.

Acknowledgments

Supported by INRCA and European Commission (ZINCAGE project no. FOOD-CT-2003-506850; coordinated by Dr. Eugenio Mocchegiani). We thank N. Gasparini for the excellent technical support and C. Sirolla for the statistical support.

References

[1] Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. Am J Cardiol 2003;91:7A–11A.
[2] Valgimigli M, Merli E, Malagutti P, Soukhomovskaia O, Cicchitelli G, Antelli A, et al. Hydroxyl radical generation, levels of tumor necrosis factor-alpha, and progression to heart failure after acute myocardial infarction. J Am Coll Cardiol 2004;43:2000–8.
[3] Li JM, Shah AM. Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. Am J Physiol Regul Integr Comp Physiol 2004;287:R1014–30.
[4] Landmesser U, Spiekermann S, Dikalov S, Tatge H, Wilke R, Kohler C, et al. Vascular oxidative stress and endothelial dysfunction in patients with chronic heart failure: role of xanthine-oxidase and extracellular superoxide dismutase. Circulation 2002;106:3073–8.
[5] Maritim AC, Sanders RA, Watkins JB. Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol 2003;17:24–38.
[6] Cai L, Wang Y, Zhou G, Chen T, Song Y, Li X, et al. Attenuation by metallothionein of early cardiac cell death via suppression of mitochondrial oxidative stress results in a prevention of diabetic cardiomyopathy. J Am Coll Cardiol 2006;48:1688–97.
[7] Maret W. Zinc coordination environments in proteins as redox sensors and signal transducers. Antioxid Redox Signal 2006;8:1419–41.
[8] Arroyo M, Laguardia SP, Bhattacharya SK, Nelson MD, Johnson PL, Carbone LD, et al. Micronutrients in African-Americans with decompensated and compensated heart failure. Transl Res 2006;148:301–8.

[9] Giacconi R, Muti E, Malavolta M, Cipriano C, Costarelli L, Bernardini G, et al. The +838 C/G MT2A polymorphism, metals, and the inflammatory/immune response in carotid artery stenosis in elderly people. Mol Med 2007;13:388–95.
[10] Karagulova G, Yue Y, Moreyra A, Boutjdir M, Korichneva. Protective role of intracellular zinc in myocardial ischemia/reperfusion is associated with preservation of protein kinase C isoforms. I. J Pharmacol Exp Ther 2007;321:517–25.
[11] Reunanen A, Knekt P, Marniemi J, Maki J, Maatela J, Aromaa A. Serum calcium, magnesium, copper and zinc and risk of cardiovascular death. Eur J Clin Nutr 1996;50:431–7.
[12] Leone N, Courbon D, Ducimetiere P, Zureik M. Zinc, copper, and magnesium and risks for all-cause, cancer, and cardiovascular mortality. Epidemiology 2006;17: 308–14.
[13] Zuo C, Qiu HY, Li Z, Xie XS, Fan JM. Relationship between cardiovascular complication and inflammation, oxidative stress of patient with maintenance hemodialysis. Sichuan Da Xue Xue Bao Yi Xue Ban 2008;39:119–21.
[14] Kerkeni M, Added F, Ben Farhat M, Miled A, Trivin F, Maaroufi K. Hyperhomocysteinaemia and parameters of antioxidative defence in Tunisian patients with coronary heart disease. Ann Clin Biochem 2008;45:193–8.
[15] Ceballos-Picot I, Trivier JM, Nicole A, Sinet PM, Thevenin M. Age-correlated modifications of copper–zinc superoxide dismutase and glutathione-related enzyme activities in human erythrocytes. Clin Chem 1992;38:66–70.
[16] Newman KP, Bhattacharya SK, Munir A, Davis RC, Soberman JE, Ramanathan KB. Macro- and micronutrients in patients with congestive heart failure, particularly African-Americans. Vasc Health Risk Manag 2007;3:743–7.
[17] Mocchegiani E, Giacconi R, Costarelli L, Muti E, Cipriano C, Tesei S, et al. Zinc deficiency and IL-6 polymorphism in old people from different European countries: effect of zinc supplementation. Zincage study. Exp Gerontol 2008;43: 433–44.
[18] Mariani E, Mangialasche F, Feliziani FT, Cecchetti R, Malavolta M, Bastiani P, et al. Effects of zinc supplementation on antioxidant enzyme activities in healthy old subjects. Exp Gerontol 2008;43:445–51.
[19] Eby GA, Halcomb WW. High-dose zinc to terminate angina pectoris: a review and hypothesis for action by ICAM inhibition. Med Hypotheses 2006;66:169–72.
[20] Giacconi R, Bonfigli AR, Testa R, Sirolla C, Cipriano C, Muti E, et al. +647 A/C and +1245 MT1A polymorphisms in the susceptibility of diabetes mellitus and cardiovascular complications. Mol Genet Metab 2008;94:98–104.
[21] Yang L, Li H, Yu T, Zhao H, Cherian MG, Cai L, et al. Polymorphisms in metallothionein-1 and -2 genes associated with the risk of type 2 diabetes mellitus and its complications. Am J Physiol Endocrinol Metab 2008;294:E987–92.
[22] Marcellini F, Giuli C, Papa R, Gagliardi C, Dedoussis G, Herbein G, et al. Zinc status, psychological and nutritional assessment in old people recruited in five European countries: Zincage study. Biogerontology 2006;7:339–45.
[23] Bamia C, Trichopoulos D, Ferrari P, Overvad K, Bjerregaard L, Tjønneland A, et al. Dietary patterns and survival of older Europeans: the EPIC-Elderly Study [European Prospective Investigation into Cancer and Nutrition]. Public Health Nutr 2007;10:590–8.
[24] Kanoni S, Dedoussis GV. Design and descriptive characteristics of the GHRAS: the Greek Health Randomized Aging Study. Med Sci Monit 2008;14:CR204–12.
[25] Cipriano C, Malavolta M, Costarelli L, Giacconi R, Muti E, Gasparini N, et al. Polymorphisms in MT1a gene coding region are associated with longevity in Italian Central female population. Biogerontology 2006;7:357–65.
[26] Malavolta M, Costarelli L, Giacconi R, Muti E, Bernardini G, Tesei S, et al. Single and three-color flow cytometry assay for intracellular zinc ion availability in human lymphocytes with Zinpyr-1 and double immunofluorescence: relationship with metallothioneins. Cytometry A 2006;69:1043–53.
[27] L'Abbé MR, Fisher PWF. Automated assay of superoxide dismutase in blood. Methods Enzymol 1990;186:232–7.
[28] Beers RF, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 1952;195:133–40.
[29] Flohé L, Gunzler WA. Assays of glutathione peroxidase. Methods Enzymol 1984;105:114–21.
[30] Winterbourn CC, Hawkins RE, Brian M, Carrell RW. The estimation of red cell superoxide dismutase activity. J Lab Clin Med 1975;85:337–41.
[31] ZINCAGE project, 2004–2007, www.Zincage.org.
[32] Kanoni S, Dedoussis G, Herbein G, Fulop T, Audrey Varin A, Jajte J, et al. Assessment of gene–nutrient interactions on inflammatory status of the elderly with the use of a Zinc Diet Score—ZINCAGE study. J Nutr Biochem 2009.
[33] Dhalla NS, Temsah RM, Netticadan T. Role of oxidative stress in cardiovascular diseases. J Hypertens 2000;18:655–73.
[34] Weber KT, Weglicki WB, Simpson RU. Macro- and micronutrient dyshomeostasis in the adverse structural remodelling of myocardium. Cardiovasc Res 2009;81: 500–8.
[35] Guo R, Ma H, Gao F, Zhong L, Ren J. Metallothionein alleviates oxidative stress-induced endoplasmic reticulum stress and myocardial dysfunction. J Mol Cell Cardiol 2009.
[36] Maret W, Krezel A. Cellular zinc and redox buffering capacity of metallothionein/thionein in health and disease. Mol Med 2007;13:315–71.
[37] Bell SG, Vallee BL. The metallothionein/thionein system: an oxidoreductive metabolic zinc link. ChemBioChem 2009;10:55–62.
[38] Nelson TL, Fingerlin TE, Moss LK, Barmada MM, Ferrell RE, Norris JM. Association of the peroxisome proliferator-activated receptor gamma gene with type 2 diabetes mellitus varies by physical activity among non-Hispanic whites from Colorado. Metabolism 2007;56:388–93.
[39] Gable DR, Matin J, Whittall R, Cakmak H, Li KW, Cooper J, et al. Common adiponectin gene variants show different effects on risk of cardiovascular disease and type 2 diabetes in European subjects. Ann Hum Genet 2007;71:453–66.
[40] Santos JL, Boutin P, Verdich C, Holst C, Larsen LH, Toubro S, et al. Genotype-by-nutrient interactions assessed in European obese women. A case-only study. Eur J Nutr 2006;45:454–62.
[41] Ordovas JM. Genetic influences on blood lipids and cardiovascular disease risk: tools for primary prevention. Am J Clin Nutr 2009.
[42] Mocchegiani E, Costarelli L, Giacconi R, Cipriano C, Muti E, Tesei S, et al. Nutrient–gene interaction in ageing and successful ageing. A single nutrient (zinc) and some target genes related to inflammatory/immune response. Mech Ageing Dev 2006;127:517–25.
[43] Pepersack T, Rotsaert P, Benoit F, Willems D, Fuss M, Bourdoux P, et al. Prevalence of zinc deficiency and its clinical relevance among hospitalised elderly. Arch Gerontol Geriatr 2001;33(3):243–53.
[44] Alissa EM, Bahjri SM, Ahmed WH, Al-Ama N, Ferns GA. Trace element status in Saudi patients with established atherosclerosis. J Trace Elem Med Biol 2006;20: 105–14.
[45] Lee DH, Folsom AR, Jacobs Jr DR. Iron, zinc, and alcohol consumption and mortality from cardiovascular diseases: the Iowa Women's Health Study. Am J Clin Nutr 2005;81:787–91.
[46] Hennig B, Meerarani P, Toborek M, McClain CJ. Antioxidant-like properties of zinc in activated endothelial cells. J Am Coll Nutr 1999;18:152–8.
[47] Meerarani P, Ramadass P, Toborek M, Bauer HC, Bauer H, Hennig B. Zinc protects against apoptosis of endothelial cells induced by linoleic acid and tumor necrosis factor alpha Am. J Clin Nutr 2000;71:81–7.
[48] Davis SR, Cousins RJ. Metallothionein expression in animals: a physiological perspective on function. J Nutr 2000;130:1085–8.
[49] Gomez NN, Fernandez MR, Zirulnik F, Gil E, Scardapane L, Ojeda MS, et al. Chronic zinc deficiency induces an antioxidant adaptive response in rat lung. Exp Lung Res 2003;29:485–502.
[50] Shen H, Oesterling E, Stromberg A, Toborek M, MacDonald R, Hennig B. Zinc deficiency induces vascular pro-inflammatory parameters associated with NF-kappaB and PPAR signalling. J Am Coll Nutr 2008;27:577–87.
[51] Cai L. Diabetic cardiomyopathy and its prevention by metallothionein: experimental evidence, possible mechanisms and clinical implications. Curr Med Chem 2007;14:2193–203.
[52] Jing MY, Sun JY, Zi NT, Sun W, Qian LC, Weng XY. Effects of zinc on hepatic antioxidant systems and the mRNA expression levels assayed by cDNA microarrays in rats. Ann Nutr Metab 2007;51(4):345–51.
[53] 't Hoen PA, Van der Lans CA, Van Eck M, Bijsterbosch MK, Van Berkel TJ, Twisk J. Aorta of ApoE-deficient mice responds to atherogenic stimuli by a prelesional increase and subsequent decrease in the expression of antioxidant enzymes. Circ Res 2003;93:262–9.
[54] Yang H, Roberts LJ, Shi MJ, Zhou LC, Ballard BR, Richardson A, et al. Retardation of atherosclerosis by overexpression of catalase or both Cu/Zn-superoxide dismutase and catalase in mice lacking apolipoprotein E. Circ Res 2004;95: 1075–81.
[55] Trichopoulou A, Bamia C, Norat T, Overvad K, Schmidt EB, Tjønneland A, et al. Modified Mediterranean diet and survival after myocardial infarction: the EPIC-Elderly Study. Eur J Epidemiol 2007;22:871–81.
[56] Dedoussis GV, Kanoni S, Mariani E, Cattini L, Herbein G, Fulop T, et al. Mediterranean diet and plasma concentration of inflammatory markers in old and very old subjects in the ZINCAGE population study. Clin Chem Lab Med 2008;46:990–6.