

# The +838 C/G MT2A Polymorphism, Metals, and the Inflammatory/Immune Response in Carotid Artery Stenosis in Elderly People

Robertina Giacconi,<sup>1</sup> Elisa Muti,<sup>1</sup> Marco Malavolta,<sup>1</sup> Catia Cipriano,<sup>1</sup> Laura Costarelli,<sup>1</sup> Gianni Bernardini,<sup>1</sup> Nazzarena Gasparini,<sup>1</sup> Erminia Mariani,<sup>2,3</sup> Vittorio Saba,<sup>4</sup> Gianfranco Boccoli,<sup>4</sup> and Eugenio Mocchegiani<sup>1</sup>

<sup>1</sup>Immunology Center (Section of Nutrition, Immunity, and Ageing), Research Department INRCA, Ancona, Italy;

<sup>2</sup>Laboratorio di Immunologia e Genetica, Istituto di Ricerca Codivilla Putti, IOR, Bologna, Italy;

<sup>3</sup>Dipartimento di Medicina Interna e Gastroenterologia, Università di Bologna, Italy; <sup>4</sup>Pathology Surgery, INRCA Hospital, Ancona, Italy

Carotid artery stenosis (CS) is a well-established risk factor for stroke. Increased proinflammatory chemokines, enhanced metallothionein (MT), and altered metal homeostasis may play roles in atherosclerosis progression and plaque destabilization. MT may sequester zinc during chronic inflammation, provoke zinc deficiency, and modulate NK cell cytotoxicity. A recent investigation of older patients with diabetes and atherosclerosis showed an association between the -209 A/G MT2A polymorphism, CS, and zinc status. In this study, we evaluated the relationship between two MT2A polymorphisms (-209 and + 838 locus), metal status, and inflammatory/immune response in older patients with CS only (the CS1 group) or with CS and previous cerebrovascular episodes (transient ischemic attack or stroke) (the CS2 group). A total of 506 individuals (188 CS1, 100 CS2, and 218 healthy controls) were studied. Atherosclerotic patients (CS1 and CS2) showed increased levels of MT, MCP-1, and RANTES, reduced NK cell cytotoxicity, and altered trace element concentrations (zinc, copper, magnesium, iron). The +838 C/G MT2A polymorphism was differently distributed in CS1 and CS2 patients, who displayed the GG genotype (C-) with significantly higher frequency than elderly controls. C- carriers showed increased MCP-1 and decreased NK cell cytotoxicity, CD56+ cells, and intracellular zinc availability along with decreased zinc, copper, and magnesium content in erythrocytes and increased iron in plasma. C- carriers also showed a major incidence of soft carotid plaques. In conclusion, the +838 C/G MT2A polymorphism seems to influence inflammatory markers, zinc availability, NK cell cytotoxicity, and trace element status, all of which may promote CS development.

Online address: <http://www.molmed.org>

doi: 10.2119/2007-00045.Giacconi

## INTRODUCTION

Carotid artery stenosis (CS) is a known risk factor for cerebrovascular events associated with oxidative stress and alterations in metal metabolism (1,2). Among the essential trace metals, zinc is an important element of more than 200 metalloenzymes, including the antioxidant superoxide dismutase (SOD) enzyme (3). In the cardiovascular system, zinc deficiency impairs the integrity of the endothelial cells (4) while favoring lipid peroxidation by enhancing oxidative-stress (5) and

therefore may play a critical role in atherogenesis (6).

Other trace elements, such as magnesium and copper, are also involved in the pathogenesis of atherosclerosis (7,8) and have both been found to be reduced in atherosclerotic patients (9,10). Indeed, magnesium, copper, and zinc have been implicated in the antioxidant defense system, and their reduced availability may lead to atherosclerosis progression (11). In contrast, increased iron (Fe) stores are associated with enhanced atherosclerosis risk (12).

Homeostasis of intracellular zinc is regulated by metallothioneins (MTs), which are critical components of an antioxidant system against cellular damage induced by reactive oxygen and nitrogen species (13). MT gene expression, leading to overall reduced zinc availability, is promoted by chronic inflammation and increases in atherosclerosis (14,15).

High MT expression and zinc depletion are also involved in the reduced innate immune response (NK cell cytotoxicity) observed during aging and the inflammatory process (16,17), as well as in atherosclerosis (18,19).

An altered production of different chemokines, including CCL2/MCP-1 (monocyte chemoattractant protein-1), CXCL8/IL-8 (interleukin 8), and CCL5/RANTES (regulated on activation, normally T-cell expressed and secreted), has also been reported in atherosclerotic le-

---

**Address Correspondence and Reprint Requests to** Eugenio Mocchegiani, Immunology Center (Section Nutrition, Immunity and Ageing), Research Department INRCA, Via Birarelli 8, 60121 Ancona, Italy, Phone: + 39-071-8004216; Fax: + 39-071-206791; E-mail: [e.mocchegiani@inrca.it](mailto:e.mocchegiani@inrca.it)

Submitted April 23, 2007; Accepted for publication May 2, 2007.

sions. Indeed, MCP-1, RANTES, and IL-8 regulate leukocyte recruitment into the arterial vessel, which leads to atherosclerosis development and vascular reocclusion (20,21).

Interestingly, increased MT expression has been found to provide protection against cardiac ischemia in diabetic mice and neuroprotection in mouse focal cerebral ischemia (22–25). However, no studies have fully examined the role played by MTs in human atherosclerosis and stroke, even though a recent study indicated a relationship between the –209 A/G MT2A polymorphism and the prevalence of ischemic cardiomyopathy in patients affected by CS and type 2 diabetes mellitus (DM2) (26). The aim of the present study was to investigate the possible association of two polymorphisms, at –209 and +838 positions of the human metallothionein II (MT2A) gene, with the incidence of CS and ischemic stroke in older patients. Relationships between MT2A polymorphism, trace metal status, higher expression of inflammatory risk factors, and innate immune response have also been investigated in CS patients.

## MATERIALS AND METHODS

### Patient and Control Populations

The studied population included older individuals born and living in Central Italy: 288 older subjects with CS admitted at the Department of Surgical Pathology (INRCA Geriatric Hospital, Ancona, Italy) for endarterectomy and as controls 218 age- and sex-matched healthy subjects living in their own homes. To study the possible association of MT polymorphisms with carotid artery disease and cerebrovascular events, CS patients were divided in two groups. The first group (indicated as CS1) included patients (n = 188) characterized by CS but without previous cerebrovascular episodes [transient ischemic attack (TIA) or stroke], while the second group (called CS2) (n = 100) was represented by patients with CS who had developed TIA or stroke in the past (Table 1). Doppler ultrasonography

**Table 1.** Baseline Characteristics of Study Subjects<sup>a</sup>

Parameters	Elderly controls (n = 218)	CS patients (n = 288)
Age (years)	72.3 ± 7.3	72.8 ± 5.5
Male	121/218 (55%)	169/288 (58.7%)
Carotid stenosis	0/218 (0%)	288/288 (100%)
TIA or stroke (TS group)	0/218 (0%)	100/288 (34.7%)
Hypertension	135/218 (62%)	211/288 (72%) <sup>b</sup>
Current smokers	150/218 (69%)	167/288 (58%) <sup>c</sup>
Body mass index (Kg/m <sup>2</sup> )	25.1 ± 3.6	25.7 ± 3.4
Total cholesterol (mg/dL)	212 ± 37	225 ± 42 <sup>c</sup>
LDL cholesterol (mg/dL)	122 ± 22	131 ± 26 <sup>c</sup>
HDL cholesterol (mg/dL)	68 ± 13	64 ± 19 <sup>b</sup>
Triglycerides (mg/dL)	121 ± 76	143 ± 64 <sup>c</sup>
Platelet count (× 10 <sup>3</sup> /mL)	235 ± 58	221 ± 54 <sup>c</sup>
Fibrinogen (mg/dL)	299 ± 73	373 ± 84 <sup>c</sup>

<sup>a</sup>Results are expressed as mean ± SD and/or percentages.

<sup>b</sup>P < 0.05 compared with age-matched controls.

<sup>c</sup>P < 0.001 compared with age-matched controls.

was performed to evaluate the CS degree and the carotid plaque morphology (soft or fibrocalcified plaques) (15,27). All patients who underwent an endarterectomy had a high degree of CS (> 80%).

Elderly controls were in good health according to the “Senieur Protocol” admission criteria for immunogerontological studies in subjects without symptoms of diabetes or history of coronary heart disease (CHD), stroke. The absence of CS in the control group was evaluated by doppler ultrasonography.

The study was approved by INRCA’s Ethical Committee. Informed consent was obtained from each recruited individual in compliance with the Italian legislation.

### Laboratory Determinations

Venous peripheral blood samples, collected after an overnight fast, underwent basal biochemical laboratory determinations and immunological studies. Serum total cholesterol, HDL and LDL cholesterol, triglycerides, platelet count, and fibrinogen concentration were determined by standard laboratory methods.

### Genotyping of –209 A/G MT2A and +838 C/G MT2A Polymorphisms

We screened a single-nucleotide polymorphism (SNP) found in the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) (PubMed Reference rs10636)

represented by a C/G transition at 1604 position of J00271 genomic sequence in the human metallothionein-II gene (MT-II) corresponding to the nucleotide +838 of the untranslated region. A PCR-restriction fragment length analysis (PCR-RFLP) was performed using the following primers:

Sense 5'-CCGCTCCCAGATGTAAAGAA-3';

Antisense : 5'-GGCATATAAAGAAAACCAGAGACA-3'.

Genomic DNA of PBMC extracted by the phenol chloroform method, according to the standard procedure, was amplified with the primers described above. PCR products were digested with Mae III (Roche, Germany) at 55°C overnight, separated on 3% agarose gel, and stained with ethidium bromide. The presence of cytosine at position +838 produced a single fragment of 157 bp (C allele), whereas the presence of guanine (G allele) generated two fragments of 62 and 95 bp, respectively. Genotyping of –209 MT2A polymorphism was performed as previously described (26).

### Multiple Immunoassay for Chemokine and Cytokine Plasma Profiles

Four-fold diluted plasma samples were assayed in duplicate using com-

mercially available multiplex bead-based immunoassay kits. IL-6, IL-8, TNF- $\alpha$ , MCP-1, and RANTES concentrations were simultaneously evaluated using multiplex reagent kits (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (28). Briefly, premixed distinct dyed beads, loaded with specific capture antibodies, were incubated with plasma samples and subsequently with fluorescently labeled detection antibodies. Data were analyzed using the Bio-Plex Manager software version 3.0 (Bio-Rad Laboratories). Values were expressed as picograms or nanograms per milliliter.

#### Zinc, Copper, Magnesium, and Iron Concentrations in Plasma and Erythrocytes

Plasma and red blood cell (RBC) zinc (Zn), copper (Cu), magnesium (Mg), and iron (Fe) concentrations were determined by a Thermo XII Series ICP-MS (Thermo Electron Corporation, Waltham, MA, USA) following the manufacturer's application note (AN\_EO604) with slight modifications. Plasma and RBC samples were diluted 1:10 and 1:26, respectively, with a diluent containing both 0.1% triton to maintain a stable emulsion with the diluted sample and 0.15 % HNO<sub>3</sub> to ensure that trace elements were maintained in solution and to favor the washout of these elements between samples. External calibration solutions containing Zn, Cu, Fe (blank to 2000 ppb), and Mg (blank to 20000 ppb) were prepared by serial dilution of a parent multielement solution (1000 ppm for Zn, Cu, and Fe; 10000 ppm for Mg) (VHG Labs, Manchester, NH, USA), using the same diluent used for the samples. Rhodium (Rh), at 200 ng/mL, was used as internal standard. Data were acquired for <sup>66</sup>Zn, <sup>65</sup>Cu, <sup>56</sup>Fe, and <sup>24</sup>Mg.

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, Nebraska, USA). A Burgener Trace nebulizer was used, because 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 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, and 35 s wash time (2 repeats per sample).

#### Flow Cytometric Analysis of Zinc Ion Availability

Zinc-free RPMI medium was obtained by treatment of RPMI with 5% Chelex 100 (Sigma-Aldrich, Milan, Italy). Thawed PBMC were divided into two equal aliquots of  $2 \times 10^5$  cells, at least one aliquot was incubated with 20  $\mu$ M Zinpyr-1 (ZP-1) (Neurobiotech, Galveston, Texas, USA) for 30 min at 37°C, 5% CO<sub>2</sub> in HEPES buffered zinc-free RPMI medium containing 1mM EDTA, as an extracellular chelator, of free zinc, which remained in the medium and/or was adsorbed to the cell membrane.

The s aliquot was incubated under the same conditions, with the addition of 50  $\mu$ M 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<sub>min</sub>) for ZP-1 fluorescence (29).

Data were reported as normalized fluorescence intensity (MFI/MFI<sub>min</sub>) and represented the zinc ion availability (30).

#### Natural Killer Cell Cytotoxicity and CD56 Subset Detection by Flow Cytometry

Natural killer (NK) cell cytotoxicity was measured using  $1 \times 10^5$  mL<sup>-1</sup> K562 target cells labeled with 100  $\mu$ Ci of <sup>51</sup>Cr and  $2 \times 10^6$  mL<sup>-1</sup> human effector PBMC. The data were expressed as lytic Unit  $20/10^7$  cells (17).

For CD56 detection, fresh PBMC ( $2 \times 10^5$ ) were stained for 30 min at 4°C with an FITC-labeled CD56 monoclonal antibody (1  $\mu$ g/ $10^6$  cells) (BD/Pharmingen, Erembodegem, Belgium) and analyzed by flow cytometry (Coulter EPICS XL; Ramsey, MN, USA). An FITC-conjugated immunoglobulin isotype was used as a

negative control. Results are expressed as percentage of positive cells.

#### Metallothioneins Determination

Thawed PBMC ( $2 \times 10^5$ ) were treated with 0.3% paraformaldehyde and stored at 4°C for two days before processing. MT determination was performed as previously reported by Yurkow and Makhijani (31) using the monoclonal mouse anti-horse metallothionein clone E9 antibody (Dakocytomation, Glostrup, Denmark). Results are expressed as mean fluorescence intensity (MFI) (30).

#### Statistical Analysis

Statistical analysis was performed with SPSS software for Windows 2000. The Kolmogorov-Smirnov test was applied to test for a normal distribution. Differences in quantitative variables were assessed using the Mann-Whitney test for nonnormal distributions and unpaired Student t-test for normal distributions. The frequencies of -209 A/G MT2A and +838 C/G MT2A genotypes were compared using the Pearson  $\chi^2$  test. Fisher exact test was performed to calculate odds ratios (OR). Results were considered significantly different when  $P < 0.05$ .

## RESULTS

#### Clinical Characteristics, Immune and Biochemical parameters

Our CS patient cohort exhibited most of the classic atherosclerosis risk factors (Table 1). The percentage of hypertensive, but not of smoking, subjects was higher in CS patients than in healthy elderly controls ( $P < 0.05$ ). CS patients showed increased levels of LDL-cholesterol, triglycerides, and fibrinogen ( $P < 0.001$ ) and reduced HDL-cholesterol and platelet count ( $P < 0.05$  and  $P < 0.001$ , respectively) compared with healthy elderly controls. The body mass index showed no statistically significant differences between patients and controls.

Compared with elderly controls, the CS patients displayed increased MT levels in peripheral blood mononuclear cells (PBMCs) ( $P < 0.01$ ), enhanced plasma

levels of MCP-1 and RANTES ( $P < 0.01$ ), and impaired NK cell cytotoxicity ( $P < 0.05$ ;  $P < 0.01$ ) (Table 2). Plasma and RBC zinc concentrations were found to be decreased ( $P < 0.01$ ) and, interestingly, we also observed significant differences in zinc availability ( $P < 0.05$ ). Furthermore, we found levels of copper and magnesium to be reduced in plasma ( $P < 0.05$ ;  $P < 0.01$ ) but not decrease in RBC, while iron levels were increased in RBC ( $P < 0.05$ ) but not in plasma. Finally, no differences were observed in IL-6, IL-8, or TNF- $\alpha$  plasma levels or CD56+ cells.

No differences were found in clinical, immune, or biochemical parameters between CS patients without previous cerebrovascular events (CS1) and CS subjects suffering from TIA or stroke (CS2) (data not shown). No sex-related differences were found (data not shown).

### +838 C/G MT2A and -209 A/G MT2A Genotype and Allele Distributions

Examination of CS patients and the healthy elderly control group revealed no significant deviations of genotypic frequencies from the Hardy Weinberg expectation when we considered the two MT2A polymorphisms (Table 3). We instead observed significantly different genotypic distribution of +838 C/G MT2A polymorphisms between patients and the control population ( $P < 0.05$ ). In particular, C- genotype frequency was higher in CS1 and CS2 patients compared with healthy controls ( $P < 0.05$ , as determined by Fisher Exact Test using the approximation of Woolf). Accordingly, a significant increment of G-allele frequency was found in CS1 (OR = 1.47,  $P = .028$ , 95% CI = 1.048-2.054) and in CS2 patients (OR = 1.793,  $P = .008$ , 95% CI = 1.160 - 2.771) compared with Age-matched controls. By contrast, CS1 and CS2 subjects showed quite similar genotypic frequencies, even though the C- genotype tended to increase in the CS2 compared with the CS1 group (73% vs 66%) with a higher OR (2.13 vs 1.52) (Table 3).

As for the -209 A/G MT2A polymorphism, no differences in genotypic distribution were found when we compared

**Table 2.** Immune and Biochemical Parameters of Studied Subjects<sup>a</sup>

Parameters	Elderly controls (n = 218)	CS patients (n = 288)
MCP-1 (pg/mL)	87 ± 31	93 ± 32 <sup>b</sup>
RANTES (pg/mL)	9249 ± 6064	11094 ± 8450 <sup>c</sup>
IL-6 (pg/mL)	21.1 ± 11	22 ± 14
IL-8 (pg/mL)	7.1 ± 3.9	7.6 ± 4.1
TNF- $\alpha$ (pg/mL)	84 ± 35	83 ± 35
MT (MFI)	20.2 ± 12.7	23.7 ± 15.2 <sup>c</sup>
NK activity (L.U. 20/10 <sup>7</sup> )	35.1 ± 5.1	20.5 ± 12.6 <sup>c</sup>
CD56+ (%)	17.8 ± 6.3	17.3 ± 6.0
Zn plasma levels ( $\mu$ M)	12.0 ± 2.1	11.1 ± 2.5 <sup>c</sup>
Cu plasma levels ( $\mu$ M)	18.0 ± 3.5	17.3 ± 4.1 <sup>b</sup>
Mg plasma levels ( $\mu$ M)	1110 ± 540	788 ± 208 <sup>c</sup>
Fe plasma levels ( $\mu$ M)	18.1 ± 6.7	18.9 ± 7.1
Zn ( $\mu$ mol/L RBC)	131 ± 36	122 ± 35 <sup>c</sup> *
Cu ( $\mu$ mol/L RBC)	8.7 ± 1.9	8.5 ± 2.0
Mg ( $\mu$ mol/L RBC)	1451 ± 776	1481 ± 670
Fe ( $\mu$ mol/L RBC)	8477 ± 2834	9056 ± 3335 <sup>b</sup>
Zn availability (MFI /MFI <sub>min</sub> )	1.35 ± 0.12	1.33 ± 0.10 <sup>b</sup>

<sup>a</sup>Results are expressed as mean ± SD. **MFI** = mean fluorescence intensity; **L.U.** = Lytic Unit.

<sup>b</sup> $P < 0.05$  compared with age-matched controls.

<sup>c</sup> $P < 0.01$  compared with age-matched controls.

**Table 3.** +838 C/G MT2A Genotypic and Allelic Frequencies in CS1 and CS2 Patients and Elderly Controls<sup>a</sup>

	Elderly controls (n = 218)		CS1 patients (without cerebrovascular episodes) (n = 188)		CS2 patients (n = 100)	
	n	Frequency	n	Frequency	n	Frequency
<b>Genotypes</b>						
GG (C-)	122	0.56	124	0.66	73	0.73
CG (C +)	81	0.37	57	0.30	22	0.22
CC (C +)	15	0.07	7	0.04	5	0.05
Total	218		188		100	
<b>Alleles</b>						
G allele	325	0.75	305	0.81	168	0.84
C allele	111	0.25	71	0.19	32	0.16
Total	436		376		200	

<sup>a</sup>Patients and controls were in Hardy Weinberg equilibrium ( $P > 0.05$ ). Significant differences of +838 C/G MT2A genotype distributions were observed between CS1 patients, CS2 patients, and elderly controls ( $\chi^2 = 10.612$ ,  $df = 4$ ,  $P = .031$ ), and between CS2 patients and elderly controls ( $\chi^2 = 8.492$ ,  $df = 2$ ,  $P = .014$ ). However, the comparison between CS1 and CS2 patients failed to reach statistical significance ( $\chi^2 = 1.498$ ,  $df = 2$ ,  $P = .23$ ).

A significant increment of C- carrier frequencies was observed in CS1 and CS2 patients compared with elderly controls (OR = 1.525;  $P = .042$ , 95% CI = 1.019-2.282 and OR = 2.13,  $P = .004$ , 95% CI = 1.27-3.56, respectively) as determined by Fisher exact test using the approximation of Woolf. Accordingly, a significant increment of G allele frequency was found in CS1 (OR = 1.47,  $P = .028$ , 95% CI = 1.048-2.054) and in CS2 patients (OR = 1.793,  $P = .008$ , 95% CI = 1.160-2.771) compared with elderly controls.

healthy age-matched controls and CA and CS2 patients. The allelic frequency was also similar among the three groups (Table 4).

#### Laboratory and Instrumental Parameters in Relation to +838 C/G MT2A Genotype

In our CS patients, to clarify the effect of the +838 C/G MT2A polymorphism on the risk of CS development, some biochemical, immune and instrumental parameters, related to +838 C/G MT2A genotype were also analyzed (Table 5). C- carriers displayed higher MCP-1 plasma levels, fibrinogen concentrations, and platelet counts than C+ carriers ( $P < 0.05$ ;  $P < 0.005$ ). Moreover, C- carriers showed lower zinc, copper, and magnesium content in erythrocytes ( $P < 0.01$ ) and increased iron ( $P < 0.05$ ) and decreased magnesium in plasma ( $P < 0.05$ ), as well as reduced NK cell cytotoxicity and CD56+ cells ( $P < 0.001$ ). Finally, compared with C+, C- subjects showed decreased availability of zinc ( $P < 0.001$ ).

Conversely, plasma levels of IL-6, IL-8, TNF- $\alpha$ , RANTES, zinc, and copper and PBMC metallothionein and RBC iron contents were not affected by the +838 C/G MT2A polymorphism.

Interestingly, the +838 C/G MT2A polymorphism affected plaque morphology in CS patients (Figure 1), as indicated by the increased incidence of carotid soft plaques in C- carriers (C- vs C+ genotypes:  $P = .048$ ; OR = 1.309; CI 95% = 0.9932-1.72, by Fisher exact test using the approximation of Woolf). In the -209 A/G MT2A genotype we found no changes in the biochemical, immune, or instrumental parameters indicated above (data not shown).

#### DISCUSSION

The induction of MTs in response to inflammatory and oxidative stimuli is associated not only with increased antioxidant cell capacity, but also with perturbed zinc homeostasis. High MT levels during aging are correlated with low zinc availability and impaired inflammatory immune response, likely contributing to the

**Table 4.** -209 A/G MT2A Genotypic and Allelic Frequencies in CS1 and CS2 Patients and Elderly Controls<sup>a</sup>

	Elderly controls (n = 218)		CS1 patients (without cerebrovascular episodes) (n = 188)		CS2 patients (n = 100)	
	n	Frequency	n	Frequency	n	Frequency
<b>Genotypes</b>						
AA (G-)	154	0.71	137	0.73	75	0.75
AG (G+)	56	0.26	48	0.25	24	0.24
GG (G+)	8	0.03	3	0.02	1	0.01
Total	218		188		100	
<b>Alleles</b>						
A allele	364	0.83	322	0.87	174	0.87
G allele	72	0.17	71	0.13	25	0.13
Total	436		376		200	

<sup>a</sup>All populations were in Hardy Weinberg equilibrium ( $P > 0.05$ ). No differences of AA genotype distribution of -209 A/G MT2A polymorphism were observed among CS1 patients, CS2 patients, and elderly controls, by using  $\chi^2$  test.

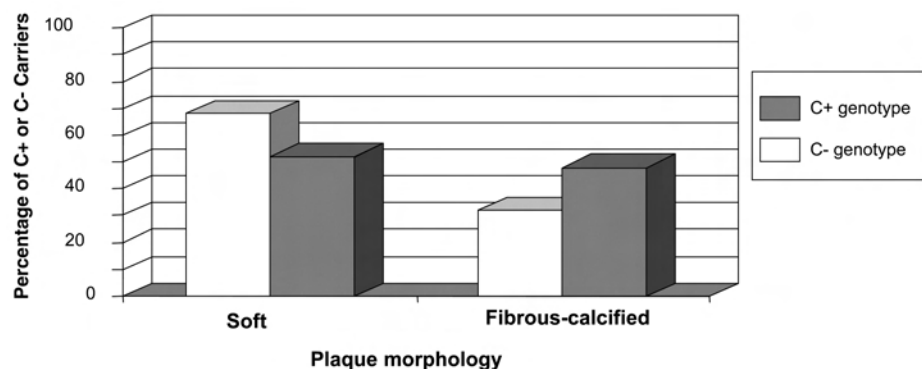
A Allele frequencies were similar among CS1 and CS2 patients and elderly controls (OR = 1.179,  $P = .43$ , 95% CI = 0.8038-1.731 and OR = 0.9209,  $P = .2$ , 95% CI = 0.8066-1.051, respectively) as determined by Fisher exact test using the approximation of Woolf.

**Table 5.** Biochemical and Immune Parameters in Relation to +838 C/G MT2A Genotype in CS Patients

	C- genotype N = 197	C+ genotype N = 91
Platelet count ( $\times 10^3$ /mL)	231 $\pm$ 45 <sup>b</sup>	211 $\pm$ 56
Fibrinogen (mg/dL)	385 $\pm$ 79 <sup>c</sup>	360 $\pm$ 88
MCP-1 (pg/mL)	99 $\pm$ 35 <sup>d</sup>	86 $\pm$ 28
RANTES (pg/mL)	10913 $\pm$ 7010	11284 $\pm$ 9892
IL-6 (pg/mL)	22.6 $\pm$ 12.7	21.5 $\pm$ 12.5
IL-8 (pg/mL)	7.9 $\pm$ 4.1	7.3 $\pm$ 4.0
TNF- $\alpha$ (pg/mL)	82 $\pm$ 33	83 $\pm$ 38
MT (MFI)	25 $\pm$ 16.1	22 $\pm$ 14.2
NK activity (L.U. 20/10 <sup>7</sup> )	15.9 $\pm$ 9.5 <sup>d</sup>	25.3 $\pm$ 15.6
CD56+ (%)	15.1 $\pm$ 5.8 <sup>d</sup>	19.5 $\pm$ 6.2
Zn plasma levels ( $\mu$ M)	10.8 $\pm$ 2.6	11.4 $\pm$ 2.3 <sup>e</sup>
Cu plasma levels ( $\mu$ M)	16.9 $\pm$ 4.6	17.6 $\pm$ 3.8
Mg plasma levels ( $\mu$ M)	756 $\pm$ 237 <sup>c</sup>	820 $\pm$ 175
Fe plasma levels ( $\mu$ M)	19.9 $\pm$ 8.4 <sup>c</sup>	17.9 $\pm$ 5.8
Zn ( $\mu$ mol/L RBC)	114 $\pm$ 40 <sup>d</sup>	130 $\pm$ 31
Cu ( $\mu$ mol/L RBC)	8.2 $\pm$ 1.8 <sup>f</sup>	8.9 $\pm$ 2.1
Mg ( $\mu$ mol/L RBC)	1325 $\pm$ 662 <sup>d</sup>	1635 $\pm$ 680
Fe ( $\mu$ mol/L RBC)	9046 $\pm$ 4828	9071 $\pm$ 1822
Zn availability (MFI /MFI <sub>min</sub> )	1.31 $\pm$ 0.09 <sup>d</sup>	1.36 $\pm$ 0.11

<sup>a</sup>Results are expressed as mean  $\pm$  SD. MFI = mean fluorescence intensity; L.U. = Lytic Unit.

<sup>b</sup> $P < 0.005$ ; <sup>c</sup> $P < 0.05$ ; <sup>d</sup> $P < 0.001$ ; <sup>e</sup> $P = .06$ ; <sup>f</sup> $P < 0.001$ ; compared with C+ genotypes.



**Figure 1.** Incidence of soft or fibrous-calcified plaques in CS patients according to +838 C/G MT2A genotype. C- genotype frequencies were higher in patients with soft plaques, 68% vs 52%,  $P = .048$ , OR = 1.309, CI 95% = 0.9932-1.726 by Fisher exact test using the approximation of Woolf.

development of age-related diseases, including atherosclerosis (16,15,26,32). Indeed, zinc deficiency has been suggested as a risk factor for atherosclerosis development (6,33), because the cation acts as a cofactor of antioxidant enzymes and protects from lipid peroxidation (34).

In the present study, we found that CS patients show increased MT levels combined with reduced zinc bioavailability in PBMCs. This finding is in agreement with the decreased plasma zinc concentration observed in patients suffering from coronary artery disease (33). In CS patients, zinc deficiency may be responsible for decreased NK cell activity and enhanced plasma concentrations of MCP-1 and RANTES. Furthermore, in CS patients zinc deficiency associated with dyshomeostasis of other trace elements (copper and magnesium in the plasma, iron in the RBCs) may cause enhanced oxidative stress (11) and induce MCP-1 production in vascular endothelial cells (35). Although some studies reported higher circulating levels of IL-6, IL-8, and TNF- $\alpha$  (36–38) to be linked to atherosclerosis, no such increases were observed in our cohort of CS patients. MCP-1 and IL-8 chemokines have been shown to be increased in atherosclerotic lesions in humans and in animal models, but our data are in agreement with those suggesting a more pathogenic role for

MCP-1 (39,40). Until now, no studies investigated the possible association of two MT2 gene polymorphisms (at the -209 and +838 positions) with carotid artery disease.

We found no association between the -209 A/G MT2A polymorphism and CS or cerebrovascular events, although in a previous report we showed involvement of this polymorphism in cardioischemic complications in diabetic patients with CS (26). This discrepancy is attributable to the fact that, in the present study, we excluded diabetic patients to avoid the confounding interference of this metabolic disease, which per se increases the incidence of stroke (41). We observed, however, that the +838 C/G MT2A polymorphism is associated with CS; in particular, the G allele (C- genotype) may be a predictor of carotid artery disease as shown by the significant ORs. Although the greater incidence of vulnerable soft plaques in C- patients might suggest an increased risk for cerebrovascular episodes compared with C+ patients, the similar C- genotype frequency in the CS2 and CS1 groups (0.73 vs 0.66, OR 2.13 vs 1.52; Table 3), seems to indicate that +838 C/G MT2A polymorphism do not affect the risk of occurrence of cerebrovascular events in subjects already affected by CS.

The most intriguing results concern the C- carriers who displayed increased

MCP-1 plasma levels and platelet counts as well as fibrinogen concentrations. These patients also showed lower magnesium status, copper and zinc content in erythrocytes, increased iron plasma levels, and limited zinc ion availability. Altogether, the alterations we found in metal trace elements and chemokine levels in our CS patients support the potential role of these factors as promoters of atherosclerosis progression (8,12).

The higher CS incidence in the C- carriers is in line with the role of MCP-1 in atherogenesis, restenosis, and plaque destabilization (42–44) as well as with the fact that patients with stroke or cardiovascular diseases show elevated MCP-1 plasma levels (45,46). The increased platelet count and high fibrinogen concentrations that we found in C- subjects fit the known association of these factors with severe CS and risk of stroke (47,48).

Finally, the observed depletion of zinc due to MT sequestration (14,49), associated with copper and magnesium deficiency and iron overload, is in line with previous studies indicating the role of metal dyshomeostasis in atherosclerosis development (6,50–52). Low magnesium concentration has been linked to increased rate of cerebrovascular events (53), copper and zinc decrease in ulcerated plaques can promote atheroma destabilization (2) and, as for iron, an overload of this element leads to higher production of reactive oxygen species with subsequent atherosclerosis progression or cardiovascular diseases (12). Furthermore, high MT levels have been linked to atheroma, especially in the case of proinflammatory genotypes (26,32). The increased MT levels and reduced zinc availability that we found in PBMCs may also occur in atherosclerotic lesions, with consequent severe inflammation, oxidative stress, and ultimately plaque weakness. In the context of C- carriers, low zinc availability can act as a further aggravating factor, considering that low zinc levels in these subjects can promote depressed immune function, as showed by the observed decreased count of

CD56+ subsets and reduced NK cell cytotoxicity (54,16).

In conclusion, the +838 C/G MT2A polymorphism seems to promote the progression of carotid artery disease to CS by modulating inflammatory markers, coagulation factors, zinc availability, NK cell cytotoxicity, and trace element status.

## ACKNOWLEDGMENTS

Supported by the European Commission (ZINCAGE project, n. FOOD-CT-2003-506850; Coordinated by Dr. E. Mocchegiani) and INRCA, Ancona, Italy. Partially supported by grants from Bologna University (RFO fund) and Ricerca Corrente IOR, Bologna, Italy. We thank Professor Stefano Sensi for his very valuable suggestions and criticisms. We acknowledge Dr Marchegiani Francesca for the revision of statistical analysis.

## REFERENCES

- Watanabe T, Yasunari K, Nakamura M, Maeda K. (2006) Carotid artery intima-media thickness and reactive oxygen species formation by monocytes in hypertensive patients. *J. Hum. Hypertens.* 20:336-40.
- Radak D, Cvetkovic Z, Tasic N, Petrovic B, Lackovic V, Djordjevic-Denic G. (2004) The content of copper and zinc in human ulcerated carotid plaque. *Srp. Arh. Celok. Lek.* 132:80-4.
- Rostan EF, DeBuys HV, Madey DL, Pinnell SR. (2002) Evidence supporting zinc as an important antioxidant for skin. *Int. J. Dermatol.* 41:606-11.
- Meerarani P, Ramadass P, Toborek M, Bauer HC, Bauer H, Hennig B. (2000) Zinc protects against apoptosis of endothelial cells induced by linoleic acid and tumor necrosis factor alpha. *Am. J. Clin. Nutr.* 71:81-7.
- Faure P, Benhamou PY, Perard A, Halimi S, Roussel AM. (1995) Lipid peroxidation in insulin-dependent diabetic patients with early retina degenerative lesions: effects of an oral zinc supplementation. *Eur. J. Clin. Nutr.* 49:282-288.
- Reiterer G et al. (2005) Zinc deficiency increases plasma lipids and atherosclerotic markers in LDL-receptor-deficient mice. *J. Nutr.* 13:2114-8.
- Kurabayashi M. (2005) Role of magnesium in cardiac metabolism. *Clin. Calcium* 15:77-83.
- Mielcarz G, Howard AN, Mielcarz B, Williams NR, Rajput-Williams J, Nigdigar SV, Stone DL. (2001) Leucocyte copper, a marker of copper body status is low in coronary artery disease. *J. Trace Elem. Med. Biol.* 15:31-5.
- Ueshima K. (2005) Magnesium and ischemic heart disease: a review of epidemiological, experimental, and clinical evidences. *Magnes. Res.* 18:275-84.
- Vlad M, Caseanu E, Uza G, Petrescu M. (1994) Concentration of copper, zinc, chromium, iron and nickel in the abdominal aorta of patients deceased with coronary heart disease. *J. Trace Elem. Electrolytes Health Dis.* 8:111-4.
- Paolisso G, Esposito R, D'Alessio MA, Barbieri M. (1999) Primary and secondary prevention of atherosclerosis: is there a role for antioxidants? *Diabetes Metab.* 25:298-306.
- Lee DH, Folsom AR, Jacobs DR Jr. (2005) Iron, zinc, and alcohol consumption and mortality from cardiovascular diseases: the Iowa Women's Health Study. *Am. J. Clin. Nutr.* 81:787-91.
- Palmiter RD. (1998) The elusive function of metallothioneins. *Proc. Natl. Acad. Sci. U. S. A.* 95:8428-30.
- Mocchegiani E, Costarelli L, Giacconi R, Cipriano C, Muti E, Tesi S, Malavolta M. (2006) Nutrient-gene interaction in ageing and successful ageing: a single nutrient (zinc) and some target genes related to inflammatory/immune response. *Mech. Ageing Dev.* 127:517-25.
- Giacconi R et al. (2004) The -174G/C polymorphism of IL-6 is useful to screen old subjects at risk for atherosclerosis or to reach successful ageing. *Exp. Gerontol.* 39:621-8.
- Mocchegiani E et al. (2002) MtmRNA gene expression, via IL-6 and glucocorticoids, as potential genetic marker of immunosenescence: lessons from very old mice and humans. *Exp. Gerontol.* 37:349-57.
- Cipriano C et al. (2005) The -308G/A polymorphism of TNF-alpha influences immunological parameters in old subjects affected by infectious diseases. *Int. J. Immunogenet.* 32:13-18.
- Brunnsgaard H, Pedersen AN, Schroll M, Skinhoj P, Pedersen BK. (2001) Decreased natural killer cell activity is associated with atherosclerosis in elderly humans. *Exp. Gerontol.* 37:127-36.
- Jonasson L, Backteman K, Ernerudh J. (2005) Loss of natural killer cell activity in patients with coronary artery disease. *Atherosclerosis* 183:316-21.
- Sheikine Y, Hansson GK. (2004) Chemokines and atherosclerosis. *Ann. Med.* 36:98-118.
- Boisvert WA. (2004) Modulation of atherogenesis by chemokines. *Trends Cardiovasc. Med.* 14:161-5.
- Schober A et al. (2002) Deposition of platelet RANTES triggering monocyte recruitment requires P-selectin and is involved in neointima formation after arterial injury. *Circulation* 106:1523-29.
- Wang J et al. (2006). Cardiac metallothionein induction plays the major role in the prevention of diabetic cardiomyopathy by zinc supplementation. *Circulation* 113:544-54.
- Wold LE et al. (2006) Metallothionein alleviates cardiac dysfunction in streptozotocin-induced diabetes: role of Ca<sup>2+</sup> cycling proteins, NADPH oxidase, poly(ADP-Ribose) polymerase and myosin heavy chain isozyme. *Free Radic. Biol. Med.* 40:1419-29.
- Trendelenburg G et al. (2002) Serial analysis of gene expression identifies metallothionein-II as major neuroprotective gene in mouse focal cerebral ischemia. *J. Neurosci.* 2002; 22:5879-88.
- Giacconi R et al. (2005) Novel -209A/G MT2A polymorphism in old patients with type 2 diabetes and atherosclerosis: relationship with inflammation (IL-6) and zinc. *Biogerontology* 6:407-13.
- Hellings WE, Ackerstaff RG, Pasterkamp G, De Vries JP, Moll FL. (2006) The carotid atherosclerotic plaque and microembolisation during carotid stenting. *J. Cardiovasc. Surg.* 47:115-26
- Mariani E, Cattini L, Neri S, Malavolta M, Mocchegiani E, Ravaglia G, Facchini A. (2006) Simultaneous evaluation of circulating chemokine and cytokine profiles in elderly subjects by multiplex technology: relationship with zinc status. *Biogerontology* 7:449-59.
- Burdette SC, Walkup GK, Spingler B, Tsien RY, Lippard SJ. (2001) Fluorescent sensors for Zn(2+) based on a fluorescein platform: synthesis, properties and intracellular distribution. *J. Am. Chem. Soc.* 123:7831-41.
- Malavolta M et al. (2006) 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.* 69:1043-53.
- Yurkow EJ, Makhijani PR. (1998) Flow cytometric determination of metallothionein levels in human peripheral blood lymphocytes: utility in environmental exposure assessment. *J. Toxicol. Environ. Health A.* 54:445-57.
- Giacconi R et al. (2006) CD14 C (-260)T polymorphism, atherosclerosis, elderly: Role of cytokines and metallothioneins. *Int. J. Cardiol.* Nov. 10 [Epub ahead of print].
- Giacconi R et al. (2006) Involvement of -308 TNF-alpha and 1267 Hsp70-2 polymorphisms and zinc status in the susceptibility of coronary artery disease (CAD) in old patients. *Biogerontology* 7 :347-56.
- Prasad AS, Bao B, Beck FW, Kucuk O, Sarkar FH. (2004) Antioxidant effect of zinc in humans. *Free Radic. Biol. Med.* 37:1182-90.
- Chen XL, Zhang Q, Zhao R, Medford RM. (2004) Superoxide, H<sub>2</sub>O<sub>2</sub>, and iron are required for TNF-alpha-induced MCP-1 gene expression in endothelial cells: role of Rac1 and NADPH oxidase. *Am. J. Physiol Heart Circ Physiol.* 286:H1001-7.
- Larsson PT, Hallerstrom S, Rosfors S, Wallen NH. (2005) Circulating markers of inflammation are related to carotid artery atherosclerosis. *Int Angiol.* 24:43-51.
- Profumo E et al. (2007) Intracellular expression of cytokines in peripheral blood from patients with atherosclerosis before and after carotid endarterectomy. *Atherosclerosis* 191(2):340-7.
- Lee SD et al. (2006) Pro-inflammatory states and IGF-I level in ischemic heart disease with low or high serum iron. *Clin. Chim. Acta.* 370:50-6.
- Gosling Jet al. (1999) MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that over-express human apolipoprotein J. *Clin. Invest.* 103:773-8.
- Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. (1998) Absence of mono-

- cyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol. Cell.* 2:275-81.
41. Kissela B, Air E. (2006) Diabetes: impact on stroke risk and poststroke recovery. *Semin. Neurol.* 26:100-7.
  42. Egashira K. (2003) Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-1. *Hypertension* 41:834-41.
  43. Ohtani K et al. (2004) Antimonocyte chemoattractant protein-1 gene therapy reduces experimental in-stent restenosis in hypercholesterolemic rabbits and monkeys. *Gene Ther.* 11:1273-82.
  44. Kobusiak-Prokopowicz M, Orzeszko J, Mazur G, Mysiak A, Orda A, Mazurek W. (2005) Kinetics of chemokines in acute myocardial infarction. *Kardiol. Pol.* 62:301-16.
  45. Arakelyan A, Petrakova J, Hermanova Z, Boyajyan A, Lukl J, Petrek M. (2005) Serum levels of the MCP-1 chemokine in patients with ischemic stroke and myocardial infarction. *Mediators Inflamm.* 3:175-9.
  46. Martinovic I et al. (2005) Elevated monocyte chemoattractant protein-1 serum levels in patients at risk for coronary artery disease. *Circ. J.* 69:1484-9.
  47. McCabe DJ et al. (2005) Increased platelet count and leucocyte-platelet complex formation in acute symptomatic compared with asymptomatic severe carotid stenosis. *J. Neurol. Neurosurg. Psychiatr* 76:1249-54.
  48. Turaj W, Slowik A, Dziedzic T, Pulyk R, Adamski M, Strojny J, Szczudlik A. (2006) Increased plasma fibrinogen predicts one-year mortality in patients with acute ischemic stroke. *J. Neurol. Sci.* 246:13-9.
  49. Cipriano C et al. (2006) Polymorphisms in MT1A gene coding region are associated with longevity in Italian Central female population. *Biogerontology* 7:357-65.
  50. Hamilton IM, Gilmore WS, Strain JJ. (2000) Marginal copper deficiency and atherosclerosis. *Biol. Trace Elem. Res.* 78:179-89.
  51. Fujioka Y, Yokoyama M. (2005) Magnesium, cardiovascular risk factors and atherosclerosis. *Clin. Calcium* 15:221-5.
  52. Qayyum R, Schulman P. (2005) Iron and atherosclerosis. *Clin. Cardiol.* 28:119-22.
  53. Amighi J et al. (2004) Low serum magnesium predicts neurological events in patients with advanced atherosclerosis. *Stroke* 35:22-7.
  54. Ravaglia G et al. (2000) Effect of micronutrient status on natural killer cell immune function in healthy free-living subjects aged  $\geq 90$  y. *Am. J. Clin. Nutr.* 71:590-8.