

Association Between Plasma Monocyte Chemoattractant Protein-1 Concentration and Cardiovascular Disease Mortality in Middle-Aged Diabetic and Nondiabetic Individuals

LORENZO PIEMONI, MD¹
 GILIOLA CALORI, MD²
 GUIDO LATTUADA, PHD³
 ALESSIA MERCALLI, PHD¹
 FRANCESCA RAGOGNA, PHD³

MARIA PAOLA GARANCINI, MD⁴
 GIACOMO RUOTOLO, MD^{2,5}
 LIVIO LUZI, MD^{1,3,6}
 GIANLUCA PERSEGHIN, MD^{1,3,6}

OBJECTIVE— Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a chemokine involved into the pathogenesis of atherosclerosis and has prognostic value in the acute and chronic phases in patients with acute coronary syndromes.

RESEARCH DESIGN AND METHODS— MCP-1/CCL2 concentration was measured in plasma fractions of 363 middle-aged overweight/obese individuals (aged 61 ± 12 years, BMI 30.1 ± 6.6 kg/m², 15% with type 2 diabetes, and 12% with impaired glucose tolerance) of a population survey carried out in 1990–1991 in Lombardy, Italy (Cremona Study), and cardiovascular disease (CVD) mortality was assessed in 2006 through Regional Health Registry files.

RESULTS— At baseline MCP-1/CCL2 was increased in individuals with type 2 diabetes ($P < 0.05$) and showed significant correlations with biochemical risk markers of atherosclerosis. After 15 years, among the 363 subjects, there were 82 deaths due to CVD. In univariate analysis age, sex, fasting glucose and insulin, fibrinogen, glucose tolerance status, smoking habit, and MCP-1/CCL2 were associated with CVD mortality. Age, sex, fasting serum glucose, MCP-1/CCL2, and smoking habit maintained an independent association with CVD mortality in multiple regression analysis. In a subgroup of 113 subjects in whom data for C-reactive protein (CRP) were available, its level was not predictive of CVD mortality.

CONCLUSIONS— In middle-aged overweight/obese individuals MCP-1/CCL2 was independently associated with CVD mortality. Further studies will be necessary to establish its role as a surrogate biomarker and as a potential therapeutic target.

Diabetes Care 32:2105–2110, 2009

Atherosclerosis is the result of an excessive proliferative and inflammatory response that includes smooth muscle cell migration and proliferation, inflammatory cell infiltration, neovascularization, production of extracellular matrix, and the accumulation of lipids (1). Monocyte chemoattractant protein-1

(MCP-1/CCL2), a member of the CC chemokine family, is involved in most of these processes (2). In culture systems, oxidized LDL (3) and shear stress (4) up-regulated MCP-1/CCL2 synthesis in human endothelial cells. In animal models, the role of MCP-1/CCL2 appears to be more evident; MCP-1/CCL2 knockout

mice placed in an LDL receptor–deficient background showed an 80% reduction of atherosclerotic plaque and a reduction in the number of macrophages in the aortic walls (5), and similar results were obtained in mice deficient in the MCP-1/CCL2 receptor (CCR2) crossed with apolipoprotein E–deficient mice (6).

If in animal models the role of MCP-1/CCL2 in atherosclerosis appeared clear, its role in vivo in humans remains unknown. MCP-1/CCL2 levels were found to be increased in aging (7), hypertension (8), hypercholesterolemia (9), and renal failure (10) and to have a prognostic value in the acute and chronic phases in patients with acute coronary syndromes (11,12). We were the first to report increased MCP-1/CCL2 plasma levels in diabetic individuals (13). In that study we found that in 207 women selected from a population survey carried out in 1990–1991 in Lombardy, Italy (Cremona Study), because a properly stored spared fraction of plasma was available, baseline MCP-1/CCL2 correlated with risk markers of cardiovascular disease (CVD). In addition, we reported that in univariate analysis MCP-1/CCL2 was significantly associated with CVD mortality 7 years later, even if in the multivariate analysis this association did not retain a significant association (13). The present study adds to the previous report because the follow-up was extended at 15 years from baseline and because fasting plasma MCP-1/CCL2 was assessed in 156 men also.

RESEARCH DESIGN AND METHODS

The 363 individuals who entered the study were selected from a population survey carried out in 1990–1991 in the Health District of Cremona (Lombardy, Italy) that was performed to determine the prevalence of diabetes in Italy according to an oral glucose tolerance test (OGTT) and World Health Organization criteria (13). Fasting plasma leptin, α -tumor necrosis factor receptor 2

From the ¹Diabetes Research Institute, Istituto Scientifico H San Raffaele, Milan, Italy; the ²Cardiovascular Department, Istituto Scientifico H San Raffaele, Milan, Italy; ³Nutrition/Metabolism, Istituto Scientifico H San Raffaele, Milan, Italy; ⁴Medical Direction, Istituto Scientifico H San Raffaele, Milan, Italy; ⁵AstraZeneca R&D, Molndal, Sweden; and the ⁶Department of Sport, Nutrition and Health, Università degli Studi di Milano, Milan, Italy.

Corresponding author: Gianluca Perseghin, perseghin.gianluca@hsr.it.

Received 23 April 2009 and accepted 12 July 2009. Published ahead of print at <http://care.diabetesjournals.org> on 29 July 2009. DOI: 10.2337/dc09-0763.

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(α -TNF-R2), and MCP-1/CCL2 concentrations were measured in patients with type 2 diabetes (known to be affected or previously undiagnosed) ($n = 99$) and patients with impaired glucose tolerance (IGT) ($n = 77$) for whom an aliquot of frozen plasma sample was available, which was stored at -80°C and had not been previously thawed. From the above-described population, 187 individuals with normal glucose tolerance (NGT) were randomly selected to be comparable to the group of patients with type 2 diabetes and IGT in terms of age and anthropometric parameters. Past medical history and clinical data of subjects were collected through a standard protocol conducted by trained interviewers. A venous blood sample was collected after a 12-h overnight fast; thereafter, a 75-g oral glucose monohydrate was given, and a further venipuncture was performed 2 h later. Anthropometric measures were obtained by the same trained individual using the same instruments for all subjects. Heart rate and systolic and diastolic blood pressures were taken twice, at the beginning and at the end of the visit, in the sitting position and after at least a 10-min rest using a full automatic noninvasive sphygmomanometer. The lowest figure was considered. Further details concerning the study protocol have been reported previously (14). Fifteen years later, vital status and time of death were ascertained through Regional Health Registry files and causes of death were classified using the ICD-8 and ICD-9 codes 401–448 (CVD), 410–414 (coronary heart disease), and 430–438 (stroke). The protocol was approved by the Ethics Committee of the Istituto Scientifico H San Raffaele.

Definition of diabetes, IGT, and metabolic syndrome

Diabetes was defined at that time according to previously known diabetes status (patients taking oral hypoglycemic agents) or according to the results of the OGTT and on the basis of World Health Organization criteria (basal plasma glucose >7.8 or >11.1 mmol/l after a 2-h oral glucose load). Patients with known diabetes did not undergo the OGTT. IGT was defined as basal plasma glucose <7.8 mmol/l and plasma glucose >7.8 but <11 mmol/l after a 2-h oral glucose load. Metabolic syndrome was defined according to the definition of the National Cholesterol Education Program Adult Treatment Program III.

Analytical determinations

Blood and serum and plasma substrates were assessed as described previously (13). Blood was collected into tubes with glycolytic inhibitor, and the glucose concentration was measured within 3–4 h in the central laboratory through the GOD-PAP glucose oxidase method (Boehringer Mannheim, Milan, Italy) with a Hitachi 705 autoanalyzer. At the same time fibrinogen, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transferase (GGT), and alkaline phosphatase (ALP) were also determined. An additional 20 ml of fasting blood was immediately centrifuged, and plasma was obtained for the assessment in the central laboratory of insulin, triglycerides, and total and HDL cholesterol. The plasma human MCP-1/CCL2 concentration was measured in 2001 using a noncommercial sandwich enzyme-linked immunosorbent assay as described previously (15); the enzyme-linked immunosorbent assay for MCP-1/CCL2 is specific for human MCP-1 and did not detect the closely related human chemokines MCP-2 and MCP-3 (16). Leptin concentration was determined as described previously (13) by a radioimmunoassay with a human kit (Linco Research, St. Charles, MO). Intra-assay and interassay coefficients of variation (CVs) were 1.5 and 1.9%, respectively. Insulin was determined by a radioimmunoassay kit (intra-assay and interassay CVs were 6.0 and 5.3%, respectively) (Technogenetics, Medgenics, Brussels, Belgium). α -TNF-R2 was measured with an enzyme immunoassay following the manufacturer's (Immunotech Beckman Coulter, Marseille, France) recommendations as described previously (13). Total cholesterol and triglycerides were measured by enzymatic methods (Boehringer Mannheim, Mannheim, Germany) with the CIBA Corning 550 Express Autoanalyzer. The HDL fraction was separated from plasma by precipitation with polyethylene glycol using a Colortest kit (Roche, Basel, Switzerland).

High-sensitivity C-reactive protein

In 2004, C-reactive protein (CRP) levels (N high-sensitivity CRP assay; Dade-Behring, Marburg, Germany) were measured in a subgroup of older individuals (≥ 65 years old; $n = 447$) within the entire population of the Cremona Study and were reported in another article (17). With respect to that assessment, we have data for 113 subjects in whom MCP-1/

CCL2 and high-sensitivity CRP (hs-CRP) were simultaneously available.

Calculation

BMI was calculated as weight in kilograms divided by the square of height in meters. Insulin sensitivity was estimated using the quantitative insulin sensitivity check index (QUICKI) obtained from fasting baseline determinations (18) and calculated as the logarithm and the reciprocal of the insulin-glucose product:

$$\text{QUICKI} = 1/[\log(I_0) + \log(G_0)]$$

Alcohol consumption was calculated as units of alcohol (glass of wine = 20 units, glass of aperitif = 30 units, and glass of liquor = 80 units).

Statistical analysis

Analyses were performed using SAS software. Concentrations are presented as averages \pm SD unless otherwise stated. ANOVA and χ^2 analysis were used for comparison between groups, and the Bonferroni adjustment was used for post hoc comparisons. Because of the skewed distribution of serum leptin, insulin, triglycerides, fibrinogen, glucose, AST, ALT, GGT, and ALP, log-transformed values were used in the analysis. Pearson correlation analysis was used for correlations. The association of the risk factors with CVD mortality after the 15-year observational period was estimated by the Cox univariate proportional hazards model. Hazard ratios (HRs) and 95% CIs are presented. A multivariate Cox proportional model (stepwise), including parameters with $P < 0.1$ on univariate analysis, was used to investigate the independent association of the risk factors with CVD mortality.

RESULTS

Anthropometric and laboratory characteristics of study subjects

Subjects with type 2 diabetes and IGT were slightly older, but post hoc testing did not reach statistical difference among groups (Table 1). BMI and waist circumference were not statistically different among groups. Waist-to-thigh ratio was significantly increased in individuals with type 2 diabetes with respect to that in individuals with NGT; this feature was more evident in women ($P = 0.0001$) than in men ($P = 0.274$). The prevalence of smokers was not different in the three groups. Systolic and diastolic blood pres-

Table 1—Anthropometric and laboratory parameters of study subjects

	Type 2 diabetes	IGT	NGT
n (female/male)	99 (53/46)	77 (42/35)	187 (112/75)
Anthropometric parameters			
Age (years)	63 ± 11	63 ± 12	60 ± 13
Body weight (kg)	77 ± 17	71 ± 16	77 ± 17
Height (cm)	161 ± 9	158 ± 10	160 ± 10
BMI (kg/m ²)	30.0 ± 6.3	28.3 ± 5.0	29.9 ± 6.0
Waist (cm)	98 ± 14	94 ± 14	95 ± 15
Waist-to-thigh ratio	1.84 ± 0.19*	1.81 ± 0.23	1.74 ± 0.24
Smoking habit n (%)	20 (20%)	15 (19%)	46 (25%)
Alcohol consumption (alcohol units)	3 ± 11	2 ± 10	2 ± 8
Systolic blood pressure (mmHg)	161 ± 24*	155 ± 20	151 ± 21
Diastolic blood pressure (mmHg)	86 ± 14*	83 ± 13	82 ± 12
Biochemical laboratory parameters			
Glucose (mmol/l)	7.27 ± 1.67*†	5.55 ± 0.83*	5.11 ± 0.50
2-h glucose (mmol/l)	12.71 ± 3.83*†	9.10 ± 0.83*	4.83 ± 1.22
Cholesterol (mmol/l)	6.00 ± 1.03	6.03 ± 1.01	6.15 ± 1.24
HDL cholesterol (mmol/l)	1.14 ± 0.34*†	1.34 ± 0.49	1.32 ± 0.39
Triglycerides (mmol/l)	1.63 ± 0.92*†	1.37 ± 0.57	1.38 ± 0.51
ALT (units/liter)	27 ± 10	28 ± 11	26 ± 8
AST (units/liter)	24 ± 11	23 ± 15	22 ± 15
GGT (units/liter)	36 ± 30	35 ± 31	27 ± 30
ALP (units/liter)	174 ± 51	176 ± 45	172 ± 45
Fibrinogen (mg/dl)	286 ± 79	297 ± 66	275 ± 61
Hormones			
Insulin (pmol/l)	131 ± 108*†	103 ± 52	95 ± 41
Leptin (ng/ml)	10.7 ± 4.5	10.0 ± 5.8	10.4 ± 5.0
MCP-1/CCL2 (pg/ml)	224 ± 275*	195 ± 171	159 ± 113
Insulin sensitivity			
QUICKI	0.127 ± 0.014*†	0.136 ± 0.014	0.138 ± 0.011

Data are means ± SD or n (%). Leptin, insulin, triglycerides, glucose, AST, ALT, GGT, and ALP are expressed as geometric means ± SD. Subjects with a previously proved diagnosis of diabetes did not undergo an OGTT. * $P < 0.05$ vs. NGT. † $P < 0.05$ vs. IGT ANOVA and Bonferroni post hoc analysis.

ures showed an increasing trend according to glucose tolerance. Systolic ($P = 0.008$) and diastolic ($P = 0.087$) blood pressures were higher in women with type 2 diabetes with respect to those in women with NGT, whereas this feature was less evident in men ($P = 0.172$ and $P = 0.130$ for systolic and diastolic pressure, respectively). As expected, fasting and 2-h serum glucose concentrations were increased in patients with type 2 diabetes and IGT in comparison with those in subjects with NGT. Total cholesterol and LDL cholesterol were not different in the groups. However, HDL cholesterol and triglycerides differed significantly in patients with type 2 diabetes, which showed unfavorable alterations of the lipid profile. The lipid profiles of subjects with IGT, on the contrary, were not different from those of individuals with NGT. Serum ALT, AST, GGT, ALP, and fibrinogen were not different in any groups. Patients with type 2 diabetes and IGT were characterized by fasting hyper-

insulinemia and by insulin resistance expressed as a significantly lower QUICKI value. Serum leptin were not different in the study groups when leptin concentration was normalized to the BMI units.

Cross-sectional analysis of baseline MCP-1/CCL2

The plasma MCP-1/CCL2 concentration was increased in type 2 diabetic women but not in those with IGT with respect to those with NGT (Table 1). MCP-1/CCL2 was not associated with any anthropometric parameters but showed a significant association with biochemical markers of atherosclerotic disease. In fact, MCP-1/CCL2 was associated with fasting ($R = 0.15$; $P = 0.007$) and 2-h plasma glucose after the glucose challenge ($R = 0.14$; $P = 0.04$), HDL cholesterol ($R = -0.21$; $P = 0.0003$), and plasma triglycerides ($R = 0.15$; $P = 0.01$). These parameters are typical markers of the insulin resistance syndrome; confirming this observation, QUICKI was also found to be

significantly associated ($R = -0.19$; $P = 0.009$). In addition, MCP-1/CCL2 showed a significant association with α -TNF-R2 ($R = 0.14$; $P = 0.01$) but no association with leptin. Even if MCP-1/CCL2 was not significantly associated with BMI, we tested whether the above-described association of MCP-1/CCL2 was independent of BMI. The results of this analysis demonstrated that all the correlations remained significant regardless of the parameter of body adiposity.

Univariate analysis after the 15-year observational period

After 15 years, among the 363 subjects, 82 deaths occurred due to CVD (22.6%) (Table 2). The CVD mortality rate was 30% in patients with type 2 diabetes (30 of 99), 19.5% in individuals with IGT (15 of 77), and 19.8% in individuals with NGT (37 of 187). In univariate analysis, age (HR 1.127 [95% CI 1.098–1.157]; $P < 0.0001$) was associated with CVD mortality, and female sex was less prone to be associated (0.429 [0.275–0.671]; $P < 0.0002$). Age- and sex-adjusted univariate analysis showed that fasting plasma glucose concentration, MCP-1/CCL2, fibrinogen, cigarette smoking, and diabetes were significantly associated with CVD mortality (Table 2). In addition, fasting insulin showed a trend to be associated with CVD mortality ($P = 0.064$). When the analysis was performed separately by sex, age, fasting plasma glucose, and MCP-1/CCL2 were significantly associated in both men and women, whereas fasting plasma insulin and smoking were significantly associated with CVD mortality only in men and serum fibrinogen was significantly associated with CVD mortality only in women.

Multivariate analysis after the 15-year observational period

Multivariate analysis was performed using only variables significant at $P < 0.1$ in univariate analysis. The analysis in the entire population (Table 3) showed that age, sex, fasting glucose, MCP-1/CCL2, and smoking were independent predictive variables of CVD mortality (Table 3).

hs-CRP and MCP-1/CCL2

For hs-CRP, data on MCP-1/CCL2 and hs-CRP were simultaneously available for only 113 subjects. These subjects were older (71 ± 4 years) than those reported in the present article, and the number of cardiovascular events was 46. No correlation between plasma MCP-1/CCL2 and

Table 2—Age- and sex-adjusted risk ratio associated with 15 years cardiovascular mortality by univariate analysis

Variable	HR (95% CI)	P
BMI	1.037 (0.992–1.083)	0.109
LDL cholesterol	0.999 (0.993–1.005)	0.769
HDL cholesterol	0.994 (0.98–1.009)	0.448
Triglycerides	1.001 (0.997–1.004)	0.723
Fasting glucose	1.010 (1.004–1.016)	0.0015
2-h glucose	1.002 (0.997–1.007)	0.367
Fasting insulin	1.011 (0.999–1.023)	0.064
Systolic blood pressure	1.005 (0.995–1.015)	0.296
Diastolic blood pressure	1.004 (0.987–1.022)	0.642
Leptin	0.998 (0.972–1.026)	0.910
α -TNF-R2	1.000 (1.000–1.000)	0.987
MCP-1/CCL2	1.001 (1.000–1.002)	0.019
Fibrinogen	1.003 (1.000–1.006)	0.021
Smoking	2.383 (1.211–4.691)	0.012
Metabolic syndrome	1.117 (0.701–1.779)	0.641
Diabetes	1.716 (1.092–2.697)	0.019

Data are HR (95% CI).

hs-CRP (Pearson $r = 0.019$; $P = 0.85$) was detected. In univariate analysis hs-CRP was not related to CVD (HR 1.029 [95% CI 0.984–1.076]; $P = 0.22$), and in this smaller cohort MCP-1/CCL2 but not hs-CRP (1.030 [0.986–1.077]; $P = 0.19$) showed a trend to be significantly associated with CVD mortality in age- and sex-adjusted multivariate analysis (1.001 [1.000–1.002]; $P = 0.0593$).

CONCLUSIONS— In the present study, we have shown that in middle-aged overweight/obese individuals, the fasting plasma MCP-1/CCL2 concentration was related to biochemical risk markers of atherosclerosis and that this concentration was higher in individuals with type 2 diabetes and IGT with respect to that in nondiabetic patients. Most importantly, when CVD mortality was assessed retrospectively 15 years after the baseline assessment, we report that MCP-1/CCL2 was independently associated with CVD mortality.

Table 3—Cox proportional hazards model of the predictors of 15 years CVD mortality by multivariate analysis

Variable	HR (95% CI)	P
Age	1.153 (1.114–1.193)	<0.0001
Sex	0.434 (0.259–0.728)	0.0006
Fasting glucose	1.009 (1.001–1.016)	0.0253
MCP-1/CCL2	1.001 (1.000–1.002)	0.0089
Cigarette smoking	2.432 (1.110–5.328)	0.035

Data are HR (95% CI). Only variables significant at $P < 0.1$ at univariate analysis were tested in the models. Only variables that remained significantly associated are shown.

CCL2. This finding was not unexpected because in vitro, advanced glycation end-products, a high glucose concentration, glycated albumin, and glycoxidized LDL enhanced MCP-1/CCL2 expression in human endothelial cells (21). More importantly, the results support the hypothesis that MCP-1/CCL2 may be involved directly in the pathogenesis of atherosclerosis in humans because its baseline serum concentration was independently associated with CVD mortality assessed 15 years after the baseline determination of its fasting plasma concentration. There are many data in the literature that support a role of MCP-1/CCL2 in the pathogenesis of both the initiation and progression of atherosclerosis. MCP-1/CCL2 was found to be highly expressed within atherosclerotic lesions (22), and its role in atherogenesis is consistent with in vitro and in vivo studies in animal models (23). In humans, elevated MCP-1/CCL2 serum levels were found to be increased not only in individuals with diabetes but also in subjects at risk because of other risk factors for atherosclerosis, such as age, hypertension, hypercholesterolemia, renal failure, and vascular disease or in individuals with overt coronary artery disease as outlined in the INTRODUCTION. Recently, a genetic variation in the MCP-1/CCL2 gene was associated with a higher serum concentration of the chemokine and a higher prevalence of myocardial infarction (24).

Clinical implications

The results of the present study are potentially clinically important. With regard to the inflammatory hypothesis of atherosclerosis, these data suggest that elevated MCP-1/CCL2 levels, serving as a direct marker of inflammatory activity, may also be a biomarker of the risk of CVD. Holding this view and taking into account the fact that the fasting MCP-1/CCL2 level predicted CVD mortality when assessed 15 years later, we suggest that this chemokine may be an early independent predictor of atherosclerosis and an early biomarker of its natural development in the history of an at-risk individual. Interestingly, within our population the MCP-1/CCL2 plasma concentration was linked more strongly with CVD than the post-challenge plasma glucose concentration, metabolic syndrome, and serum circulating lipids (Table 2). Another clinical implication is that because treatment of several components of the insulin resistance syndrome (adiposity, dyslipidemia,

and hypertension) had beneficial effects in preventing cardiovascular disease, if subclinical inflammation is indeed another facet of the insulin resistance syndrome, anti-inflammatory treatment may also be beneficial. A recent randomized clinical trial tested the administration of 20 mg/day rosuvastatin in nondiabetic individuals with anthropometric features similar to those of the subjects in our study (age, sex, and BMI) who had LDL cholesterol levels in the normal range but higher circulating hs-CRP levels. In this study, a 44% reduction of major cardiovascular events along with a significant reduction of hs-CRP circulating levels was reported (25), suggesting that the effects of this statin may, at least partly, be mediated through anti-inflammatory properties. In addition, it is known that pharmacological treatment with hydroxymethylglutaryl-CoA reductase inhibitors may lower MCP-1/CCL2 levels (9). Thus, elevated MCP-1/CCL2 levels could identify patients more likely to benefit from aggressive statin treatment, although we must emphasize that whether treatments to reduce circulating levels of MCP-1/CCL2 are effective in reducing the onset of CVD is yet to be determined. Based also on our results, the hypothesis that the development of pharmacological tools able to target anti-inflammatory pathways may be a potential approach to reduce rates of vascular events may be true. More potent prospective studies are clearly needed to address these issues.

Study limitations and strengths

The population in which MCP-1/CCL2 plasma concentrations were obtained was small, and larger studies are needed to confirm our results. Conversely, our observational period was 15 years, and we had a high number of events (82 CVD deaths in 363 individuals) relative to the entire population. An additional limitation was that the distribution of MCP-1/CCL2 plasma concentrations in the individuals with CVD mortality and those without overlapped considerably, suggesting that plasma MCP-1/CCL2 levels alone will not be helpful in predicting CVD mortality. Therefore, we are exploring circulating levels of MCP-1/CCL2 as a systemic surrogate, trying to test whether these may be linked to a local event (atherosclerosis). However, when MCP-1/CCL2 is considered along with other classic risk factors, it may eventually be useful as a surrogate biomarker in patients with IGT and diabetes.

Another limitation of the study is that we did not obtain serial plasma MCP-1/CCL2 concentrations. The association we report in the present work is between one single assessment of the plasma chemokine concentration and the CVD mortality assessed 15 years later, but we have no data about the circulating levels of the chemokine during the observational period.

Finally, we tested MCP-1/CCL2 along with a standard inflammatory marker such as fibrinogen because that biomarker was measured in 1990–1991. hs-CRP is a more reliable marker, but, unfortunately, we only had simultaneous availability of MCP-1/CCL2 and hs-CRP for 113 subjects. Even if MCP-1/CCL2 remained associated with CVD mortality in this subset of individuals, suggesting an independent role of MCP-1/CCL2, a rigorous comparison remains to be done.

In summary, in the present study, we report that in middle-aged individuals the plasma MCP-1/CCL2 concentration was related to biochemical risk markers of atherosclerosis and, in particular, to both fasting and post-OGTT plasma glucose concentrations. In the multivariate analysis MCP-1/CCL2 was associated with increased CVD mortality assessed 15 years later.

Acknowledgments— This work was supported by grants from Italian Minister of Health (030.5/RF96.305 and 030.5/RF98.49), Ministero dell'Istruzione, Università e Ricerca Cofin (9806409093), and the Italian National Research Council (CNR 97.00485.CT04). The financial support of the European Association for the Study of Diabetes is also gratefully acknowledged.

No potential conflicts of interest relevant to this article were reported.

Parts of this study were presented in abstract form at the 69th Scientific Sessions of the American Diabetes Association, New Orleans, Louisiana, 5–9 June 2009.

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