Fasting Plasma Leptin, Tumor Necrosis Factor-α Receptor 2, and Monocyte Chemoattracting Protein 1 Concentration in a Population of Glucose-Tolerant and Glucose-Intolerant Women

Impact on cardiovascular mortality

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OBJECTIVE — Leptin and tumor necrosis factor (TNF)-α are associated with insulin resistance and cardiovascular disease. In vitro studies suggested that these effects may be mediated via overproduction of monocyte chemoattracting protein (MCP)-1/CCL2, which is a chemokine involved in the pathogenesis of atherosclerosis.

RESEARCH DESIGN AND METHODS — In this study, fasting plasma leptin, soluble TNF-α receptor 2 (TNF-α-R2), and MCP-1/CCL2 concentrations were measured in 207 middle-aged women (age 61 ± 12 years, BMI 30.1 ± 6.6 kg/m^2), including 53 patients with type 2 diabetes, 42 with impaired glucose tolerance, and 112 with normal glucose tolerance, to assess cross-sectionally their relationship with markers of atherosclerosis and, longitudinally over 7 years, whether their circulating levels were associated with cardiovascular disease (CVD) mortality.

RESULTS — At baseline, leptin and TNF-α-R2 were not different among groups; meanwhile, MCP-1/CCL2 was increased in type 2 diabetes ($P < 0.05$). All showed significant associations with biochemical risk markers of atherosclerosis. In a univariate analysis, age, fasting insulin, leptin, and MCP-1/CCL2 were associated with CVD mortality at 7 years. When a multivariate analysis was performed, only age, leptin, and insulin retained an independent association with CVD mortality, with leptin showing a protective effect (hazard ratio 0.88; $P < 0.02$).

CONCLUSIONS — In middle-aged women, MCP-1/CCL2, leptin, and TNF-α-R2 were all related to biochemical risk markers of atherosclerosis. MCP-1/CCL2 concentration was the only one to be increased in type 2 diabetes with respect to nondiabetic women and the only one to be associated with increased risk of CVD mortality after a 7-year follow-up period in the univariate analysis. In the multivariate analysis, neither MCP-1/CCL2 nor TNF-α-R2 was associated with CVD mortality, and inspection of the data showed that leptin, in both the univariate and multivariate analysis, was associated with a protective effect.

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; CVD, cardiovascular disease; γGT, γ-glutamyl transferase; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; MCP, monocyte chemoattracting protein; QUICKI, quantitative insulin sensitivity check index; TNF, tumor necrosis factor; TNF-α-R2, TNF-α receptor 2.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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 chemoattracting protein (MCP)-1/CCL2, a member of the CC chemokine family, is involved in most of these processes (2). In the human endothelial cell culture system, oxidized LDL (3) and shear stress (4) upregulated MCP-1/CCL2 synthesis, suggesting that the plaque formation associated with dyslipidemia and hypertension may be related to MCP1/CCL2. Moreover, MCP-1/CCL2 knockout mice placed in a 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 of 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 appears clear, its role in vivo in humans remains unknown. Excess adipose tissue is a typical feature of the metabolic syndrome, and it is now believed that it may also be relevant in the pathogenesis of atherosclerosis (7). Leptin and tumor necrosis factor (TNF)-α are adipocyte-derived products involved in the pathogenesis of insulin resistance and obesity (8). They were suggested to be the key factors mediating the adipose tissue deleterious effects on the in vivo metabolic homeostasis. In vitro, both leptin and TNF-α stimulate endothelial MCP-1/CCL2 secretion (9,10); leptin may do so by inducing accumulation of reactive oxygen species, which are recognized proinflammatory factors involved in the

pathogenesis of atherosclerosis (10). In this study, we explored the relationships among fasting plasma MCP-1/CCL2, leptin, and α-TNF concentration in vivo in humans. To achieve this goal, we used two approaches: *1*) we correlated known risk factors of atherosclerosis with circulating MCP-1/CCL2, leptin, and TNF-α receptor 2 (TNF-α-R2) in a cross-sectional fashion in 207 middle-aged women with or without normal glucose tolerance; and *2*) we assessed whether these circulating levels were associated with cardiovascular disease (CVD) mortality assessed in the same group after a 7-year period.

RESEARCH DESIGN AND METHODS

Study cohort and follow-up

The 207 women who participated in the study were selected from a population survey carried out in 1990–1991 in the Health District of Cremona (Lombardy, Italy), performed to determine the prevalence of diabetes in Italy according to the oral glucose tolerance test and World Health Organization criteria (11). Fasting plasma leptin, TNF-α-R2, and MCP-1/CCL2 concentrations were measured in patients with type 2 diabetes (known to be affected or previously undiagnosed) ($n = 53$) and patients with impaired glucose tolerance (IGT) ($n = 42$) for whom an aliquot of frozen plasma sample was available and properly stored since 1990–1991 for the assay. A total of 112 individuals with normal glucose tolerance (NGT) were randomly selected from the above-described population to be compared with 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 taken by the same trained person using the same instruments for all subjects. Heart rate and systolic and diastolic blood pressures were taken twice (at the beginning and 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 (11,12). Seven years later, vital status and time of death were ascertained on 28 February 1997 through Regional Health Registry files, and causes of death were classified using ICD-8 and -9 codes 401–448 (CVD), 410–414 (CHD), and 430–438 (stroke).

Definition of diabetes and IGT

Diabetes was defined according to a previously known diabetes status (patients on oral hypoglycemic agents) or according to the results of the oral glucose tolerance test and to 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 oral glucose tolerance test. A total of 18 subjects were diagnosed on this occasion, 37 were on diet treatment, 36 were on oral hypoglycemic agents, and 8 were on insulin therapy. 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.

Analytical determinations

Blood and serum and plasma substrates were assessed as previously described (11). Blood was collected into tubes with a glycolytic inhibitor, and glucose concentration was measured within 3–4 h in the central laboratory with the GOD-PAP glucose oxidase method (Boehringer Mannheim, Milan, Italy) on a Hitachi 705 autoanalyzer. At the same time, fibrinogen, aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyl transferase (γGT), and alkaline phosphatase (ALP) were also determined. An additional 20 ml fasting blood was immediately centrifuged, and plasma was obtained for assessment of insulin, triglycerides, and total and HDL cholesterol in the central laboratory. Leptin concentrations were determined as previously described (13,14) by radioimmunoassay with a human kit (Linco Research, St. Charles, MO). Intra- and interassay coefficients of variations (CVs) were 1.5 and 1.9%, respectively. Insulin was determined by a radioimmunoassay (intra- and interassay CVs were 6.0 and 5.3%, respectively) (Technogenetics kit; Medgenics, Brussels, Belgium). Plasma human MCP-1/CCL2 concentration was measured using a sandwich enzyme-linked immunosorbent assay as previously described (15); the enzyme-linked immunosorbent assay for MCP-1/CCL2 is specific for human MCP-1 and does not detect the closely related human chemokines MCP-2 and MCP-3 (16). TNF-α-R2 was measured with an enzyme immunoassay following manufacturer (Immunotech Beckman Coulter, Marseille, France) recommendations as previously described (14). 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 PEG using a Colortest kit (Roche, Basel, Switzerland).

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 (17) 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 the Statistical Analysis System (SAS) software. Concentrations are presented as means ± SD unless differently stated. ANOVA and χ^2 analysis were used for comparison between groups, and Bonferroni adjustment was used for post hoc comparisons. Because of the skewed distribution of serum leptin, insulin, triglycerides, fibrinogen, glucose, AST, ALT, γGT, and ALP, log-transformed values were used in the analysis. Pearson's correlation analysis was used for correlations. The association of the investigated risk factors with CVD mortality after the 7-year observational period was estimated by the Cox proportional hazard model. Hazard ratios (HRs) and 95% CIs are presented. χ^2 for trend was used to investigate the presence of a trend in the proportion of CVD mortality in classes of tertiles of plasma leptin concentrations.

Table 1—*Anthropometric and laboratory parameters of study groups*

	Type 2 diabetes	IGT	NGT
Subjects (*n*)	53	42	112
Anthropometric parameters			
Age (years)	65 ± 11	64 ± 12	60 ± 12
Body weight (kg)	73.5 ± 17.7	63.5 ± 13.2	71.3 ± 17.9
Height (cm)	155.0 ± 6.4	150.9 ± 5.9	152.5 ± 5.7
BMI (kg/m^2)	30.6 ± 7.1	28.6 ± 5.2	30.5 ± 6.7
Waist (cm)	94 ± 14	90 ± 13	92 ± 14
Waist-to-thigh ratio	1.76 ±0.16*	1.68 ± 0.20	1.63 ± 0.19
Actual smoking habit [*n* (%)]	8 (15)	2 (5)	19 (17)
Alcohol consumptoin (alcohol units)	0.5 ± 1.9	0.3 ± 1.2	0.5 ± 2.0
Heart rate (bpm)	82.4 ± 17.4*	80.9 ± 12.1	76.0 ± 11.8
Systolic blood pressure (mmHg)	163 ± 22*	156 ± 21	151 ± 23
Diastolic blood pressure (mmHg)	85 ± 14	81 ± 13	80 ± 13
Biochemical laboratory parameters			
Fasting blucose (mmol/l)	7.66 ± 1.89*†	5.50 ± 0.56*	4.94 ± 0.56
2-h glucose (mmol/l)	14.54 ± 3.49*†	8.99 ± 0.83*	5.00 ± 1.28
Cholesterol (mmol/l)	6.05 ± 0.88	6.28 ± 0.91	6.31 ± 1.22
HDL cholesterol (mmol/l)	1.19 ± 0.36*	1.40 ± 0.39	1.42 ± 0.34
LDL cholesterol (mmol/l)	4.06 ± 0.83	4.19 ± 0.83	4.24 ± 1.11
Triglycerides (mmol/l)	1.55 ± 0.90*	1.41 ± 0.58	1.30 ± 0.47
ALT (units/l)	25 ± 11	24 ± 7	25 ± 9
AST (units/l)	22 ± 11	20 ± 10	20 ± 12
γGT (units/l)	29 ± 20*	25 ± 17	21 ± 14
ALP (units/l)	174 ± 56	183 ± 53	177 ± 45
Fibrinogen (mg/dl)	293 ± 88	315 ± 63	286 ± 65
Insulin sensitivity			
QUICKI	0.125 ± 0.011*†	0.134 ± 0.009*	0.140 ± 0.011
Hormones			
Insulin (pmol/l)	140 ± 94*	109 ± 50	90 ± 46
Leptin (ng/ml)	14.1 ± 7.8	15.7 ± 10.0	15.5 ± 9.9
Leptin-to-BMI ratio	0.51 ± 0.20	0.63 ± 0.34	0.59 ± 0.30
TNF-α-R2 (ng/ml)	1.26 ± 0.0	1.26 ± 0.0	1.25 ± 0.0
MCP-1/CCL2 (pg/ml)	266 ± 365*	206 ± 212	149 ± 48

Leptin, insulin, triglycerides, fibrinogen, glucose, AST, ALT, γGT, and ALP are expressed as geometric means ± SD. All other data are *n*, *n* (%), or means ± SD. Subjects with previously proved diagnosis of diabetes did not undergo the oral glucose tolerance test. *$P < 0.05$ vs. NGT; †$P < 0.05$ vs. IGT.

RESULTS

Anthropometric and laboratory characteristics of study subjects

As summarized in Table 1, subjects with type 2 diabetes and IGT were slightly older, but post hoc testing did not reach statistical difference among groups. 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 women with NGT. The prevalence of smokers was not different in the three groups. Heart rate was significantly higher in women with type 2 diabetes and IGT than in women with NGT. Systolic and diastolic blood pressures also showed a trend for increasing values according to glucose tolerance, but only systolic blood pressures were higher in women with type 2 diabetes with respect to women with NGT. As expected, fasting and 2-h serum glucose concentrations were increased in patients with type 2 diabetes and IGT compared with women with NGT. Total and LDL cholesterol were not different in the groups; meanwhile, HDL cholesterol and triglycerides significantly differed in patients with type 2 diabetes, showing unfavorable alterations of the lipid profile. The lipid profile of subjects with IGT, on the contrary, was not different from that of women with NGT. Serum ALT, AST, ALP, and fibrinogen were not different in all groups. γGT was slightly increased in women with type 2 diabetes with respect to women with NGT. Patients with type 2 diabetes were characterized by fasting hyperinsulinemia; meanwhile, patients with both type 2 diabetes and IGT showed marked insulin resistance, expressed as a significantly lower QUICKI value.

Cross-sectional analysis of baseline data: leptin

Plasma leptin was not different in the study groups when leptin concentration was normalized to the BMI. Fasting serum leptin strongly correlated with BMI ($R = 0.62$; $P = 0.0001$), the waist circumference ($R = 0.62$; $P = 0.0001$), and the waist-to-thigh ratio ($R = 0.27$; $P = 0.0001$) in a similar fashion in the three study groups. Leptin was strongly associated with markers of insulin sensitivity; in fact, it was directly proportional to fasting serum insulin levels ($R = 0.51$; $P = 0.0001$) and inversely associated with the QUICKI ($R = -0.46$; $P = 0.0001$). Leptin was also associated with the circulating levels of TNF-α-R2 ($R = 0.15$; $P = 0.009$), regardless of glucose tolerance status. Leptin was directly associated with 2-h blood glucose levels ($R = 0.19$; $P = 0.0018$). Also, a proatherosclerotic lipid profile was associated with the serum leptin concentration: in fact, the serum HDL cholesterol concentration was inversely related to the leptin levels ($R = -0.15$; $P = 0.028$); meanwhile, LDL cholesterol ($R = 0.18$; $P = 0.0005$) and triglyceride concentrations ($R = 0.13$; $P = 0.017$) were directly proportional to the leptin levels. Among the proatherosclerotic risk factors, the systolic blood pressure was proportionally associated with increased serum leptin levels ($R = 0.15$; $P = 0.03$); meanwhile, the diastolic blood pressure showed a less pronounced degree of association ($R = 0.11$; $P = 0.09$). In addition, leptin levels were associated with the serum fibrinogen concentration ($R = 0.29$; $P = 0.0001$) regardless of diagnosis of type 2 diabetes, IGT, or NGT. Leptin was not significantly associated with MCP-1/CCL2 circulating levels ($R = 0.025$; $P = 0.74$). Even if the majority of the individuals included in the study were overweight or obese within a narrow range of BMIs, the correlation analysis demonstrated a significant relationship between leptin and BMI. We therefore tested whether the above-described relationships of leptin with the markers of atherosclerosis were independent of BMI. We observed that the association of leptin

with insulin, QUICKI, LDL cholesterol, and triglycerides remained significant regardless of BMI; meanwhile, the association with TNF-α-R2, 2-h blood glucose levels, HDL cholesterol, systolic and diastolic blood pressure, and fibrinogen was lost when normalized for BMI.

Cross-sectional analysis of baseline data: MCP-1/CCL2

Plasma MCP-1/CCL2 concentration was increased in type 2 diabetic women, but not in women with IGT with respect to women 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 triglyceride ($R = 0.15$; $P = 0.01$) measurements. These parameters are typical markers of the insulin resistance syndrome, and, confirming this observation, QUICKI was also found to be significantly associated ($R = -0.19$; $P = 0.009$). In addition, MCP-1/CCL2 also showed a significant association with TNF-α-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 associations of MCP-1/CCL2 were independent of BMI. The results of this analysis demonstrated that all the correlations remained significant regardless of the parameter of body adiposity.

Cross-sectional analysis of baseline data: TNF-α-R2

Fasting plasma TNF-α-R2 was not different in patients with type 2 diabetes, IGT, or NGT. Even if a relationship with BMI ($R = 0.07$; $P = 0.21$) was not found, TNF-α-R2 was associated with age ($R = 0.36$; $P = 0.0001$), waist ($R = 0.12$; $P = 0.04$), and weight-to-thigh ratio ($R = 0.20$; $P = 0.0004$). A common denominator with the markers of atherosclerosis typical of the metabolic syndrome was also reflected by the associations with plasma glucose 2 h after the glucose challenge ($R = 0.22$; $P = 0.0008$), HDL cholesterol ($R = 0.14$; $P = 0.01$), triglycerides ($R = 0.15$; $P = 0.08$), and moreover with fasting plasma insulin ($R = 0.21$; $P = 0.0003$) and QUICKI ($R = 0.12$; $P = 0.04$). Even if TNF-α-R2 was not significantly associated with BMI, we tested whether the above-described associations of TNF-α-R2 were independent of BMI. The results of this analysis demonstrated that all the correlations remained significant regardless of the parameter of body adiposity.

Table 2—*Cox proportional hazard models of the predictors of 7-year CVD mortality by univariate analysis in the 207 women overall*

	Mortality for CVD	
	Hazard ratio (95% CI)	*P*
Age	1.15 (1.08–1.22)	<0.001
BMI	0.96 (0.87–1.06)	0.48
LDL Cholesterol	0.99 (0.98–1.009)	0.47
HDL cholesterol	1.01 (0.98–1.04)	0.69
Triglycerides	1.00 (0.99–1.01)	0.68
Fasting glucose	1.01 (0.99–1.02)	0.45
Insulin	1.03 (1.01–1.05)	0.04
Systolic blood pressure	1.02 (1.00–1.04)	0.12
Heart rate	1.02 (0.99–1.04)	0.24
Leptin	0.92 (0.85–0.99)	0.03
MCP1/CCL2	1.05 (1.03–1.07)	0.014
TNF-α-R2	1.00 (0.99–1.01)	0.40
Fibrinogen	1.00 (0.99–1.01)	0.75
Actual smoking habit	3.56 (0.35–35.9)	0.28
Glucose intolerance (IGT + diabetes) vs. NGT	1.15 (0.43–3.06)	0.78

Hazard ratios are age adjusted.

Univariate analysis after the 7-year observational period

After 7 years, the CVD mortality rate was 18.9% in patients with type 2 diabetes (10 of 53), 9.5% in women with IGT (4 of 42), and 2.7% in women with NGT (3 of 112). Univariate analysis showed that age, fasting plasma insulin, fasting plasma leptin, and fasting plasma MCP-1/CCL2 were significantly associated with CVD (Table 2). In Fig. 1, the impact of leptin on CVD mortality is more clearly evident when CVD mortality is shown by tertiles of leptin. Tertile cutoffs were 12.0 and 22.0 ng/ml. CVD mortality was higher in the tertiles of low leptin concentrations (10 deaths; 14.7%) and progressively decreased in the tertiles of intermediate (4 deaths; 5.6%) and higher (3 deaths; 4.5%) leptin concentrations (χ^2 for trend, $P = 0.03$) (Fig. 1).

Multivariate analysis after a 7-year observational period

Multivariate analysis was performed using only variables significant at $P < 0.1$ in a univariate analysis; BMI was also included because of its strong relationship with serum leptin concentration. The analysis in the entire population (Table 3) showed that age ($P < 0.001$), fasting plasma insulin ($P = 0.008$), and serum leptin ($P = 0.02$) were independent predictive variables; when the analysis was performed excluding individuals with NGT and maintaining patients with type 2 diabetes and IGT pooled together, age ($P < 0.001$), fasting plasma insulin ($P = 0.005$), and serum leptin ($P = 0.05$) were confirmed to be significantly associated with CVD mortality. Whereas age (HR

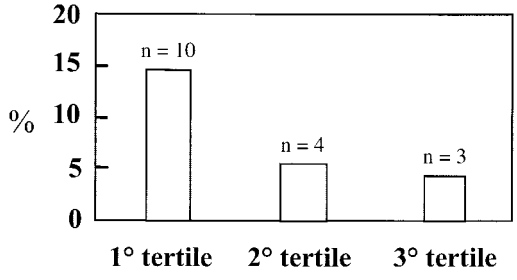

Figure 1—*CVD mortality by tertiles of leptin. Tertile cutoffs were 12.0 and 22.0 ng/ml. CVD mortality in women was higher in the tertiles of low leptin concentrations (14.7%) and progressively decreased in the tertiles of intermediate (5.6%) and higher (4.5%) leptin concentrations (χ^2 for trend,* P = *0.03).*

Table 3—*Cox proportional hazard models of the predictors of 7-year CVD mortality by multivariate analysis*

	7-year mortality for CVD					
	General population		Glucose intolerant		Glucose tolerant	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	1.14 (1.10–1.22)	<0.001	1.20 (1.08–1.32)	<0.001	1.13 (1.04–1.24)	0.006
BMI	1.05 (0.92–1.19)	0.46	1.14 (0.98–1.34)	0.11	0.86 (0.6–1.15)	0.310
Leptin	0.88 (0.80–0.97)	0.02	0.86 (0.74–1.00)	0.05	0.91 (0.7–1.07)	0.27
Insulin	1.03 (1.01–1.05)	0.008	1.03 (1.01–1.06)	0.005	0.99 (0.87–1.14)	0.94

Only variables significant at $P < 0.1$ at univariate analysis were tested in the models. Glucose-intolerant individuals are patients with type 2 diabetes and IGT pooled together.

1.14) and fasting plasma insulin (HR 1.03) were independent risk factors for CVD mortality, fasting serum leptin (HR 0.88) was an independent protective factor.

CONCLUSIONS — In the present study, we have shown that in middle-aged women, MCP-1/CCL2, leptin, and TNF-α-R2 were all independently related to biochemical risk markers of atherosclerosis, but circulating MCP-1/CCL2 concentration was the only one to be increased in women with type 2 diabetes and IGT with respect to nondiabetic patients. Moreover, the univariate analysis demonstrated that CVD mortality after a 7-year follow-up period was associated with increased MCP-1/CCL2 and decreased leptin; in the multivariate analysis, lower leptin retained an independent association with CVD mortality.

Pathophysiological implications

Adipose tissue has been cited as playing a crucial role in the development of the metabolic syndrome and of modulating the increased risk to develop atherosclerosis and cardiovascular events in affected individuals. The link between increased adipogenesis and development of atherosclerosis is still not well understood; adipocyte-derived growth factors and cytokines might be potential candidates of the effects on the vascular wall (18), and leptin, the TNF-α system, and MCP-1/CCL2 could regulate each other in an autocrine-paracrine-endocrine fashion, resulting in atherosclerosis. In the line of this working hypothesis, recent works have also suggested the existence of *1*) a leptin-MCP-1/CCL2 axis and *2*) a TNF-α-MCP-1/CCL2 axis. The leptin receptor with the longest cytoplasmic domain (Ob-Rb) has been found to be expressed in endothelial cells (19), and leptin has been shown to increase the accumulation of reactive oxygen species and to induce the secretion of MCP-1/CCL2 (10). In our study, serum leptin did not show a significant association with MCP-1/CCL2, and their impact on CVD mortality after a 7-year observational period demonstrated discordant effects in the univariate analysis (MCP-1/CCL2 was associated with increased CVD mortality, and leptin in contrast was associated with reduced CVD mortality). The in vitro studies, besides the leptin-MCP-1/CCL2 pathway, suggested that a TNF-α-MCP-1/CCL2–related pathway may represent the link to adipocytes/atherosclerosis. In this study, we measured the levels of soluble TNF-α-R2, which has been validated as a sensitive indicator of activation of the TNF-α system (20). Even if baseline TNF-α-R2 was not associated with CVD mortality 7 years later, we observed that its level correlated with leptin, confirming the existence of the leptin/TNF-α axis, and moreover with MCP-1/CCL2. On the basis of our study, the understanding of the relationships among leptin, TNF-α, and MCP-1/CCL2 is difficult: we might speculate that both the increased activity of the TNF-α system and the typical biochemical modification of insulin resistance may promote the MCP-1/CCL2 expression in endothelial cells, resulting in atherosclerosis. The lack of association of leptin with MCP-1/CCL2 and the fact that the results of the longitudinal approach failed to detect a significant deleterious effect of leptin on CVD mortality would suggest that the association between leptin levels and atherogenic phenotypes shown in the correlative studies and in the cross-sectional analysis of our baseline determinations does not play a primary role in the development of CVD and could be secondary to other factors. The inspection of our data (the multivariate analysis in Table 3 and the tertiles of leptin in Fig. 1) suggests that a relative leptin deficiency in women might contribute to increased CVD mortality, and this is in contrast with previous studies in which leptin was shown to be a risk marker of first-ever hemorrhagic stroke (21), myocardial infarction (22), and coronary events (23). In our opinion, this discrepancy may be explained by two factors. *1*) Our study is the first in which the population of interest was represented by women (previous data were extrapolated in men), and Cnop et al. (24) demonstrated, in contrast with men, a lack of association between parameters of the insulin resistance syndrome and leptin levels in women; the lack of stringent association between leptin and CVD mortality in women may also be similarly explained. *2*) Our study originally included individuals with IGT and overt type 2 diabetes, and recently it was demonstrated that patients with type 2 diabetes, also during metformin treatment, are characterized by reduced circulating leptin levels in the fasting condition (25), even if type 2 diabetes is a condition typically characterized by increased risk of CVD mortality, suggesting that the relationship between fasting leptin and CVD mortality in patients with type 2 diabetes is more complex than previously described and yet to be understood.

MCP-1/CCL2 and diabetes

If these results underscored the hypothesis that, in vivo, the leptin-MCP-1/CCL2 pathway may not be relevant, a peculiar role of MCP-1/CCL2 may not be excluded. In fact, MCP-1/CCL2 was associated with biochemical markers of the metabolic syndrome such as fasting and 2-h plasma glucose after glucose challenge, plasma triglyceride, HDL cholesterol, and QUICKI. Plasma MCP-1/CCL2 levels were found to be increased in aging (26), hypertension (27), hypercholesterolemia (28), renal failure (29), and vascular disease (29,30). This study represents the first report of increased MCP-1/CCL2 plasma levels in diabetes that was recently recognized as an equivalent of atherosclerosis (7). This finding was not unexpected because, in vitro, advanced glycation end product, high glucose concentration, glycated albumin, and glycoxidized LDL enhances MCP-1/CCL2 expression in

human endothelial cells (10,31). In one of the above-mentioned reports, a level above the 75th percentile was also associated with increased risk of death and myocardial infarction (30). With this respect, our work also showed that in the univariate analysis, baseline MCP-1/CCL2 was significantly associated with CVD mortality 7 years later, even if in the multivariate analysis this association disappeared. Interestingly, in contrast with the strong association of MCP-1/CCL2 with biochemical markers of the insulin resistance syndrome, other important risk factors such as systolic and diastolic blood pressure were also not associated.

Limitations of the study

The in vitro and in vivo proatherosclerotic biological effects of MCP-1/CCL2 along with the recent epidemiological findings in patients with acute coronary syndromes in which the long-term clinical outcome was associated with increased MCP-1/CCL2 plasma levels (30) supported the hypothesis that the circulating levels of this chemokine might represent an attractive surrogate biomarker of atherosclerosis in humans. MCP-1/CCL2 is produced by all cell types present in atheroma, and the macrophages and the endothelial cells are considered the primary sources (32). We must emphasize that MCP-1/CCL2 is also produced by vascular smooth muscle cells (32), keratinocytes (33), fibroblasts (34), mesangial cells (35), tubular epithelial cells (35), and lymphocytes in response to proinflammatory stimuli. Therefore, we cannot exclude that, in some individuals, peculiar undetectable conditions might have influenced the circulating levels of MCP-1/CCL2, inducing its production from some nonatheroma-related sources. As a consequence, the above-mentioned results regarding MCP-1/CCL2, leptin, and TNF-α-R2 must be considered exploratory and a more robust validation using prospective cohorts is required.

Nevertheless, the results of the present study are potentially clinically important. Treatment of several components of the insulin resistance syndrome (adiposity, dyslipidemia, and hypertension) had beneficial effects in preventing type 2 diabetes (36) and cardiovascular disease (37). Therefore, if subclinical inflammation is indeed another facet of the insulin resistance syndrome, anti-inflammatory treatment may also be beneficial. Accordingly, it has been suggested that the effects of aspirin or statins may, at least partly, be mediated through anti-inflammatory properties. Furthermore, treatment aimed at improving insulin resistance, whether nonpharmacological, such as exercise and weight reduction, or pharmacological, such as estrogen replacement (38), HMG-CoA reductase inhibitor therapy (28), or thiazolidinediones (39), may lower MCP-1/CCL2 levels and thus provide additional therapeutic benefits. Alternatively, if elevated MCP-1/CCL2 levels were merely a marker of prevalent or developing atherosclerosis, these treatment strategies would then be clinically fruitless. More potent prospective studies are clearly needed to address these issues.

In conclusion, in the present study, we showed that in middle-aged women, MCP-1/CCL2, leptin, and TNF-α-R2 were all independently related to biochemical risk markers of atherosclerosis. MCP-1/CCL2 concentration was the only one to be increased in women with type 2 diabetes and IGT with respect to nondiabetic individuals, and in the univariate analysis, CVD mortality after a 7-year follow-up period was associated with its increased circulating levels. In the multivariate analysis, both MCP-1/CCL2 and TNF-α-R2 were not associated with increased CVD mortality, and inspection of the data showed a leptin-related protective effect in both the univariate and multivariate analysis.

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References

1. Ross R: Atherosclerosis: an inflammatory disease. *N Engl J Med* 340:115–126, 1999
2. Shin WS, Szuba A, Rockson SG: The role of chemokines in human cardiovascular pathology: enhanced biological insights. *Atherosclerosis* 160:91–102, 2002
3. Li D, Mehta JL: Antisense to LOX-1 inhibits oxidized LDL-mediated: upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. *Circulation* 101:2889–2895, 2000
4. Yu H, Zeng Y, Hu J, Li C: Fluid shear stress induces the secretion of monocyte chemoattractant protein-1 in cultured human umbilical vein endothelial cells. *Clin Hemorheol Microcirc* 26:199–207, 2002
5. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ: Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 2:275–281, 1998
6. Dawson TC, Kuziel WA, Osahar TA, Maeda N: Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis* 143:205–211, 1999
7. National Cholesterol Education Program Expert Panel: ATP III final report: third report of the National Cholesterol Education Program (NCEP) Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults final report. I. Background and introduction. *Ciculation* 106:3157–3160, 2002
8. Havel PJ: Control of energy homeostasis and insulin action by adipocyte hormones: leptin, acylation stimulating protein, and adiponectin. *Curr Opin Lipidol* 13:51–59, 2002
9. Yamashiro S, Kamohara H, Yoshimura T: MCP-1 is selectively expressed in the late phase by cytokine-stimulated human neutrophils: TNF-alpha plays a role in maximal MCP-1 mRNA expression. *J Leukoc Biol* 65:671–679, 1999
10. Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, Brownlee M: Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J Biol Chem* 276:25096–25100, 2001
11. Garancini MP, Calori G, Ruotolo G, Manara E, Izzo A, Ebbli E, Bozzetti AM, Boari L, Lazzari P, Gallus G: Prevalence of NIDDM and impaired glucose tolerance in Italy: a OGTT-based population study. *Diabetologia* 38:306–313, 1995
12. Garancini MP, Calori G, Manara E, Izzo A, Ebbli E, Galli L, Boari L, Gallus G: An Italian population-based study of the prevalence of diabetes: some methodological aspects. *Diabete Metab* 19:116–120, 1993
13. Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, Vanzulli A, Testolin G, Pozza G, Del Maschio A, Luzi L: Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a ^{1}H-^{13}C NMR spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* 48:1600–1606, 1999
14. Perseghin G, Scifo P, Pagliato E, Battezzati

A, Soldini L, Benedini S, Testolin G, Del Maschio A, Luzi L: Gender factors affect fatty acids-induced insulin resistance in nonobese humans: effects of oral steroidal contraception. *J Clin Endocrinol Metab* 86: 3188–3196, 2001
15. Piemonti L, Leone BE, Nano R, Saccani A, Monti P, Maffi P, Bianchi G, Sica A, Peri G, Melzi R, Aldrighetti L, Secchi A, Di Carlo V, Allavena P, Bertuzzi F: Human pancreatic islets produce and secrete MCP-1/CCL2: relevance in human islet transplantation. *Diabetes* 51:55–65, 2002
16. Peri G, Milanese C, Matteucci C, Ruco L, Zhou D, Sozzani S, Coletta I, Mantovani A: A new monoclonal antibody (5D3–F7) which recognizes human monocyte-chemotactic protein-1 but not related chemokines: development of a sandwich ELISA and in situ detection of producing cells. *J Immunol Methods* 147:249–257, 1994
17. Perseghin G, Caumo A, Caloni M, Testolin G, Luzi L: Incorporation of the fasting plasma FFA concentration into QUICKI improves its association with insulin sensitivity in nonobese individuals. *J Clin Endocrinol Metab* 86:4776–4781, 2001
18. Loskutoff DJ, Samad F: The adipocyte and hemostatic balance in obesity: studies of PAI-1. *Arterioscler Thromb Vasc Biol* 18: 1–6, 1998
19. Sierra-Honigmann MR, Nath AK, Murakami C, Garcia-Cardena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR: Biological action of leptin as an angiogenic factor. *Science* 281: 1683–1686, 1998
20. Nophar Y, Kemper O, Brakebusch C, Englemann H, Zwang R, Aderka D, Holtmann H, Wallach D: Soluble forms of tumor necrosis factor receptors (TNF-Rs): the cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *EMBO J* 9:3269–3278, 1990
21. Söderberg S, Ahren B, Stegmayr B, Johnson O, Wiklund PG, Weinehall L, Hallmans G, Olsson T: Leptin is a risk marker for first-ever hemorragic stroke in a population-based cohort. *Stroke* 30:328–337, 1999
22. Söderberg S, Ahren B, Jansson JH, Johnson O, Hallmans G, Asplund K, Olsson T: Leptin is associated with increased risk of myocardial infarction. *J Intern Med* 246: 409–418, 1999
23. Wallace AM, McMahon AD, Packard CJ, Kelly A, Shepherd J, Gaw A, Sattar N: Plasma leptin and the risk of cardiovascular disease in the West of Scotland Coronary Prevention Study (WOSCOPS). *Circulation* 104:3052–3056, 2001
24. Cnop M, Landchild MJ, Vidal J, Havel PJ, Knowles NG, Carr DR, Wang F, Hull RL, Boyko EJ, Retzlaff BM, Walden CE, Knopp RH, Kahn SE: The concurrent accumulation of intra-abdominal and subcutaneous fat explains the association between insulin resistance and plasma leptin concentrations: distinct metabolic effects of two fat compartments. *Diabetes* 51:1005–1015, 2002
25. Sivitz WI, Wayson SM, Bayless ML, Larson LF, Sinkey C, Bar RS, Haynes WG: Leptin and body fat in type 2 diabetes and monodrug therapy. *J Clin Endocrinol Metab* 88:1543–1553, 2003
26. Inadera H, Egashira K, Takemoto M, Ouchi Y, Matsushima K: Increase of circulating levels of monocyte chemoattractant protein-1 in aging. *J Interferon Cytokine Res* 19:1179–1182, 1999
27. Parissis JT, Venetsanou KF, Kalantzi MV, Mentzikof DD, Karas SM: Serum profile of granulocyte-macrophage colony-stimulating factor and C-C chemokines in hypertensive patients with or without significant hyperlipidemia. *Am J Cardiol* 85:777–779, 2000
28. Garlichs CD, John S, Schmeisser A, Eskafi S, Stumpf C, Karl M, Goppelt-Struebe M, Schmieder R, Daniel WG: Upregulation of CD40 and CD40 ligand (CD154) in patients with moderate hypercholesterolemia. *Circulation* 104:2395–2400, 2001
29. Papayianni A, Alexopoulos E, Giamalis P, Gionanlis L, Belechri AM, Koukoudis P, Memmos D: Circulating levels of ICAM-1, VCAM-1 and MCP-1 are increased in haemodialysis patients: association with inflammation, dyslipidemia and vascular events. *Nephrol Dial Transplant* 17:435–441, 2002
30. de Lemos JA, Morrow DA, Sabatine MS, Murphy SA, Gibson CM, Antman EM, McCabe CH, Cannon CP, Braunwald E: Association between plasma levels of monocyte chemoattractant protein-1 and long-term clinical outcomes in patients with acute coronary syndromes. *Circulation* 107:690–695, 2003
31. Ihm CG, Park JK, Hong SP, Lee TW, Cho BS, Kim MJ, Cha DR, Ha H: A high glucose concentration stimulates the expression of monocyte chemotactic peptide 1 in human mesangial cells. *Nephron* 79:33–37, 1998
32. Reape TJ, Grooth PH: Chemokines and atherosclerosis. *Atherosclerosis* 147:213–225, 1999
33. Giustizieri ML, Mascia F, Frezzolini A, De Pita O, Chinni LM, Giannetti A, Girolomoni G, Pastore S: Keratinocytes from patients with atopic dermatitis and psoriasis show a distinct chemokine production profile in response to T cell-derived cytokines. *J Allergy Clin Immunol* 107:871–877, 2001
34. Brenier-Pinchart MP, Vigan I, Jouvin-Marche E, Marche PN, Pelet E, Gross U, Ambroise-Thomas P, Pelloux H: Monocyte chemotactic protein-1 secretion and expression after Toxoplasma gondii infection in vitro depend on the stage of the parasite. *FEMS Microbiol Lett* 214:45–49, 2002
35. Lazzeri E, Lasagni L, Serio M, Romagnani S, Romagnani P: Cytokines and chemokines in nephropathies and renal transplant. *G Ital Nefrol* 19:641–649, 2002
36. Eriksson KF, Lindgarde F: Prevention of type 2 (non-insulin-dependent) diabetes mellitus by diet and physical exercise: the 6-year Malmo feasibility study. *Diabetologia* 34:891–898, 1991
37. Hansson GK: Atherosclerosis: cell biology and lipoproteins. *Curr Opin Lipidol* 9:73–75, 1998
38. Stork S, Baumann K, von Schacky C, Angerer P: The effects of 17-βestradiol on MCP1 serum levels in postmenopausal women. *Cardiovasc Res* 53:642–649, 2002
39. Ghanim H, Garg R, Aljada A, Mohanty P, Kumbkarni Y, Assian E, Hamouda W, Dandona P: Suppression of nuclear factor-kappaB and stimulation of inhibitor kappaB by troglitazone: evidence for an anti-inflammatory effect and a potential antiatherosclerotic effect in the obese. *J Clin Endocrinol Metab* 86:1306–1312, 2001