ORIGINAL RESEARCH

Evaluation of metalloproteinase 2 and 9 levels and their inhibitors in combined dyslipidemia

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Abstract

Purpose: To evaluate the distribution of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and their specific inhibitors in a sample of patients affected by mild dyslipidemia but not yet treated with antihyperlipidemic drugs.

Methods: One hundred and sixty-eight Caucasian patients aged ≥ 18 yr of either sex with combined dyslipidemia and who had never previously taken lipid-lowering medications were evaluated. As a control population, we enrolled 179 Caucasian healthy subjects, aged ≥ 18 yr of either sex. We evaluated body mass index (BMI), fasting plasma glucose (FPG), fasting plasma insulin (FPI), homeostasis model assessment (HOMA index), systolic blood pressure (SBP), diastolic blood pressure (DBP), total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), triglycerides (Tg), lipoprotein(a) Lp(a), plasminogen activator inhibitor-1 (PAI-1), homocysteine (Hct), fibrinogen (Fg), high sensitivity C-reactive protein (Hs-CRP), adiponectin (ADP), MMP-2, MMP-9, tissue inhibitors of metalloproteinase-1 (TIMP-1), and tissue inhibitors of metalloproteinase-2 (TIMP-2).

Results: TC, Tg, and LDL-C were higher ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively) in the dyslipidemic group, while HDL-C levels were lower ($P < 0.01$) com-

pared with the control group. Increases of PAI-1, Hct, Fg, and Hs-CRP ($P < 0.01$, $P < 0.05$, $P < 0.05$, and $P < 0.05$, respectively) were present in the dyslipidemic group, while ADP level was lower ($P < 0.01$) in the dyslipidemic patients compared with controls. MMP-2, MMP-9, TIMP-1, and TIMP-2 levels were higher ($P < 0.0001$) in the dyslipidemic group.

Conclusions: Combined hyperlipidemic patients have increased levels of prothrombotic and microinflammatory parameters and higher levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 than control subjects. The prognostic importance of this observation has to be evaluated in adequately designed prospective studies.

List of Abbreviations

MMPs	metalloproteinases
AMI	acute myocardial infarction
TIMPs	tissue inhibitors of metalloproteinases
MMP-9	matrix metalloproteinase-9
MMP-2	matrix metalloproteinase-2
AHA	American Heart Association
ILIB	International Lipid Information Bureau
LPL	lipoprotein lipase
ULN	upper limit of normal
FPG	fasting plasma glucose

FPI	fasting plasma insulin
HOMA index	homeostasis model assessment
TIMP-1	tissue inhibitors of metalloproteinase-1
TIMP-2	tissue inhibitors of metalloproteinase-2
BMI	body mass index
CsV	coefficients of variation
BP	blood pressure
TC	total cholesterol
Tg	triglycerides
HDL-C	high density lipoprotein-cholesterol
LDL-C	low density lipoprotein-cholesterol
PAI-1	plasminogen activator inhibitor-1
Fg	fibrinogen
Hct	homocysteine
Hs-CRP	high sensitivity C-reactive protein
Lp(a)	lipoprotein (a)
ELISA	enzyme-linked immunosorbent assay
ADP	adiponectin
SBP	systolic blood pressure
DBP	diastolic blood pressure
ox-LDL	oxidated LDL
ACS	acute coronary syndrome
TNF- α	tumor necrosis- α
CAD	coronary artery disease

Scientific interest in metalloproteinases (MMPs) and their inhibitors has grown rapidly in the last few years, especially since it has been postulated that they could be relevant targets for treating atherothrombotic cardiovascular disease.¹ Some data suggest that circulating MMPs levels are elevated in patients with acute myocardial infarction (AMI), unstable angina, and also after coronary angioplasty.² Other studies showed that there is an increase of MMP concentration in macrophages, endothelium fibrous cap and vascular smooth muscle cells in the atherosclerotic plaque and some MMPs and tissue inhibitors of metalloproteinases (TIMPs) appear to be elevated more in patients with AMI and unstable angina than in healthy people.³⁻⁵

Increased *in vitro* and *in vivo* levels of MMPs were found in coronary vessels after revascularization. A possible role for MMPs in restenosis has been suggested, although more studies are needed to define the

action of these enzymes in the complex mechanism of restenosis.⁶

Little information is available in regard to a possible role for MMPs in subjects at high cardiovascular risk.⁷ We have previously shown that matrix metalloproteinase-9 (MMP-9) remains elevated in diabetic patients⁸ 3 months after an acute coronary syndrome.⁹

Moreover, there is some evidence that they are also slightly increased in conditions associated with an augmented risk of developing cardiovascular disease, such as uncomplicated hypertension,¹⁰ type 1 diabetes,¹¹ type 2 diabetes,¹² obesity¹³ and familial hypercholesterolemia.¹⁴

The aim of this study was to evaluate the distribution of matrix metalloproteinase-2 (MMP-2), MMP-9 and their specific inhibitors in a large sample of patients affected by mild mixed dyslipidemia not yet treated with antihyperlipidaemic drugs.

Materials and Methods

Study design

This multicenter case-control trial was conducted at the Department of Internal Medicine and Therapeutics, University of Pavia (Pavia, Italy); and in the "G. Descovich" Atherosclerosis Study Center, Department of Internal Medicine, Aging and Kidney disease, University of Bologna (Bologna, Italy). The study protocol was approved at each site by institutional review boards and was conducted in accordance with the Declaration of Helsinki and its amendments. All patients provided written informed consent to participate.

Subjects began a controlled-energy diet (near 600 Kcal daily deficit) based on American Heart Association (AHA) recommendations¹⁵ containing 30% of calories as saturated fat (< 7% of energy), trans fat to < 1% of energy, and maximum cholesterol content of 300 mg/day. Standard dietary advice was given by a dietitian and/or specialist doctor. A dietitian and/or

specialist physician periodically provided instruction on dietary intake recording as part of a behaviour modification program and then used the subject's food diaries later for counselling. Individuals were encouraged to increase their physical activity by walking briskly or cycling for 20 to 30 min, 3 to 5 times per week. Changes in physical activity throughout the study were not assessed.

Study population

Caucasian patients aged ≥ 18 yr of either sex were eligible for inclusion in the study if they had combined dyslipidemia [defined by International Lipid Information Bureau (ILIB)],¹⁶ and had never previously taken lipid-lowering medications. 168 patients, identified after review of case notes and/or computerized clinic registers, were contacted by the investigators in person or by telephone.

Patients were excluded if they had genetic conditions affecting lipid metabolism (eg, familial hypercholesterolemia, type III hyperlipidemia, lipoprotein lipase (LPL) deficiency, etc.); a history of microalbuminuria or nephrotic syndrome; impaired hepatic function (plasma aminotransferase and/or gamma-glutamyltransferase level > upper limit of normal [ULN] for age and sex); impaired renal function (serum creatinine concentration > ULN for age and sex); thyroid diseases; endocrine or metabolic disease; history of alcohol or drug abuse; neoplastic, infectious or autoimmune disease; poor mental condition; or taking any other drug able to influence lipid metabolism. Patients with serious cardiovascular disease (New York Heart Association class I-IV congestive heart failure or a history of myocardial infarction or stroke) or cerebrovascular conditions within 6 months before study enrollment also were excluded. As controls we enrolled 179 Caucasian healthy subjects, aged ≥ 18 of either sex. Subjects with infective or inflammatory disorders were excluded, as were those taking anti-inflammatory medications.

Assessments

Before starting the study, all patients underwent an initial screening assessment that included medical history, physical examination, vital signs, 12-lead ECG, fasting plasma glucose (FPG), fasting plasma insulin (FPI), homeostasis model assessment (HOMA index), blood pressure, lipid profile, coagulation, fibrinolytic, inflammation parameters, MMP-2, MMP-9, tissue inhibitors of metalloproteinase-1 (TIMP-1), and tissue inhibitors of metalloproteinase-2 (TIMP-2).

Plasma parameters were determined after 12-h overnight fast, determined 2 h after lunch. Venous blood samples were taken for all patients between 08.00 and 09.00 and were drawn from an antecubital vein with a 19-gauge needle without venous stasis. Plasma was obtained by addition of Na₂-EDTA, 1 mg/ml, and centrifugation at 3000 g for 15 min at 4°C. Immediately after centrifugation, plasma samples were frozen and stored at -80°C for no more than 3 months. All measurements were performed in a central laboratory.

Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. The estimate of insulin resistance was calculated by HOMA index with the formula: FPI (mU/ml) x FPG (mmol/L)/22.5, as described by Matthews and coworkers.¹⁷

Blood pressure (BP) measurements were obtained from each patient (using the right arm) in the seated position, using a standard mercury sphygmomanometer (Erkameter 3000, ERKA, Bad Tolz, Germany) (Korotkoff I and V) with a cuff of appropriate size. Blood pressure was measured by the same investigator at each visit, in the morning, after the patient had rested for ≥ 10 min in a quiet room. Three successive BP readings were obtained at 1-minute intervals, and the mean of the 3 readings was calculated.

Plasma glucose concentration was measured by the glucose-oxidase method (GOD/PAP, Roche Diagnostics, Mannheim, Germany) with intra- and interassay coefficients of variation (CsV) of < 2%.¹⁸ Plasma

insulin was assayed with Phadiaseph Insulin RIA (Pharmacia, Uppsala, Sweden) by using a second antibody to separate the free and antibody-bound ^{125}I -insulin (intra- and interassay CsV: 4.6 and 7.3%, respectively).¹⁹ Total cholesterol (TC) and triglycerides (Tg) levels were determined using enzymatic techniques^{20,21} on a clinical chemistry analyzer (HI-TACHI 737; Hitachi, Tokyo, Japan); intra- and inter-assay CsV were 1.0 and 2.1 for TC measurement, and 0.9 and 2.4 for Tg measurement, respectively. High density lipoprotein-cholesterol (HDL-C) level was measured after precipitation of plasma apo B-containing lipoproteins with phosphotungstic acid²² intra- and interassay CsV were 1.0 and 1.9, respectively; low density lipoprotein-cholesterol (LDL-C) level was calculated by the Friedewald formula.²³

Plasminogen activator inhibitor-1 (PAI-1) was assayed with a commercial two-stage indirect enzymatic assay (Spectrolyse, Biopool AB, Umea, Sweden) intra- and interassay CsV were 5.9%.²⁴ Fibrinogen (Fg) was determined according to Clauss. The intra-assay CV for the Fg method was less than 5%.²⁵

Homocysteine (Hct) was measured by a modified procedure of Araki and Sako²⁶ with high pressure liquid chromatography and fluorescence detection. The intra-assay CV of the method was 2.5%. High sensitivity C-reactive protein (Hs-CRP) was measured with latex-enhanced immunonephelometric assays on a BN II analyzer (Dade Behring, Newark, Delaware, USA). The intra- and interassay CsV were 5.7% and 1.3% respectively.²⁷

Lipoprotein (a) [Lp(a)] was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) method, that is insensitive to the presence of plasminogen, using the commercial kit Macra-Lp(a) (SDI, Newark, Delaware, USA);^{28,29} the intra- and inter-assay CsV of this method were 5% and 9%, respectively.

The adiponectin level was determined using ELISA kits (B-Bridge International, Inc., Sunnyvale, CA, USA). The intra-assay CsV were 3.6% for low and 3.3% for high control samples, while the inter-

assay CsV were 3.2% for low and 7.3% for high control samples.³⁰

Matrix metalloproteinase-2, MMP-9, TIMP-1, and TIMP-2 levels were determined by a two-site ELISA method using commercial reagents (Amersham Biosciences, Uppsala, Sweden). The intra- and inter-assay CsV for measuring MMP-2 levels were 5.4%, and 8.3%, respectively.³¹ The intra- and interassay CsV to evaluate MMP-9 levels were 4.9%, and 8.6%.³² The intra- and interassay CsV for measuring TIMP-1 levels were 9.3%, and 13.1%, respectively,³³ while those for measuring TIMP-2 levels were 5.4%, and 5.9%, respectively.³⁴

Statistical Analysis

Non-parametric tests were employed in the statistical analysis of the data because data were not normally distributed (Kolmogorov-Smirnov test). Mann-Whitney U test was used to compare two independent groups. A $P < 0.05$ was considered statistically significant. All tests were two-sided. Statistica 6.0 (Statsoft, Inc. 2003, Tulsa, OK, US.) was used for statistical computations.

Results

Study sample

A total of 347 patients were enrolled in this trial. The characteristics of the patient population at study entry are shown in Table 1.

Body mass index

No BMI change was observed in dyslipidemic patients compared to control group.

Glycemic control

No FPG, FPI, and HOMA index variations were observed in the dyslipidemic group compared with control.

TABLE 1. Baseline data.

	Control group	Dyslipidemic group
n	179	168
sex (M/F)	86/93	41/43
age (years)	48.3 ± 6.1	51.3 ± 7.2
Current smoking (n)	43	37
Comb. Dysl. Dur. (y)	-	7.4 ± 2.3
BMI (Kg/m ²)	25.9 ± 0.8	26.2 ± 1.3
FPG (mg/dl)	85.5 ± 7.6	87.8 ± 8.5
FPI (μU/ml)	7.9 ± 1.8	8.3 ± 1.9
HOMA index	1.7 ± 0.8	1.8 ± 0.9
SBP (mmHg)	125.3 ± 5.2	128.4 ± 6.5
DBP (mmHg)	77.8 ± 5.3	80.6 ± 6.8
TC (mg/dl)	186.7 ± 9.4	223.4 ± 15.7*
LDL-C (mg/dl)	116.2 ± 5.8	148.5 ± 7.2*
HDL-C (mg/dl)	49.2 ± 5.1	38.4 ± 4.2**
Tg (mg/dl)	106.4 ± 27.3	182.6 ± 39.7**
Lp(a) (mg/dl)	8.3 ± 13.2	9.1 ± 14.3
PAI-1 (ng/ml)	17.3 ± 2.4	36.1 ± 7.8**
Hct (μmol/l)	7.4 ± 1.9	12.3 ± 3.1*
Fg (mg/dl)	316.7 ± 46.2	394.8 ± 49.1*
Hs-CRP (mg/l)	0.6 ± 0.3	1.4 ± 0.6*
ADP (μg/ml)	9.3 ± 2.8	5.7 ± 1.0*

Data are means ± SD; * $P < 0.05$ vs control group; ** $P < 0.01$ vs control group

Comb. Dysl. Dur. : combined dyslipidemia duration

BMI: body mass index; FPG: fasting plasma glucose; FPI: fasting plasma insulin; HOMA index: homeostasis model assessment index; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; LDL-C: low density lipoprotein-cholesterol; HDL-C: high density lipoprotein-cholesterol; Tg: triglycerides; Lp(a): lipoprotein(a); PAI-1: plasminogen activator inhibitor-1; Hct: homocysteine; Fg: fibrinogen; Hs-CRP: high sensitivity C-reactive protein; ADP: adiponectin.

Blood pressure control

No systolic blood pressure (SBP) or diastolic blood pressure (DBP) variations were present in dyslipidemic patients with respect to controls.

Lipid profile and lipoprotein variables

Values of TC and LDL-C were higher in the dyslipidemic group than in the control group while a decrease of HDL-C levels ($P < 0.01$) was present in dyslipidemic patients compared with controls. An increase in Tg ($P < 0.01$) was observed in the dyslipidemic group compared with control, while no change was observed in Lp(a) value.

Coagulation, fibrinolytic and inflammation parameters

An increase in PAI-1 ($P < 0.01$) was present in dyslipidemic patients compared with control baseline values. Increases in Hct, Fg, and Hs-CRP were observed in the dyslipidemic group compared with controls, while ADP decreased in the dyslipidemic group.

Enzymatic characterization

MMP-2, MMP-9, TIMP-1 and TIMP-2 levels quantified in control and dyslipidemic group are reported in Table 2. An increase was observed for MMP-2, MMP-9, TIMP-1 and TIMP-2 levels ($P < 0.0001$) in the dyslipidemic group compared with control.

TABLE 2. MMP-2, MMP-9, TIMP-1, and TIMP-2 levels.

	Control group	Dyslipidemic group
MMP-2 levels, means (ng/ml) $\pm$ DS, median (ng/ml) [IQR]	642.7 $\pm$ 273.8 651.4 [428.6-823.9]	1242.7 $\pm$ 141.4 1272.8 [1225.1-1415.8]*
MMP-9 levels, means (ng/ml) $\pm$ DS, median (ng/ml) [IQR]	51.3 $\pm$ 14.8 57.5 [41.5-73.6]	506.3 $\pm$ 58.7 512.6 [463.9-552.4]*
TIMP-1 levels, means (ng/ml) $\pm$ DS, median (ng/ml) [IQR]	162.7 $\pm$ 53.9 164.8 [132.4-191.6]	496.2 $\pm$ 42.6 505.8 [457.2-539.8]*
TIMP-2 levels, means (ng/ml) $\pm$ DS, median (ng/ml) [IQR]	78.6 $\pm$ 5.2 79.3 [73.2-84.7]	104.7 $\pm$ 7.3 105.1 [99.4-112.5]*

Data are means $\pm$ SD, median, and interquartile range [IQR] ; * $P < 0.0001$ vs control group.

MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; TIMP-1: tissue inhibitors of metalloproteinase-1; TIMP-2: tissue inhibitors of metalloproteinase-2.

Correlations

Stepwise multilinear regression analysis was undertaken to establish if the degree of dyslipidemia could best correlate with coagulation, fibrinolytic, inflammation parameters, and with MMPs changes. Predictors of change in MMP-2 and MMP-9 were TC, and LDL-C concentration ($r = 0.65$, $P < 0.01$, and $r = 0.63$, $P < 0.01$, respectively), Hs-CRP value ($r = 0.64$, $P < 0.05$), and ADP value ($r = -0.66$, $P < 0.05$). Other correlation analysis did not indicate various patterns of associations in PAI-1, Hct and Fg value with any other parameters.

Discussion

The relationship between cholesterolemia and cardiovascular risk is well known. The most common hyperlipidemia in the general population and, in particular, in subjects with cardiovascular disease is combined dyslipidemia.³⁵ This could be partly related to a different distribution less effective HDL subclasses,³⁶ but also to other factors

In this study, carried out on subjects affected by acquired mixed dyslipidemia, we observed that the serum levels of MMP-2, MMP-9 and their tissue inhibitors are macroscopically higher than in control subjects. In particular, MMP-2 is doubled and TIMP-1 tripled, MMP-9 is 10 times higher than controls, while TIMP-2 increased by 30%. In mixed dyslipidemic patients we found higher plasma levels of PAI-1, Hct,

Fg and Hs-CRP, and a lower level of ADP than in non dyslipidemic subjects, revealing a prothrombotic and micro-inflammatory pattern, usually associated to an increased cardiovascular disease risk.³⁷ As in our previous observation,³⁸ dyslipidemic patients have increased levels of prothrombotic and microinflammatory parameters, but this increase is not quantitatively proportional to that observed in MMPs and TIMPs. Predictors of change in MMP-2 and MMP-9 were TC, and LDL-C concentration, Hs-CRP value, and ADP value. A link between lipids concentration and MMP was known from previous in vitro studies.

There are data supporting that oxidated LDL (ox-LDL) upregulates MMP-9 expression and reduces TIMP-1 expression in monocyte-derived macrophages. Furthermore, HDL abrogate ox-LDL-induced MMP-9 expression. Thus, ox-LDL may contribute to macrophage-mediated matrix breakdown in the atherosclerotic plaques, thereby predisposing them to plaque disruption and/or vascular remodeling.³⁹

More recent studies suggest that interaction of primary monocytes with ox-LDL and pro-inflammatory cytokines may contribute to vascular remodelling and plaque rupture.⁴⁰

We found a correlation between Hs-CRP and MMPs levels. Association of MMPs and other inflammatory parameters, and particular CRP are well known both in patients with acute coronary syndrome

(ACS) and in healthy subjects with an increased risk for cardiovascular diseases.^{41,42}

Adiponectin belongs to the family of cytokines, molecules secreted from adipose tissue, that directly contribute to obesity and vascular diseases. Physiological concentrations of human recombinant ADP suppress tumour necrosis factor- α (TNF- α), induce endothelial adhesion molecule expression, transformation from macrophage to foam cell, and TNF- α expression in macrophages.^{43,44} Decreases in ADP levels were observed in patients with coronary artery disease (CAD).⁴⁵

Recent data suggest a direct role of ADP in atherosclerotic plaque stability through interactions with MMPs and their inhibitors. ADP seems have a negative relationship with MMP-9/TIMP-1 ratio in patients with ACS. The MMP-9/TIMP-1 ratio is an independent predictor of the stability of atherosclerotic plaque and the severity of coronary atherosclerosis.⁴⁶

We measured ADP rather than other cytokines. Recently, the anti-inflammatory role of ADP and its role in cardiovascular protection has been accumulated and its biological characteristics elucidated. ADP measurement is feasible with small samples and presents little individual variability. ADP concentrations are independent of circadian rhythm and represent a possible target for prevention of cardiovascular disease.^{47,48}

Extracellular matrix is a dynamic structure that requires constant synthesis and degradation by MMPs.⁴⁹ This is tightly controlled by TIMPs. Therefore, increased extracellular matrix protein synthesis, diminished MMP activity, and/or increased TIMP activity, might contribute to vascular collagen deposition and fibrosis.⁵⁰ This appears to be important in patients during the acute phase of a cardiovascular event, and also in patients at increased risk for cardiovascular events.^{10,12} MMPs are increased in some genetic dyslipidemias, such as familial hypercholesterolemia¹⁴ and familial combined hyperlipoproteinemia.³⁸ However, their concentrations are even higher in the metabolic syndrome, where dyslipidemia is acquired, and

in subjects where metabolic syndrome overlaps with familial combined hyperlipoproteinemia,³⁸ and in obese patients.¹³

This study has some limitations. On the basis of our observation, we could not conclude that higher plasma MMPs and TIMPs levels were associated with greater cardiovascular risk, because we did not collect data on vascular damage related to these levels. Moreover, in a cross-sectional study, we were unable to attribute a prognostic importance to our observation. Genetic dyslipidemias were excluded on a clinical rather than molecular basis. However, our aim was to evaluate if some laboratory markers of vascular remodelling were elevated in mixed hyperlipidemic subjects as occurs in other patients at increased cardiovascular risk.

In conclusion, patients with combined hyperlipidemia have higher plasma levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 than controls. The prognostic importance of this observation needs to be evaluated in prospective studies.

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