Evaluation of metalloproteinase 2 and 9 levels and their inhibitors in diabetic and healthy subjects


Abstract

Objectives. – We hypothesized that molecules active in vascular remodeling (i.e. MMPs and their TIMPs) could be modified in diabetic patients, as indirect markers of the diabetes related generalized abnormality of vascular activity. To test this hypothesis, we measured the plasma levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in type 2 diabetic patients and in healthy subjects.

Methods. – We enrolled 181 diabetic patients and 165 controls. We measured body mass index (BMI), glycosylated hemoglobin (HbA1c), fasting plasma glucose (FPG), fasting plasma insulin (FPI), homeostasis model assessment index (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), and plasma levels of MMP-2, MMP-9, TIMP-1, and TIMP-2.

Results. – A significant increase (P<0.0001) of BMI, HbA1c, FPG, FPI, HOMA index, SBP, 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), and plasma levels of MMP-2, MMP-9, TIMP-1, and TIMP-2.

Conclusion. – Plasma levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 are increased in diabetic patients which may reflect abnormal extracellular matrix (ECM) metabolism.

© 2007 Elsevier Masson SAS. All rights reserved.
1. Introduction

The matrix metalloproteinases (MMPs) are a family of molecules that are associated with the breakdown of constituents of the extracellular matrix (ECM). Both MMPs and their tissue inhibitors (TIMPs) are involved in the regulation of the ECM metabolism [1]. ECM is a dynamic structure that requires constant synthesis and degradation by MMPs [2] and this is tightly controlled by TIMPs [3]. Either MMP-2 or -9 are synthesized and secreted locally in atherosclerotic lesions, predominantly by monocyte-derived macrophages and endothelial cells [4] and may participate in rupture of the atherosclerotic plaque [5]. Li et al. [6] found that MMPs and their inhibitors are involved in the process of cardiac remodeling in patients with heart failure, with a selective down-regulation of various TIMPs, along with the up-regulation of MMP-9 and gelenolytic activity. Type 2 diabetic patients are at high risk for acute coronary events due to an increased propensity of their atherosclerotic plaques to ulceration and overlying thrombosis [7]. The most common extracellular pathology in diabetes is the thickening of the basement membrane as a result of the deposition of ECM proteins [8]. The expression and activity of MMPs in diabetes thus far have been reported predominantly in relation to macrovascular and microvascular complications [9–11]. However, there is no data available about the values or the role of MMPs and TIMPs in yet untreated type 2 diabetic patients. After an attentive analysis of the available bibliography on MMPs and TIMPs by the consultation of Pub-Med and ENBASE scientific databases, we hypothesized that molecules active in vascular remodeling (i.e. MMPs and their TIMPs) could be modified in diabetic patients, as indirect markers of the diabetes related generalized abnormality of vascular activity. To test this hypothesis, we measured the plasma levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in type 2 diabetic patients and in healthy subjects.

2. Methods

2.1. Study design

This multicenter case–control trial was conducted at the Department of Internal Medicine and Therapeutics, University of Pavia (Pavia, Italy); the “G. Descovich” Atherosclerosis Study Center, “D. Campanacci” Clinical Medicine and Applied Biotechnology Department, University of Bologna (Bologna, Italy); the Department of Medicine and Aging Sciences, University of Chieti (Chieti, Italy); and the Department of Endocrinology and Metabolism, Section of Metabolic Diseases and Diabeties, University of Pisa (Pisa, 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.

2.2. Study population

Caucasian patients aged ≥18 of either sex were eligible for inclusion in the study if they had type 2 diabetes mellitus according to the American Diabetes Association (ADA) criteria [12] (duration, ≤ 6 months), diet and exercise-treated and who had not previously taken oral hypoglycaemic agents.

There were 181 overweight patients (body mass index [BMI], 25.2–28.0 kg/m²) [13]. Suitable patients, identified from 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 a history of ketoacidosis or had unstable or rapidly progressive diabetic retinopathy, nephropathy, or neuropathy; impaired hepatic function (defined as plasma aminotransferase and/or gamma-glutamyltransferase level higher than the upper limit of normal [ULN] for age and sex), impaired renal function (defined as serum creatinine level higher than the ULN for age and sex), severe anemia, and necrotic, infectious or autoimmune disease. Patients with serious cardiovascular disease (CVD) (e.g. 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 control population we enrolled 165 healthy Caucasian subjects, aged ≥18 of either sex. Subjects with infective or inflammatory disorders were excluded, as were those taking anti-inflammatory medications. Diabetic patients comprised 107 men (59%) and 74 women (41%) aged 54.1 years, while healthy control subjects comprised 93 men (56%) and 72 women (44%) aged 49.5 years. There were no significant differences between centers in sex distribution, age, and diabetes duration. All patients provided written informed consent to participate.
2.3. Diet and exercise

All diabetic patients had received dietary advice prior to enrolling in the study and were taking a controlled-energy diet (~600 kcal daily deficit), based on ADA recommendations [14], that contained 50% of calories from carbohydrates, 30% from fat (6% saturated), and 20% from proteins, with a maximum cholesterol content of 300 mg/d, and 35 g/d of fiber.

Each center’s standard diet advice was given by a dietitian and/or specialist physician. All individuals were also encouraged to increase their physical activity by walking briskly or riding a stationary bicycle for 20–30 min, three to five times per week. The recommended changes in physical activity throughout the study were not assessed.

2.4. Laboratory methods

Before starting the study, all patients underwent an initial screening assessment that included a medical history, physical examination, vital signs, a 12-lead electrocardiogram, measurements of fasting plasma glucose (FPG), fasting plasma insulin (FPI), homeostasis model assessment (HOMA index), blood pressure (BP), lipid profile, coagulation, fibrinolytic, and inflammation parameters, MMP-2, MMP-9, TIMP-1, and TIMP-2. All plasmatic parameters were determined after a 12-h overnight fast, determined 2 hours 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. We used plasma obtained by addition of Na$_2$EDTA, 1 mg/ml, and centrifuged at 3000 × g for 15 min at 4 °C. Immediately after centrifugation, the plasma samples were frozen and stored at −80 °C for no more than 3 months. All measurements were performed in a central laboratory.

BMI was calculated by the investigators 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 (μU/ml) × FPG (mmol/l)/22.5, as described by Matthews et al. [15]. 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. BP 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-min intervals, and the mean of the three readings was calculated.

Plasma glucose was assayed by glucose-oxidase method (GOD/PAP, Roche Diagnostics, Mannheim, Germany) with intra- and inter-assay coefficients of variation (CsV) of < 2% [16]. 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 inter-assay CsV: 4.6% and 7.3%, respectively) [17]. Total cholesterol (TC) and triglycerides (Tg) levels were determined using fully enzymatic techniques [18,19] on a clinical chemistry analyzer (HITACHI 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 acid [20] intra- and inter-assay CsV were 1.0 and 1.9, respectively; low density lipoprotein-cholesterol (LDL-C) level was calculated by the Friedewald et al. [21] formula.

Plasminogen activator inhibitor-1 (PAI-1) was assayed with a commercial two-stage indirect enzymatic assay (Spectrolyse, Biopool AB, Umeå, Sweden) intra- and inter-assay CsV were 5.9% [22]. Fibrinogen (Fg) was determined according to Clauss. The intra-assay CV for the Fg method was less than 5% [23]. Homocysteine (Hct) was measured by a modified procedure of Araki and Sako [24] 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 use of latex-enhanced immunonephelometric assays on a BN II analyzer (Dade Behring, Newark, Delaware, USA). The intra- and inter-assay CsV were 5.7% and 1.3%, respectively [25]. 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) [26,27]; the intra- and inter-assay CsV of this method were 5% and 9%, respectively.

MMP-2, MMP-9, TIMP-1, and TIMP-2 levels were determined by a two-site ELISA methods using commercial reagents (Amersham Biosciences, Uppsla, Sweden). The intra- and inter-assay CsV for measuring MMP-2 levels were 5.4% and 8.3%, respectively [28]. The intra- and inter-assay CsV to evaluate MMP-9 levels were 4.9%, and 8.6% [29]. The intra- and inter-assay CsV for measuring TIMP-1 levels were 9.3%, and 13.1%, respectively [30], while those for measuring TIMP-2 levels were 5.4%, and 5.9%, respectively [31].

2.5. 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 value of less than 0.05 was considered statistically significant. All tests were two-sided. Statistica 6.0 (Statsoft, Inc. 2003, Tulsa, OK, USA) was used for statistical computations.

3. Results

A total of 346 patients were enrolled in the trial. The characteristics of the patient population at study entry were shown in Table 1. Significant BMI change ($P < 0.0001$) was observed in diabetic patients respect to controls as reported in detail in Table 1.

HbA$_1c$, FPG, FPI, and HOMA index increase ($P < 0.0001$) was present in the diabetic group compared to the control baseline value (Table 1). Systolic blood pressure (SBP) and diasto-
fic blood pressure (DBP) value ($P < 0.0001$) was higher in patients with diabetes with respect to controls as reported in Table 1. A significant TC, LDL-C, Tg and Lp(a) increase ($P < 0.0001$) was observed in the diabetic group while a significant decrease of HDL-C levels ($P < 0.0001$) was present in diabetic patients with respect to healthy subjects (Table 1).

Significant PAI-1, Hct, Fg, and hs-CRP increase ($P < 0.0001$) was present in patients with diabetes compared to the control baseline value (Table 1).

MMPs, TIMP-1, and TIMP-2 levels quantified in control and diabetic group are reported in Table 2. MMP-2, and MMP-9 levels were significantly higher in diabetic patients than in healthy subjects ($P < 0.0001$). Significant increase was observed for TIMP-1, and TIMP-2 levels ($P < 0.0001$) in patients with diabetes compared to controls. Correlation analyses did not indicate various patterns of associations in MMP-2 and MMP-9, TIMP-1, and TIMP-2 with any other parameters in the control and diabetic groups.

### Table 1

<table>
<thead>
<tr>
<th>Data at baseline in control and diabetic groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>n</td>
</tr>
<tr>
<td>Sex (M/F)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Diab. duration (years)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>HbA$_1c$ (%)</td>
</tr>
<tr>
<td>FPG (mg/dl)</td>
</tr>
<tr>
<td>HOMA index</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
</tr>
<tr>
<td>Tg (mmol/l)</td>
</tr>
<tr>
<td>Lp(a) (µmol/l)</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
</tr>
<tr>
<td>Hct (µmol/l)</td>
</tr>
<tr>
<td>Fg (µmol/l)</td>
</tr>
<tr>
<td>Hs-CRP (ng/ml)</td>
</tr>
</tbody>
</table>

Data are mean ± S.D. * $P < 0.001$ vs. control. BMI: body mass index; HbA$_1c$: glycated hemoglobin; 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: hemocytene; Fg: fibrinogen; hs-CRP: C-reactive protein.

### Table 2

<table>
<thead>
<tr>
<th>MMPs, TIMP-1, and TIMP-2 levels in controls and diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>MMP-2 levels, means (ng/ml) ± DS, median (ng/ml) [IQR]</td>
</tr>
<tr>
<td>MMP-9 levels, means (ng/ml) ± DS, median (ng/ml) [IQR]</td>
</tr>
<tr>
<td>TIMP-1 levels, means (ng/ml) ± DS, median (ng/ml) [IQR]</td>
</tr>
<tr>
<td>TIMP-2 levels, means (ng/ml) ± DS, median (ng/ml) [IQR]</td>
</tr>
</tbody>
</table>

Data are mean ± S.D, median, and interquartile range [IQR], * $P < 0.0001$ vs. control. MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; TIMP-1: tissue inhibitors of metalloproteinase-1; TIMP-2: tissue inhibitors of metalloproteinase-2.

### 4. Discussion

The interest of the scientific community in MMPs has been rapidly increasing during the last years, especially since it has been postulated that they could be relevant targets for atherothrombotic CVD treatment [32]. CVD in diabetic patients is widely accelerated by impaired glucose metabolism that leads to an amplified risk for vascular events [33]. Recent studies show evidence that high glucose induces dysregulation of the MMP/TIMP system in two key vascular cells, endothelial cells and macrophages [34]. In fact, high glucose significantly amplifies MMPs expression and activity, leading to an upset in the balance between ECM synthesis and degradation [35]. Diabetes is also associated with plaque vulnerability and a high risk for acute coronary events due to overriding thrombosis; therefore the MMP/TIMP system may provide a novel mechanism to account for the increased incidence of acute vascular events in diabetic patients [36].

Metalloproteinases facilitate fibrous cap degeneration and plaque disruption. Moreover they enhance platelet agreeability and blood hypercoagulability. Thus, they seem to favor thrombotic obstruction following atherosclerotic plaque rupture [33]. Both MMP-2 and MMP-9 may be involved in rupture of the atherosclerotic plaque.

Many data in literature show evidence that MMPs are involved in the development of micro and macroangiopathy.

In a study by Hojo et al. [37], it has been observed that plasma levels of MMP-2 and MMP-1 are significantly increased in patients with acute coronary syndrome, during the subacute phase; these findings may suggest the role of MMPs in the degradation of ECM that leads to ventricular remodeling after myocardial infarction. The involvement of MMPs in the development of ventricular remodeling in patients with coronary artery disease is also in agreement with the results from Kameda et al. In fact, they found out increased levels of MMP-2 and MMP-9 [38]. Previous epidemiological data from Inokubo et al. [39], also showed that both MMP-9 and TIMP-1 plasma level are markedly increased in patients affected by acute coronary syndrome. Data from a study of Cipollone et al. [40], provide the evidence that overexpression of MMP-2 and MMP-9 in diabetic atherosclerotic plaques is related to plaque vulnerability that leads to increased risk of ischemic events.

In fact, circulating MMP levels are elevated in patients with acute myocardial infarction, unstable angina, and also after coronary angioplasty, which is related to late loss index after the procedure: these observations from a study of Ikeda and Shimada [41], suggest that MMP expression may be not only related to instability of the plaque, but also to the formation of restenotic lesions by promoting migration of macrophages and...
vascular smooth muscle cells and formation of thrombus. From literature that has been published so far, we realize that the most analyzed MMPs have been the gelatinases A and B, that are MMP-2 and MMP-9; the interest for these proteinases is mainly due to the widespread presence of the two enzymes in almost all tissues, compared to the other classes [42]. There are many studies that analyze the relationship between MMPs and their inhibitors with hypertension that is one of the most important cardiovascular risk factors. In our recent study, we demonstrated significant high levels of MMP-2, MMP-9, and TIMP-1 in hypertensive patients compared to normotensive control subjects [43]. These results are in agreement with the data from the study of Wallace et al., that confirm the presence of significantly increased levels of MMP-9 in hypertensive patients; furthermore this study highlights increased plasma levels of MMP-2 in hypertensive patients, as compared to healthy controls [44]. Different data comes out from the study of Zervoudaki et al. [45], in which they observe a significant reduction of plasma concentrations of MMP-9 and MMP-2 in patients with essential hypertension, as compared to normotensive subjects. This difference between the results may be due to the limited number of the study population in the last study, as compared to the previous ones (42 patients with never-treated essential hypertension and 25 normotensive control subjects).

There is little data in the literature about a possible connection between altered plasma concentrations of MMPs and obesity. In a study from Chavey et al. [46], authors observed significantly increased levels of MMP-2 and MMP-9 in obese mice, as compared to the control group. Studies in humans have not yet been carried out that can confirm or belies these findings.

There are even less data about the levels and activity of MMPs in diabetic patients without any evidence of significant vascular disease or organ damage, and there are no studies at all about their role in type 2 diabetic patients that have never been treated. Plasma levels of MMP-9 and TIMP-1 were significantly elevated in type 1 diabetic patients compared to controls, in a study from Maxwell et al. [10], but no significant difference in plasma MMP-2 between diabetic patients and controls was observed. These results are in disagreement with what has been observed in a study from Derosa et al., that has been carried out in type 1 diabetic children and adolescents. Their findings highlighted significantly increased levels of MMP-2 in diabetic patients as compared to the control population, but there were no significant differences between the two groups regarding MMP-9 concentrations [47]. The apparently discrepancy between data from the two different studies may be ascribed to the non-homogeneity of the study populations as well as to the limited number of them: 43 subjects, median age 27.0 years, were recruited in the study of Maxwell et al., while 25 children and adolescents, median age 10.6 years, were recruited by Derosa et al. In the meantime however, these findings allow us to postulate that MMP-2 may be a good index of severity and stability of microangiopathy, while MMP-9 may be a marker of macroangiopathy.

In our study we observed significantly increased levels of MMP-9 in type 2 diabetic patients that were only treated with diet, as compared to healthy controls. These findings are qualitatively and quantitatively in line with the results from Death et al. In fact in their study on cultured endothelial cells and monocyte-derived macrophages, they found out that high glucose exposure could induce not only the expression but also the gelatinolitic activity of MMP-9 [34]. Enhanced MMP-9 activity was also observed by Uemura et al., in their study on type 1 and type 2 diabetic rats [36]. In contrast with our results are the findings from the study of Baugh et al., where no significant difference in MMP-9 production was observed between controls and type 2 diabetes groups [48]. It is possible that this difference between the results is still in this case due to the limited number of the study population (22 type 2 diabetic patients and 18 control subjects), but it is also important to note that even if they all were in good glycaemic control, 13 of the 22 patients with type 2 diabetes were taking hypoglycemic therapy. Conflicting results are also observed by Lee et al., in a study on patients with type 2 diabetes from Korea, where no significant difference was found between the levels of MMP-9 in the diabetic group and the control group [49]. In this case the discordant findings may be related not only to the different ethnic origins (Caucasian vs. Asian), but also to the fact that three from the diabetic patients were taking antiaggregant therapy, whereas 24 were taking statins, whose efficacy in decreasing plasma levels of MMP-9 has been demonstrated in a previous study from Kalela et al. [50]. As far as MMP-2 concentrations are concerned, we observed that they are markedly increased in diabetic patients as compared to a healthy control group. Similar results are confirmed by the above cited study of Lee et al. and also by that of Death et al.

In our study we also demonstrated that TIMP-1 and TIMP-2 plasma concentration are significantly higher in diabetic patients as compared to the values of the control group. These data can confirm what has already been observed in the above cited study of Lee et al., as far as TIMP-1 levels are concerned, but the above cited studies of Death et al. and Baugh et al. found out no significant differences between TIMP-1 levels in diabetic patients as compared to healthy subjects.

To our best knowledge, there are yet no data in the literature that may confirm our findings about TIMP-2 plasma levels.

Of course, our data have to be interpreted cautiously because of the relatively small sample of studied patients, and also because the plasma level of other MMPs (not dosed in our study) could be significantly altered in diabetic patients.

In conclusion, our study observed that plasma levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 are increased in diabetic patients compared healthy subjects and this may reflect abnormal ECM metabolism.

References