

# Increased Circulatory MMP-2 and MMP-9 Levels and Activities in Patients with Type 1 Diabetes Mellitus

MING-YUH SHIAU, M.S.<sup>1,2</sup>, SHIH-TZER TSAI, M.D.<sup>3</sup>, KAN-JEN TSAI, Ph.D.<sup>4</sup>, MING-LIH HAUNG, M.D.<sup>5</sup>,  
YI-TING HSU, M.S.<sup>2</sup>, AND YIH-HSIN CHANG, Ph.D.<sup>4</sup>

## Abstract

**Background:** Type 1 diabetes mellitus (T1DM) is an autoimmune disorder of unknown etiology. It has been suggested that metalloproteinases (MMPs) play important roles in the development and complications of autoimmune disorders. This article presents our research on the expression or activities of MMPs in Taiwanese T1DM patients.

**Methods:** Levels and activities of plasma MMP-2 and MMP-9 in patients with T1DM were investigated and compared to those of control individuals by enzyme-linked immunosorbent assay and zymography.

**Results:** Circulatory levels and activities of MMP-2 and MMP-9 in patients with T1DM were significantly higher than those in control subjects.

**Conclusions:** MMP expression and activities are significantly increased in patients with T1DM. Our results not only document plasma MMPs levels and activities in the Taiwanese population (with a very low type 1 diabetic incidence), but also suggest that MMP expression and activity are elevated before the onset of complications in diabetic patients.

**Key Words:** Matrix metalloproteinase-2, matrix metalloproteinase-9, type 1 diabetes mellitus, diabetes.

## Introduction

TYPE 1 DIABETES MELLITUS is a metabolic disease that exhibits hyperglycemia due to an inability to secrete insulin. The disease process is believed to be due to an autoimmune response that causes selective destruction of insulin-secreting islet  $\beta$ -cells. Clinical manifestations of T1DM include very low insulin secretion, absolute requirement for exogenous insulin, low age of onset, and high prevalence of autoantibodies directed against antigenic determinants of the  $\beta$ -cells. The incidence of T1DM is much lower among Taiwanese than among Caucasians (1). The average annual incidence of T1DM in Taipei is approximately 1.5 per 100,000 (1). Taiwanese and Caucosoid patients with T1DM display different clinical manifestations, such as the prevalence of different autoantibodies and genetic traits (2, 3). Therefore, it is

likely that several genetic and environmental factors contribute to the autoimmune process in different ethnic groups. A high proportion of T1DM patients of long duration have a tendency to develop diabetic nephropathy with the accumulation of extracellular matrix (ECM) proteins within the renal interstitium and progressive tubular atrophy.

Matrix metalloproteinases are a family of proteolytic enzymes that play crucial roles in normal remodeling of connective tissue and the degradation of basement membrane and surrounding ECM in various physiologic situations. MMPs are also involved in numerous pathological conditions, including atherosclerosis, inflammation, tumor growth and metastasis. Evidence from metabolic studies of experimental animal models demonstrates that enhanced production of collagen is a key event in the development of diabetic glomerular ECM abnormalities. Recently it has been proposed that MMPs participate in the process of concurrent reduced degradation and the enlargement of mesangial area during the pathogenesis of diabetic nephropathy (4–6).

Matrix metalloproteinase-2 (MMP-2) gene downregulation is observed in glomeruli from type 2 diabetic patients (7). An MMP induction and activation system in human arterial vasculature is downregulated in diabetes (8). However, contradictory observations suggest that MMP-2 protein and enzyme activity in kidney samples from diabetic patients are elevated (9). Increased plasma matrix metalloproteinase-9 (MMP-9) activity in the left ventricle of alloxan-induced diabetic wild-type mice is

<sup>1</sup>Institute of Medicine, Chung Shan Medical University, Taichung, <sup>2</sup>Hung Kuang University, Taichung, <sup>3</sup>Division of Endocrinology and Metabolism, Veterans General Hospital-Taipei, <sup>4</sup>School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, <sup>5</sup>Department of Hematology, Show Chwan Memorial Hospital, Changhua, Taiwan, Republic of China.

Address all correspondence to Yih-Hsin Chang, Ph.D., Professor/Chair, School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung 402, Taiwan, Republic of China; e-mail: cyh@csmu.edu.tw

Accepted for publication April 2006.

also documented (10). In human subjects, circulating MMP-9 levels are increased in type 2 diabetic patients with coronary artery disease, and elevated serum MMP-9 concentrations are linked to premature coronary atherosclerosis (11). It is proposed that increased plasma levels of MMP-9 occur before the development of renal microvascular complications in patients with type 2 diabetic mellitus (12).

Expression and activities of MMPs in diabetes have been studied predominantly in microenvironmental renal and cardiac tissues, with conflicting results. Discrepancies exist among diabetic patients with different ethnic backgrounds, which reveals that ethnicity and environmental factors are involved in the diabetic pathogenesis (13, 14). Therefore, we wanted to determine whether MMP expression or activity is altered in Taiwanese T1DM patients, a population with the lowest range of diabetic incidence (1), and to clarify the reported conflicts as well.

## Materials and Methods

### Study Subjects

Two hundred and one consecutive T1DM patients attending the diabetic clinic of the Division of Endocrinology and Metabolism, Veterans General Hospital-Taipei, were recruited. The average residual C-peptide level (measured by using the Human C-Peptide RIA kit, Linco Research Inc., St. Charles, MO, USA) of the T1DM patients was  $0.13 \pm 0.04$  nM/L (Table 1). One hundred and eighty-nine healthy control subjects were also recruited from the Physical Check-up Unit, Taichung Veterans General Hospital. Fasting blood samples from all the study subjects were collected from a median cubital vein and drawn into a vacutainer containing 1.8 mg/mL edetic acid (EDTA), then centrifuged within 1 h at 3,000 rpm for 5 min, and the plasma was stored at  $-80^{\circ}\text{C}$  until analysis. The demographic characteristics and clinical manifestations of each patient were filed for statistical analysis (Table 1). Written consent was obtained from all the study subjects after the nature of the procedure was explained. Protocol for this study was reviewed and approved by the Ethics Committee of Hung Kuang University.

### Zymographic Analysis of MMP-2 and MMP-9 Activities

MMP-2 and MMP-9 activities were detected using gelatin zymography (15). In brief, ten-fold diluted sera containing 20  $\mu\text{g}$  of total proteins, quantified by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., CA, USA), were loaded into each

**TABLE 1**  
*Demographic Characteristics and Biochemical Information on Subjects in This Study*

	Study Subjects	
	Control (n=189)	T1DM (n=201)
Sex (M/F)	99/90	85/116
Age (yr)	58.2 $\pm$ 10.6	14.4 $\pm$ 0.6
Age of onset (yr)	-	9.6 $\pm$ 0.5
Duration (yr)	-	4.5 $\pm$ 0.2
C-peptide (nM/L)	-	0.13 $\pm$ 0.04
BMI (kg/m <sup>2</sup> )	24.52 $\pm$ 3.40	25.34 $\pm$ 3.16
Fasting glucose (70–110 mg/dL)*	95.69 $\pm$ 6.79	180.38 $\pm$ 70.65
Systolic pressure (120–140 mm Hg)*	125.42 $\pm$ 19.18	135.22 $\pm$ 18.19
Diastolic pressure (70–90 mm Hg)*	79.20 $\pm$ 10.38	79.99 $\pm$ 10.51
Blood urea nitrogen (6–22 mg/dL)*	15.86 $\pm$ 5.09	17.58 $\pm$ 8.07
Creatinine (0.6–1.4 mg/dL)*	1.11 $\pm$ 0.42	1.04 $\pm$ 0.44
Cholesterol (125–240 mg/dL)*	201.18 $\pm$ 37.51	198.02 $\pm$ 42.12
HDL-C (>35 mg/dL)*	58.35 $\pm$ 13.88	46.52 $\pm$ 13.43
TC/HDL-C	3.59 $\pm$ 0.90	4.51 $\pm$ 1.31
Triglycerides (20–200 mg/dL)*	142.54 $\pm$ 125.52	184.51 $\pm$ 153.74
Uric acid (2.4–7.2 mg/dL)*	6.56 $\pm$ 1.64	6.12 $\pm$ 1.88

\*Numbers in parenthesis indicate the normal reference range of each biochemical test.

\*\*Data are presented as mean  $\pm$  standard deviation.

BMI = body mass index, HDL-C = high-density lipoprotein cholesterol, TC = total cholesterol.

well on 10% gelatin zymography gels. Proteins were initially separated under nonreducing condition at 15 mA through the stacking gel, and the current was increased to 20 mA during the rest of separation. The gels were then rinsed twice in 2.5% Triton X-100 and incubated overnight (16 h) in substrate buffer containing 50 mM Tris-HCl and 5 mM CaCl<sub>2</sub>. The activated gels were stained by Coomassie blue R-250 followed by destaining in solution containing 55% methanol and 7% acetic acid. Areas of digestion were visualized as non-stained regions of the gel.

### Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of MMP-2 and MMP-9 in sera were measured in duplicate using a commercial ELISA kit according to the manufacturer's protocols (R&D Systems Inc., Minneapolis, MN, USA). In brief, 100  $\mu\text{L}$  of MMP-2 or MMP-9 assay diluent was added to each well and then 50  $\mu\text{L}$  of standard or control samples. After 2 h of incubation at room temperature on a constant shaker (500  $\pm$  50 rpm), the reaction solution was aspirated and the

wells were washed 4 times with wash buffer; 200  $\mu$ L of MMP-2 or MMP-9 conjugate was then added to each well and incubated for another 2 hr on the shaker at room temperature. Then the aspiration/wash steps were repeated as mentioned above, followed by adding 200  $\mu$ L of substrate solution to each well. The microplate was allowed to stand for 30 min at room temperature in the dark. After adding 50  $\mu$ L of stop solution, the optical density at 450 nm of each well was determined. The MMP-2 and MMP-9 concentrations for each sample were calculated from the standard curve. The MMP-2 assay recognized both pro- and active-forms of MMP-2. Intra-assay and inter-assay coefficients of variation (CV) were 4.8% and 7.7%, respectively. Minimum detectable dose (MDD) of MMP-2 assay was 0.16 ng/mL. The MMP-9 assay recognized both pro- and active-forms of MMP-9. The intra-assay and inter-assay CV were 2.3% and 7.5%, respectively. The MDD was less than 0.156 ng/mL. According to the manufacturer's brochure, no significant cross-reactivity or interference to various proteins, including mutual cross-reactivity between MMP-2 and MMP-9, was observed.

### Statistical Analysis

Data analysis started with descriptive statistics, including mean and standard deviation for continuous variables, and frequency for categorical variables. The Mann-Whitney test was applied for comparing the MMP-2 and MMP-9 levels and activities between patients and control subjects. An alpha level of 0.05 was used for all statistical tests.

### Results and Discussion

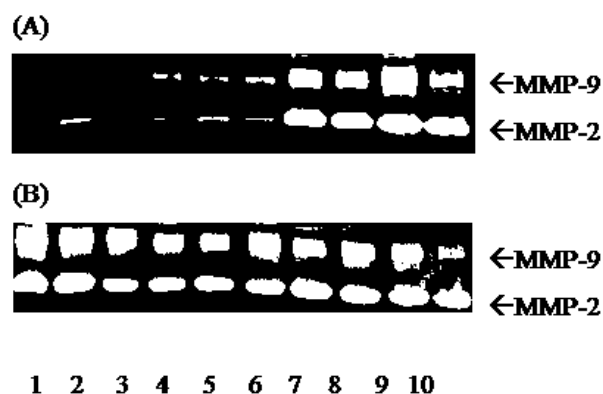
A high proportion of diabetic patients of long duration develop various diabetic complications. The most devastating diabetic complications are those that affect blood vessels and the most important microvascular diabetic complications are diabetic nephropathy and retinopathy. Diabetic nephropathy is the single most common disorder leading to renal failure. Thus, a better understating of diabetic pathogenesis and nephropathy pathophysiology could lead to the design and development of urgently needed new treatment approaches.

MMPs are one of the numerous factors that have been implicated in diabetic nephropathy. Although the MMP induction/expression system, including MMPs and tissue inhibitors of matrix metalloproteinase (TIMPs), is implicated in diabetic complications, experimental data suggest that localized renal MMP-2 and MMP-9 are the major MMPs that take part in the pathogenesis of diabetic nephropathy (4–6). Nevertheless, results re-

garding MMP-2 and MMP-9 levels and activities in diabetic complications remain controversial.

MMP-2 and MMP-9 activities in sera from our study subjects were analyzed by zymography. Representative results of the zymographic data are shown in Fig. 1. The data showed that both MMP-2 and MMP-9 activities in sera from patients with T1DM were significantly higher than those from control subjects. When the MMP-2 and MMP-9 expression levels were quantitatively measured by ELISA, the MMP-2 level in control subjects and T1DM patients was  $146.1 \pm 34.2$  and  $241.5 \pm 73.7$  ng/mL, respectively (Table 2). The MMP-9 level in control subjects and T1DM patients was  $51.4 \pm 57.1$  and  $305.3 \pm 180.6$  ng/mL, respectively (Table 2). Both MMP-2 and MMP-9 levels were significantly higher in sera from all patients, compared to those from control subjects. No significant correlation between MMP levels or activities with patients' demographic or clinical data was found.

The major observations of our study are that MMP-2 and MMP-9 levels and activities are higher in T1DM patients compared to those of control individuals. In addition to ELISA, our study also measured MMP activities by zymography, which can provide information complementary to the results of direct enzyme assay by solution methods. Both ELISA and zymography results confirmed that MMP levels and activities were elevated in diabetic patients. In addition, we had recruited 201 T1DM patients from the Taiwanese population, with one of the lowest diabetic incidences worldwide (1), as well as a relatively large pool of control subjects. Therefore, our data should be sufficient to faithfully reflect and represent the MMP alterations in Taiwanese diabetic patients.



**Figure.** Representative SDS-PAGE zymogram showing that MMP-2 and MMP-9 activities were elevated in patients with T1DM. MMP-2 and MMP-9 activities in sera from study subjects corresponds to the upper and lower white lytic bands, respectively. (A) Lanes 1–6, healthy control individuals; lanes 7–10, T1DM subjects; (B) T1DM subjects.

TABLE 2

Levels of MMP-2 and MMP-9 in Control Subjects and Type 1 Diabetes Mellitus Patients

	Study Subjects	
	Control (n=189)	T1DM (n=201)
MMP-2 (ng/mL)*	146.1±34.2	241.5±73.7**
MMP-9 (ng/mL)*	51.4±57.1	305.3±180.6**

\*Data are presented as mean ± standard deviation.

\*\* $p < 0.05$  by Mann-Whitney test, compared with the levels of control subjects.

MMP = metalloproteinase.

Our results support earlier observations that both MMP-2 and MMP-9 levels in diabetic subjects are higher than in nondiabetic subjects (16). Nevertheless, contradictory results exist. Though Maxwell et al. reported significantly increased concentrations of plasma MMP-9, they found no significant difference in plasma MMP-2 between diabetic patients and controls (13). On the other hand, Derosa et al. found that MMP-2 levels and activities were significantly higher in type 1 diabetic patients and T1DM patients with complications than in nondiabetic subjects (14). No significant differences were observed for MMP-9 level and activity in that study.

Several factors, such as methodological variations and statistical power, may contribute to the discrepancies of the results between the above studies and our observations. The MMP assay systems used in Maxwell's study can only recognize free and complexed MMPs; they cannot provide information on the active MMP forms (13). Therefore, the increased plasma MMP-9 and TIMP-1 levels in their study reflect increased synthesis. Their study population is perhaps too small (43 patients and 35 control individuals were recruited) to investigate the relationship between MMPs and diabetic duration or metabolic control. Because the T1DM patients recruited in that study had normal renal function without microalbuminuria, the authors suggest that diabetic environment can alter gene expression of both MMPs and TIMP-1 prior to the onset of clinically apparent complications.

Derosa et al. (14) used 2 ELISA kits to differentiate MMP levels and activity. The authors concluded that the observation of significant difference in MMP-2 levels and activity, in contrast to MMP-9 levels and activity between T1DM and control subjects, might be due to the youth and the good metabolic control of their patients. The study was designed to longitudinally and retrospectively detect the MMP levels and activity at T1DM onset and 5-year follow-up in a fixed population. Therefore, compared to the study of Maxwell et al. (13),

Derosa's study should be able to more faithfully reflect the temporal alteration of MMP levels and activity during disease progression in diabetic patients. Nevertheless, the number of Derosa's study subjects is even smaller: only 25 T1DM patients, and only 15 of the 25 developed microangiopathy during follow-up. Therefore, whether the investigative power of their study is sufficient to truly represent the relationship between MMPs and diabetes needs further clarification.

There are several limitations to our study. First of all, the age of our diabetic patients and control subjects (mean age  $14.4 \pm 0.6$  and  $58.2 \pm 10.6$  years old respectively, Table 1) was not matched. Neither Maxwell (13) nor Derosa (14) found a correlation between MMP-2, MMP-9 and age. Derosa et al. indicated that though both MMP-2 and MMP-9 levels were slightly increased in all study subjects at 5-year follow-up compared with that of their baseline levels, the increasing trend has no statistical significance. Accordingly, MMP levels of our older control group should have been higher than that of the younger diabetic subjects. The age mismatch between the T1DM and control subjects might not affect the validity of our data, since our results demonstrated significantly higher MMP levels in the much younger T1DM patients.

Secondly, pro-MMPs are actually inactive in serum, but would be activated during the zymography renaturing step and become fully active in the gels. Kleiner et al. (15) indicated that zymography is an extremely sensitive electrophoretic technique that has been extensively used in the qualitative evaluation of both active and latent forms of gelatinases present in tumors. The electrophoretic process can efficiently separate these enzymes from endogenous inhibitors, and both active and latent forms of MMPs would show the same degree of digestion in the assay. Only pro forms of MMPs can be detected in zymography gel when MMP levels are low ( $\sim \leq 200$  pg). The active form cannot be detected until the MMP levels are higher than approximately 400 pg. When the MMP levels are high, latent and active forms of these enzymes are present in approximately a 7:1 ratio. The authors also concluded that it is not biochemically unreasonable, using zymography, to determine enzyme activity and hence total enzyme protein. Thus, the assay can be used to quantitate not only total enzyme, but also the proportion of enzyme present in both the latent and active forms. This ability distinguishes zymography from whole solution methods such as ELISA, which quantitate total enzyme but do not distinguish between different molecular weight forms, and from solution activity assays, which can only quantify the active forms of the enzymes (15). Accordingly, it is suggested that the zymography results may reveal the MMP activities

in study subjects because of the much higher MMP levels in our study subjects than the detection limit, and the relatively stable proportion of pro- and active-MMPs in circulation.

Thirdly, the anticoagulant EDTA used in blood collection may affect the MMP levels and activities in our study. Jung et al. (17) demonstrated that MMP-9 concentration is decreased significantly with increasing amounts of EDTA during blood collection, whereas MMP-2 is increased. They also revealed that EDTA can inhibit the gelatinolytic activities. Mannello et al. (18) indicated that MMP-2 level did not differ in serum and heparin plasma but was lower in EDTA plasma, while activities of MMP-9 were 2- to 10- fold higher in serum than in heparin and EDTA plasma. Nevertheless, different anticoagulants used in blood sample collection did not influence the proportion of active-to-latent MMPs. Although the anticoagulant of this study was EDTA, which is reported to affect circulatory MMP levels or activities, we do not think that EDTA affected the observation regarding the significantly increased MMP-2 and MMP-9 of T1DM patients in our study, since EDTA was applied to the blood collection process of all study subjects. Therefore, if EDTA did cause alterations to circulatory MMP levels and activities, this variable became a controlled factor in our study.

And finally, the average duration of our T1DM patients in this study was  $4.5 \pm 0.2$  years (Table 1); only 15 patients (7.5%) suffered T1DM for more than 10 years. Most of the patients had no symptoms of diabetic nephropathy or other complications. Therefore, the number of patients with complications in our study was not sufficient to reflect and represent the correlation between MMPs and diabetic complications, due to limited investigation power from an insufficient number of patients with complications. The results of our study suggest that the MMP levels would be elevated before the development of diabetic nephropathy or other complications (4).

In summary, our study not only provides data on systemic MMP levels in a population with possibly the lowest type 1 diabetic incidence, but also suggests that MMP expression and activity is elevated before the onset of complications in diabetic patients, due to the relatively short duration and lack of complications among our study subjects. Whether there is a correlation or interactions between autoimmune responses and MMP system awaits further investigation, as does the role of MMPs in the T1DM pathogenesis.

### Acknowledgments

This work was supported by grants NSC94-2320-B-040-022 from the National Science Council, Taiwan, Republic of China.

### References

1. Chuang LM, Tsai ST, Juang JH, et al. Genetic epidemiology of type 1 diabetes mellitus in Taiwan. *Diabetes Res Clin Prac* 2000; 50(Suppl 2):S41–S47.
2. Shiau MY, Tsai ST, Hwang J, et al. Relationship between autoantibodies against glutamic acid decarboxylase, thyroglobulin/ thyroid microsome and DNA topoisomerase II in the clinical manifestation of patients with type 1 diabetes mellitus in Taiwan. *Eur J Endocrinol* 2000; 142(6):577–585.
3. Chang YH, Shiau MY, Tsai CT, Lan MS. Autoantibodies against IA-2, GAD and topoisomerase II in type 1 diabetic patients. *Biochem Biophys Res Commun* 2004; 320(3):802–809.
4. Zaoui P, Cantin JF, Alimardani-Bessette M, et al. Role of metalloproteases and inhibitors in the occurrence and progression of diabetic renal lesions. *Diabetes Metab* 2000; 26:25–29.
5. Marti HP. Role of matrix metalloproteinases in the progression of renal lesions [French]. *Presse Med* 2000; 29(14):811–817.
6. Diamant M, Hanemaaijer R, Verheijen JH, et al. Elevated matrix metalloproteinase-2 and -9 in urine, but not in serum, are markers of type 1 diabetic nephropathy. *Diabetes Med* 2001; 18:423–424.
7. Del Prete D, Anglani F, Forino M, et al. Down-regulation of glomerular matrix metalloproteinase-2 gene in human NIDDM. *Diabetologia* 1997; 40:1449–1454.
8. Portik-Dobos V, Anstadt MP, Hutchinson J, et al. Evidence for a matrix metalloproteinase induction/activation system in arterial vasculature and decreased synthesis and activity in diabetes. *Diabetes* 2002; 51:3063–3068.
9. Romanic AM, Burns-Kurtis CL, Ao Z, et al. Upregulated expression of human membrane type-5 matrix metalloproteinase in kidneys from diabetic patients. *Am J Physiol Renal Physiol* 2001; 281:F309–F317.
10. Camp TM, Tyagi SC, Senior RM, et al. Gelatinase B (MMP-9) an apoptotic factor in diabetic transgenic mice. *Diabetologia* 2003; 46:1438–1445.
11. Noji Y, Kajinami K, Kawashiri MA, et al. Circulating matrix metalloproteinase and their inhibitors in premature coronary atherosclerosis. *Clin Chem Lab Med* 2001; 39:380–384.
12. Ebihara I, Nakamura T, Shimada N, Koide H. Increased plasma matrix metalloproteinase-9 concentrations precede development of microalbuminuria in non-insulin-dependent diabetes mellitus. *Am J Kidney Dis* 1998; 32:544–550.
13. Maxwell PR, Timms PM, Chandran S, Gordon D. Peripheral blood level alterations of TIMP-1, MMP-2 and MMP-9 in patients with type 1 diabetes. *Diabet Med* 2001; 18(10):777–780.
14. Derosa G, Avanzini MA, Geroldi D, et al. Matrix metalloproteinase 2 may be a marker of macroangiopathy in children and adolescents with type 1 diabetes. *Diabetes Care* 2004; 27(1):273–274.
15. Kleiner ED, Stetler-Stevenson WG. Quantitative zymography: detection of pictogram quantities of gelatinases. *Anal Biochem* 1994; 218:325–329.
16. Das A, McGuire PG, Eriqat C, et al. Human diabetic neovascular membranes contain high levels of urokinase and matrix metalloproteinase enzymes. *Invest Ophthalmol Vis Sci* 1999; 40(3):809–813.
17. Jung K, Laube C, Lein M, et al. Kind of sample as preanalytical determinant of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinase 2 in blood. *Clin Chem* 1998; 44:1060–1062.
18. Mannello F, Luchetti F, Canonico B, Papa S. Effect of anticoagulants and cell separation media as preanalytical determinants on zymographic analysis of plasma matrix metalloproteinases. *Clin Chem* 2003; 49:1956–1957.