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# Plasma levels and zymographic activities of matrix metalloproteinases 2 and 9 in type II diabetics with peripheral arterial disease

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**Abstract:** Deregulation of matrix metalloproteinases (MMPs) is an important factor contributing to the development of vascular lesions. Plasma levels and zymographic activities of MMP-2 and MMP-9 were investigated in type II diabetics with ( $n = 51$ ) or without ( $n = 42$ ) peripheral artery disease (PAD) and in normal volunteers ( $n = 23$ ). Plasma MMP-2 levels were higher in type II diabetics with ( $p < 0.01$ ) or without ( $p > 0.05$ ) PAD in comparison with normal volunteers. Similarly, type II diabetics with ( $p < 0.0001$ ) or without ( $p > 0.05$ ) PAD had higher plasma MMP-9 levels than normal volunteers. Plasma zymographic activities of both MMP-2 and MMP-9 were positively correlated with their plasma levels. Plasma MMP-2 zymographic activity was higher in type II diabetics with PAD than type II diabetics without PAD ( $p > 0.05$ ). Plasma MMP-9 zymographic activity was higher in type II diabetics with ( $p < 0.0001$ ) or without ( $p < 0.0001$ ) PAD in comparison with normal volunteers. Together, these results indicate that increased plasma levels and zymographic activities of MMP-2 and MMP-9 may contribute to PAD in type II diabetics. In particular, plasma MMP-9 may be a useful marker for the development of vascular disease in type II diabetics.

**Key words:** MMP-2; MMP-9; type II diabetes; vascular disease

## Introduction

Matrix metalloproteinases (MMPs) are a family of  $Zn^{2+}$ -dependent enzymes that catalyze proteolysis of many connective tissue proteins of the extracellular matrix (ECM) such as collagen, gelatin, fibronectin, laminin, elastin, and proteoglycans.<sup>1,2</sup> Other MMP substrates are not components of the ECM. MMPs have been implicated in the degradation of myelin basic protein,<sup>3</sup> and interleukin-1 $\beta$ ,<sup>4</sup> as well as proteolytic processing of tumor necrosis factor- $\alpha$ .<sup>5</sup> MMPs are secreted as proenzymes by many cell types,<sup>6</sup> including leukocytes, macrophages, astrocytes, neurons, and microglia, and are widely distributed in tissues and biological fluids such as blood and urine.<sup>7</sup> They are involved in many physiological processes, including tissue remodeling during development and platelet aggregation.<sup>8</sup> MMPs also have roles in

pathophysiological processes such as inflammation, tissue repair, myocardial injury, vascular diseases, tumor invasion, and metastasis.<sup>9–11</sup>

Mechanisms have been identified for regulation of both expression and activity of MMPs. Transcription of MMP genes is modulated by growth factors, cytokines, and free radicals.<sup>12–14</sup> However, MMP-2 differs from other MMPs in that it is constitutively expressed. Activity of MMPs is negatively regulated by tissue inhibitors of matrix metalloproteinases (TIMPs).<sup>15</sup> The in vivo balance between MMPs and TIMPs dictates the level of MMP activity.<sup>16</sup>

Deterioration of MMP regulation contributes to the development of arterial lesions, in part, by facilitating monocyte invasion.<sup>17</sup> Gelatin zymography studies have shown that MMPs, especially MMP-2 (72-kDa gelatinase A)<sup>18,19</sup> and MMP-9 (92-kDa gelatinase B),<sup>20</sup> are involved in remodeling processes associated with atherogenesis.<sup>21,22</sup> MMPs are synthesized in atherosclerotic plaques<sup>23</sup> and are present at elevated levels in rupture-prone shoulder regions of arterial blood vessels.<sup>24</sup> Increased MMP activity has also been correlated with cardiovascular pathologies.<sup>25,26</sup> Since vascular complications such as acute coronary artery syndrome and peripheral arterial disease (PAD) are significantly more common among diabetics,<sup>27</sup> it is

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hypothesized that MMPs are preferentially activated in diabetics with a history of PAD. The aim of the present study was to determine whether plasma levels and zymographic activities of MMP-2 and MMP-9 differed between type II diabetics with or without PAD.

## Materials and methods

### Patients

Heparin plasma samples were provided by the Angiology Unit of the University of Catania between October 2002 and March 2003. Subjects enrolled in the present study included 42 type II diabetics without PAD (group 1), 51 type II diabetics with PAD (group 2), and 23 normal volunteers (group 3). Informed consent was obtained from all patients, and peripheral blood sample collection was approved by the Institutional Review Board. PAD was diagnosed for patients having either an ankle/brachial index (ABI) of less than 0.9 or an absence of one or more arteries of the lower legs as determined by the ecoduplex technique (Apogée CX 800 ATL-Philips electronic probe; 7–10 mHz). The clinical characteristics of enrolled subjects are summarized in Table 1. The mean ages of groups 1, 2, and 3 were  $65 \pm 15$ ,  $68 \pm 11$ , and  $69 \pm 13$  years, respectively. Individuals with arterial hypertension, chronic renal failure, or a history of ischemic coronary artery disease were excluded. Venous blood samples collected from each subject were deposited into lithium heparin-coated plastic tubes and kept at room temperature until centrifugation. Samples were centrifuged at 1500 RPM for 30 min within 60 min after collection. Plasma was then removed and stored at  $-80^\circ\text{C}$  until analysis.

### MMP-2 and MMP-9 ELISA

ELISA was performed according to the manufacturer's instructions (Amersham Biosciences, UK). In brief, plasma samples were diluted with assay buffer to a total volume of 100  $\mu\text{l}$  then incubated for 2 h at

room temperature. Sulphuric acid was then added at a final concentration of 1 mM to stop the reaction. Absorbance of ELISA samples at 450 nm was measured by spectrophotometry (ThermoLabsystems, Finland). Protein levels are expressed in ng/ml.

The assay recognizes the precursor of MMP-2 but not the active form of MMP-2. It does not cross-react with MMP-1, -2, -7, -8, -9 and MT1-MMP. Regarding the specificity of the MMP-9 ELISA system, no detectable cross-reactivity was observed with proMMP-1, proMMP-2, proMMP-3, TIMP-1 and TIMP-2.

### Gelatin zymography

MMP-2 and MMP-9 gelatinolytic activities were measured by zymography. Zymography was performed as described by Lorenzl et al and Gruber et al with slight modifications.<sup>28,29</sup> Protein concentrations of plasma samples were quantified with the Bio-Rad Protein Assay (Bio-Rad, USA). Aliquots containing 100  $\mu\text{g}$  of protein were mixed with sample buffer then subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without boiling or reduction. Gels contained 7.5% (w/v) polyacrylamide copolymerized with 0.2% (w/v) gelatin. After electrophoresis, gels were washed twice for 30 min in 2% (v/v) Triton X-100 then incubated at  $37^\circ\text{C}$  for 20 h in incubation buffer [50 mM Tris, 5 mM  $\text{CaCl}_2$ , 1  $\mu\text{M}$   $\text{ZnCl}_2$ , and 0.01% (w/v) sodium azide (pH = 7.5)]. After incubation, gels were fixed in 20% trichloroacetic acid (Sigma, Germany) for 30 min then stained in 0.5% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad) for 90 min. Gels were then washed for 60 min in destaining solution (35% (v/v) ethanol and 10% (v/v) acetic acid). Gelatinolytic activity generated clear bands against the blue background of the gel. Gelatinases A and B (Chemicon International, USA) were used as positive controls to indicate sizes of MMP-2 and MMP-9 present in plasma samples, respectively. Identities of MMP-2 and MMP-9 were

**Table 1** Clinical characteristics of the individuals studied.

Clinical characteristic	Diabetic patients without PAD	Diabetic patients with PAD	Normal volunteers
Age (years)	$65 \pm 15$	$68 \pm 11$	$69 \pm 13$
Sex (M/F)	12/8	16/9	10/5
Fasting glucose (g/dl)	$6.5 \pm 1.1$	$6.3 \pm 1.0$	$3.5 \pm 1.3$
Duration of diabetes (years)	$5.3 \pm 1.6$	$5.4 \pm 1.9$	Not applicable
Total cholesterol (mg/dl)	$198.2 \pm 7.2$	$201.3 \pm 2.7$	$165.3 \pm 4.6$
Triglycerides (mg/dl)	$113.5 \pm 7.9$	$110.3 \pm 7.2$	$98.5 \pm 6.4$
HbA <sub>1c</sub> (%)	$6.5 \pm 1.1$	$6.3 \pm 1.0$	$3.5 \pm 1.3$
BMI ( $\text{kg}/\text{m}^2$ )	<27	<27	<27
ABI	1.0–1.3	0.6–0.9	>1.0

The mean value of each characteristic is presented together with its corresponding standard deviation. Both Student's *t*-test and ANOVA tests were performed to analyze the statistical significance of differences between groups versus normal volunteers for each clinical characteristic. Statistical significance was observed only for ABI ( $p < 0.005$ ).

also confirmed by demonstrating that their zymographic activities were inhibited by addition of EDTA to the incubation buffer at a final concentration of 10 mM. EDTA inhibits MMP-2 and MMP-9 zymographic activities by chelating  $Zn^{2+}$ . Quantity One software (Bio-Rad) was used for densitometry analysis of MMP-2 and MMP-9 zymographic activities. Zymographic activities are expressed in pixels.

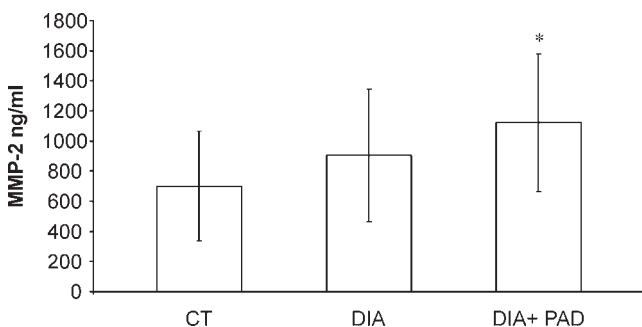
### Statistical analysis

Mean values are presented together with their corresponding standard deviations. SPSS software was used for statistical analysis. The data are normally distributed and were expressed as mean  $\pm$  standard error of the mean (SEM). Student's *t*-tests were performed to analyze the statistical significance of differences between each group. ANOVA was used to compare the parameters among PAD, non-PAD and control groups. If ANOVA analysis showed significant differences, an unpaired *t*-test using Bonferroni correction was performed in order to analyze which groups had significantly different values and to calculate *p*-values. A difference among groups was defined to be statistically significant if the corresponding *p*-value was less than 0.05. Correlations between variables were determined by Spearman rank-order correlation coefficients (*r*-values).

## Results

### MMP-2 and MMP-9 plasma levels

Mean plasma MMP-2 levels in type II diabetics with or without PAD and in normal volunteers are shown in Figure 1. Mean plasma MMP-2 levels were higher in type II diabetics with (1121  $\pm$  456 ng/ml) or without (903  $\pm$  440 ng/ml) PAD in comparison with normal volunteers (701  $\pm$  362 ng/ml). The only statistically significant difference in mean plasma MMP-2 levels



**Figure 1** Plasma MMP-2 levels in normal volunteers (CT) and in type II diabetics with (DIA + PAD) or without (DIA) PAD. Plasma MMP-2 levels were measured by ELISA as described in 'Materials and methods'. Mean values are presented together with their corresponding standard deviations. A statistically significant increase in the mean MMP-2 level was observed in type II diabetics with PAD in comparison with normal volunteers (\*).

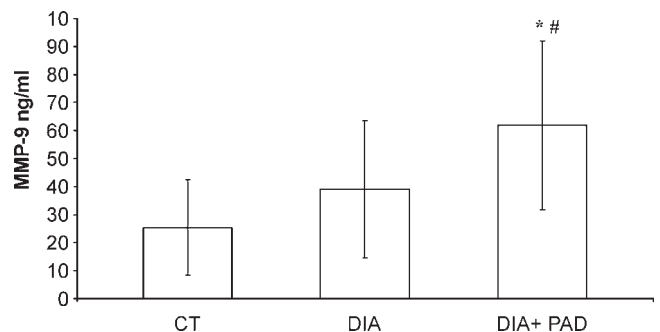
between the three groups was between type II diabetics with PAD and normal volunteers ( $p < 0.01$ ).

Mean plasma MMP-9 levels were slightly higher in type II diabetics without PAD (39  $\pm$  24 ng/ml) in comparison with normal volunteers (25  $\pm$  17 ng/ml), but this difference was not statistically significant. In contrast, a statistically significant increase in mean plasma MMP-9 levels was present in type II diabetics with PAD (62  $\pm$  30 ng/ml) in comparison with normal volunteers ( $p < 0.0001$ ) (Figure 2). Furthermore, there was a statistically significant difference in mean plasma MMP-9 levels between type II diabetics with and without PAD ( $p < 0.01$ ).

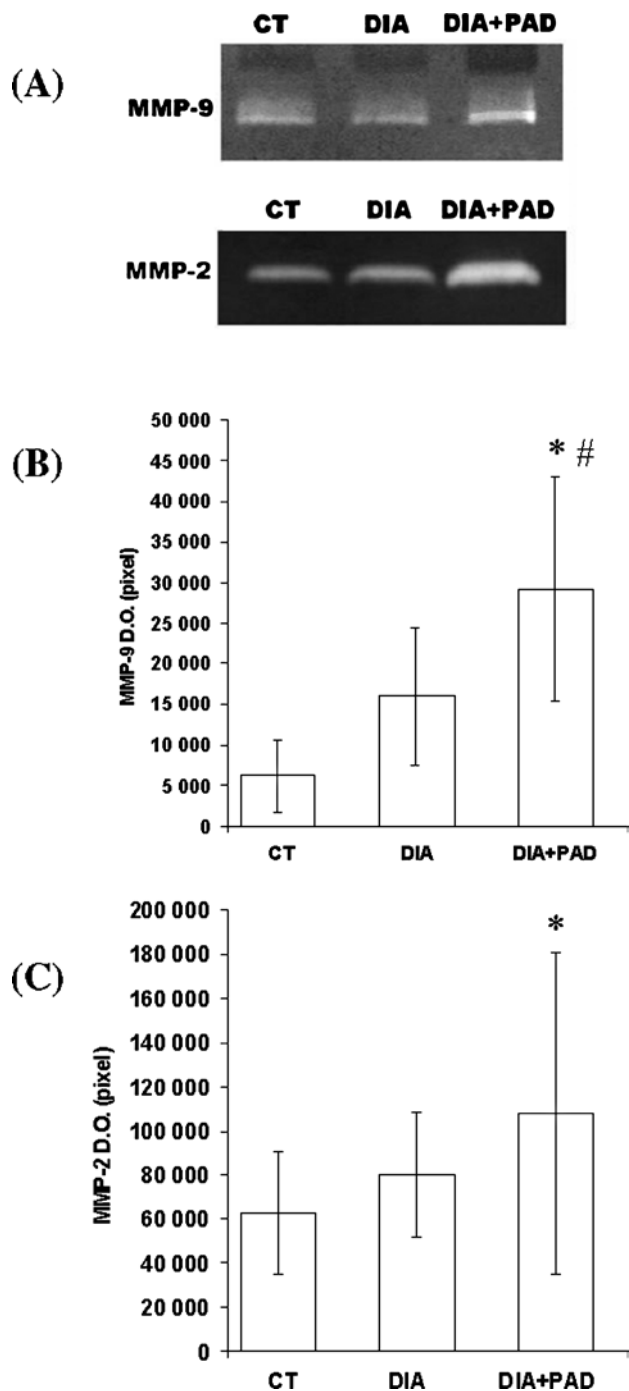
### MMP-2 and MMP-9 zymographic activities

Representative SDS-PAGE zymograms indicating plasma MMP-2 and MMP-9 zymographic activities in normal volunteers (CT) and type II diabetics with (DIA + PAD) or without (DIA) PAD are shown in Figure 3A. Results of densitometry analysis of MMP-9 and MMP-2 zymographic activities are indicated in Figures 3B and 3C, respectively.

Statistically significant differences in mean plasma MMP-9 zymographic activities were observed in type II diabetics with (29 213  $\pm$  13 797 pixels) or without (15 984  $\pm$  8522 pixels) PAD in comparison to normal volunteers (6182  $\pm$  4513 pixels) ( $p < 0.0001$ ). The difference in mean plasma MMP-9 zymographic activities between type II diabetics with PAD and type II diabetics without PAD was also statistically significant ( $p < 0.0001$ ). Mean plasma MMP-2 zymographic activities were higher in type II diabetics with (107 714  $\pm$  72 799 pixels) or without (80 361  $\pm$  28 078 pixels) PAD in comparison with normal volunteers (62 856  $\pm$  27 527 pixels). The only statistically significant difference in mean plasma MMP-2 zymographic activities between the three



**Figure 2** Plasma MMP-9 levels in normal volunteers (CT) and in type II diabetics with (DIA + PAD) or without (DIA) PAD. Plasma MMP-9 levels were measured by ELISA as described in 'Materials and methods'. Mean values are presented together with their corresponding standard deviations. A statistically significant increase in the mean MMP-9 level was observed in type II diabetics with PAD in comparison with both normal volunteers (\*) and type II diabetics without PAD (#).



**Figure 3** Plasma MMP-2 and MMP-9 zymographic activities in normal volunteers (CT) and in type II diabetics with (DIA + PAD) and without (DIA) PAD. (A) Representative analysis of MMP-2 and MMP-9 zymographic activities. MMP-9 zymographic activity was detected at 92 kDa (upper panel) and MMP-2 zymographic activity was detected at 72 kDa (lower panel). (B) Mean MMP-9 zymographic activities are presented together with their corresponding standard deviations. A statistically significant increase in mean MMP-9 zymographic activity was observed in type II diabetics with PAD in comparison with both normal volunteers (\*) and type II diabetics without PAD (#). (C) Mean MMP-2 zymographic activities are presented together with their corresponding standard deviations. A statistically significant increase in mean MMP-2 zymographic activity was observed in type II diabetics with PAD in comparison with normal volunteers (\*).

groups was between type II diabetics with PAD and normal volunteers ( $p < 0.05$ ).

### Relationships between plasma levels and activities of MMP-2 and MMP-9

Plasma levels and zymographic activities of MMP-2 and MMP-9 in type II diabetics with or without PAD were analyzed to determine whether correlative relationships were present (Table 2). MMP-2 plasma levels did not correlate with either MMP-9 plasma levels or zymographic activities in type II diabetics with or without PAD. MMP-2 zymographic activities also did not correlate with either MMP-9 plasma levels or zymographic activities in type II diabetics with or without PAD. However, zymographic activities of MMP-2 and MMP-9 did positively correlate with MMP-2 and MMP-9 plasma levels, respectively, among type II diabetics with and without PAD (Table 2).

### Discussion

It is well known that type II diabetics have a high risk of developing cardiovascular diseases. These conditions include PAD, which is considered to be a common manifestation of atherosclerosis. We have previously observed increased levels of proinflammatory cytokines and soluble adhesion molecules associated with systemic inflammation in patients with PAD.<sup>30</sup> Furthermore, increased expression of non-traditional markers of subclinical inflammation, including C-reactive protein (CRP) and interleukin-6 (IL-6), has been associated with type II diabetes.<sup>31,32</sup>

Several studies have investigated the potential involvement of MMPs in atherosclerosis. For example, expression of MMP-2 was shown to be increased within atherosclerotic plaques.<sup>33,34</sup> Additionally, smooth muscle cells and inflammatory macrophages surrounding plaques prone to acute disruption exhibited increased levels and activities of MMP-9. These results suggest that MMP-2 and MMP-9 released during macrophage activation may be important causes of vascular damage.

Other studies have implied that expression and activity of MMPs may be increased among type II diabetics. Death et al showed that exposure to a high concentration of glucose induced increased expression and activity of MMP-1 and MMP-2 in endothelial cells and MMP-9 in macrophages.<sup>11</sup> Plasma levels of MMP-9 and other markers of inflammation were decreased in type II diabetics by treatment with rosiglitazone, which has been introduced recently for the management of diabetes.<sup>35,36</sup> These effects were evident both after long-term treatment as well as after only two weeks of treatment. The decrease in plasma MMP-9 levels strictly correlated with decreased

**Table 2** Correlative analysis of MMP-2 and MMP-9 levels and zymographic activities in type II diabetics with (DIA + PAD) or without (DIA) PAD.

	MMP-2 activity		MMP-2 protein		MMP-9 activity		MMP-9 protein	
	DIA <i>r</i> -values	DIA + PAD <i>r</i> -values	DIA <i>r</i> -values	DIA + PAD <i>r</i> -values	DIA <i>r</i> -values	DIA + PAD <i>r</i> -values	DIA <i>r</i> -values	DIA + PAD <i>r</i> -values
MMP-2 activity	–	–	0.67 ( <i>p</i> < 0.001)	0.80 ( <i>p</i> < 0.0001)	0.13 ( <i>p</i> > 0.05)	0.30 ( <i>p</i> > 0.05)	0.20 ( <i>p</i> > 0.05)	0.02 ( <i>p</i> > 0.05)
MMP-2 protein	0.67 ( <i>p</i> < 0.001)	0.80 ( <i>p</i> < 0.0001)	–	–	0.33 ( <i>p</i> > 0.05)	0.36 ( <i>p</i> > 0.05)	0.12 ( <i>p</i> > 0.05)	0.31 ( <i>p</i> > 0.05)
MMP-9 activity	0.13 ( <i>p</i> > 0.05)	0.30 ( <i>p</i> > 0.05)	0.33 ( <i>p</i> > 0.05)	0.36 ( <i>p</i> > 0.05)	–	–	0.65 ( <i>p</i> < 0.001)	0.76 ( <i>p</i> < 0.0001)
MMP-9 protein	0.20 ( <i>p</i> > 0.05)	0.02 ( <i>p</i> > 0.05)	0.12 ( <i>p</i> > 0.05)	0.31 ( <i>p</i> > 0.05)	0.65 ( <i>p</i> < 0.001)	0.76 ( <i>p</i> < 0.0001)	–	–

Each *r*-value is presented together with its corresponding *p*-value.

plasma glucose levels and was independent of effects of rosiglitazone on metabolism.

In the present study, plasma levels and zymographic activities of MMP-2 and MMP-9 were studied in type II diabetics with or without PAD and in normal volunteers. Statistically significant differences in plasma MMP-2 levels and zymographic activities were only observed between type II diabetics with PAD in comparison with normal volunteers. However, statistically significant differences in plasma MMP-9 levels and zymographic activities were observed between type II diabetics with PAD in comparison with both type II diabetics without PAD and normal volunteers.

Portik-Dobos et al have reported that MMP-9 levels are lower within blood vessels isolated from type II diabetics than from normal individuals.<sup>37</sup> Our findings suggest that MMP-9 is released from blood vessels into the bloodstream in type II diabetics to a greater extent than in healthy individuals, which may contribute to increased chronic local inflammation of blood vessels among type II diabetics. Additionally, the endothelium in blood vessels from diabetics may activate MMP-producing cells in the circulation.

In conclusion, results of the present study suggest that plasma MMP-9 may be a useful marker for development of macrovascular complications, such as PAD, in type II diabetics. Additional studies are required to further characterize the effects of MMP-9 on the development and treatment of cardiovascular diseases in type II diabetics.

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