Urinary albumin excretion and progression of renal disease with impaired fibrinolytic activity in type 2 diabetes mellitus

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Background
Diabetic nephropathy is one of the major causes of end-stage renal disease. As impaired fibrinolysis can increase renal fibrosis, we investigated the relationship of impaired fibrinolysis, as assessed by the ratio of plasminogen activator inhibitor-1 (PAI-1) to tissue-type plasminogen activator (t-PA) with urinary albumin excretion (UAE) and renal disease progression in type 2 diabetes.

Patients and methods
A total of 50 patients with type 2 diabetes and 10 healthy control individuals were included in the study. Participants were subdivided according to UAE. Group 1 (G1) represented control individuals. Group 2 (G2) included patients without albuminuria. Group 3 (G3) included patients with microalbuminuria. Group 4 (G4) included patients with macroalbuminuria. Creatinine clearance and UAE were calculated. PAI-1 and t-PA were measured using an enzyme-linked immunosorbent assay kit, and the PAI-1/t-PA ratio was calculated as an index of impaired fibrinolysis.

Results
PAI-1 was highly elevated significantly in G4 when compared with the other groups, with a mean of 87.40 ± 17.03 IU/ml against 28.00 ± 6.98 IU/ml in G1, 46.4 ± 7.99 IU/ml in G2, and 64.10 ± 18.26 IU/ml in G3 ($P < 0.001$). Also, the serum level of t-PA in G4 was highly elevated significantly when compared with G1 and G2 with means of 16.85 ± 5.63 IU/ml against 7.95 ± 1.91 IU/ml and 10.45 ± 2.63 IU/ml, respectively ($P < 0.001$). The ratio of PAI-1/t-PA in G4 was significantly higher when compared with G1 (mean of 5.94 ± 2.81 against 3.54 ± 0.43; $P = 0.01$). PAI-1 and t-PA showed a significant positive correlation with UAE. Receiver operating characteristics curve analysis revealed that only PAI-1 and t-PA were significant discriminated factors for microalbuminuria and macroalbuminuria ($P < 0.001$).

Conclusion
Serum levels of PAI-1 and t-PA and the PAI-1/t-PA ratio were significantly increased in diabetic patients with higher UAE. Impaired fibrinolysis and increased UAE were associated with renal disease progression.

Keywords:
diabetic nephropathy, impaired fibrinolysis, urinary albumin excretion

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Introduction

It is well known that vascular injury and cardiovascular diseases are the main causes of death in diabetic patients [1] and diabetes is one of the most important causes of end-stage renal disease [2].

Fibrosis is the hallmark for the development of end-stage kidney disease. This process occurs as a result of abnormal deposition of extracellular matrix (ECM) in the basement membranes and interstitial tissues, associated with necrosis of the normal parenchymal cells and or apoptosis [3]. Replacement and degradation of the normal ECM by abnormal ECM is due to an increase in the proteolytic activity that is represented in the plasminogen activation system and matrix metalloproteinases [4].

Plasminogen activator inhibitor-1 (PAI-1) is a single-chain glycoprotein [5], the main inhibitor of the plasminogen activation system. It regulates intravascular fibrin deposition through the inhibition of the serine proteases tissue-type plasminogen activator (t-PA) and urokinase type PA and plasmin-mediated matrix metalloproteinase activation [6]. Also, PAI-1 is a component of ECM that binds to vitronectin (also known as protein S) [3]. This binding stabilizes PAI-1 in its active form and leads to the continuous inhibition of plasmin formation and matrix metalloproteinase

activation [7]. In addition, PAI-1 has the ability to promote fibrosis through its effect on inflammatory cell recruitment, matrix turnover, fibroblast activation, and deposition [8].

In the nondiseased kidney, PAI-1 is almost absent; increased expression was found in many chronic kidney diseases leading to renal fibrosis and end-stage kidney failure [3]. Tumor growth factor-β and the renin–angiotensin–aldosterone system are the main inducers of PAI-1 expression, and their inhibition decreases PAI-1 expression in the glomerulus [9,10].

The resultant fibrosis and vascular injury that occurs in the kidney may be one of the most important factors that lead to glomerular capillary leakage of proteins and resultant albuminuria [11]. The increase in urinary albumin excretion (UAE) reflects the vascular damage all over the body [12], which may lead to the progression of diabetic nephropathy in type 2 diabetic patients [13].

The aim from our study was to detect the relation of impaired fibrinolysis, as assessed by the ratio of the PAI-1 to the t-PA to UAE and the progression of renal disease in type 2 diabetes.

Patients and methods

Participants

A total of 50 patients with type 2 diabetes who attended the outpatient clinic of the Department of Internal Medicine, Kasr Al Aini Hospital, Cairo University, and 10 healthy age-matched control individuals were included in the study. All diabetic patients met the diagnostic criteria of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [14]. The study was performed from December 2013 to August 2014. Participants were subdivided into four groups with regard to UAE. Group 1 represented the control group. Group 2 included patients with type 2 diabetes without albuminuria. Group 3 included patients with type 2 diabetes with microalbuminuria. Group 4 included patients with type 2 diabetes with macroalbuminuria.

All patients had diabetes of type 2 of 8–10 years' duration.

Exclusion criteria

Patients with end-stage renal failure, congestive heart failure, cerebral infarction, cerebral hemorrhage, and liver diseases were excluded.

Ethical aspects

Research protocols were approved by the medical ethics committee of Kasr al Aini Medical School, Cairo University. All participants provided a written informed consent after the research protocols were explained carefully to them. Informed consent was obtained from all the study participants and their approval was taken by signature.

Procedures

All participants underwent a complete screening panel, including history taking and physical examination. Laboratory investigation included the following: fasting blood sugar, 2-h postprandial blood sugar, hemoglobin A_{1C} (HbA1c), total cholesterol, triglycerides, and serum creatinine. All laboratory investigations were performed by an autoanalyzer on Roche Diagnostic Mannheim GmbH (Germany). Creatinine clearance and UAE were calculated. PAI-1 and t-PA were measured using the enzyme-linked immunosorbent assay kit, and the PAI-1/t-PA ratio was calculated as an index of impaired fibrinolysis.

Creatinine clearance was calculated using the Cockroft-Gault equation in mg/dl [15].

(140-age) [Wt (kg)]/72 (serum creatinine in mg/dl). If the patient is female, multiply the above by 0.85.

UAE was calculated as 24-h urine collection. No albuminuria was considered if the albumin excretion in the urine was less than 30 mg/24 h. Microalbuminuria was considered if albumin excretion in the urine was in the range between 30 and 300 mg/24 h. Macroalbuminuria was considered if the albumin excretion was more than 300 mg/24 h.

PAI-1 (IU/ml) and t-PA (IU/ml) kits were supplied from Hyphen BioMed (USA). The complete enzyme-linked immunosorbent assay kit was used for tissue-PAI-1 and for t-PA antigen.

Specimen collection

Blood was collected on EDTA through a clean venipuncture avoiding any blood activation; it was then decanted after a 20-min centrifugation at 2500 *g* to avoid platelet activation, stored frozen at -20°C or below for up to 6 months, and thawed for 15 min at 37°C just before use. To avoid diurnal variation, PAI-1 and t-PA were measured on fasting samples collected at morning.

The Zymutest PAI-1 antigen kit is a one-step, two-site immunoassay for measuring human tissue-PAI-1 in the

plasma. The measurement of PAI-1 was based on the method described by Declerck *et al.* [16]. The Zymutest t-PA kit is a two-site immunoassay for measuring human t-PA in the plasma. The measurement of t-PA was based on the method described by Juhan-Vague *et al.* [17].

Statistical analysis

Data was analyzed through statistical package of social science software program, version 18 (SPSS). Statistical Package for the Social Science; SPSS Inc., Chicago, Illinois, USA.

Data were summarized using mean and SD for quantitative variables and frequency and percentage for qualitative variables.

A comparison between groups was performed using the one-way analysis of variance test for quantitative variables with the post-hoc Tukey's test for pairwise comparisons and the χ^2-test for qualitative variables. The Spearman correlation coefficient was calculated to test the association between quantitative variables. Receiver operating characteristics (ROC) analysis was used to test the discriminatory ability of certain measures to predict microalbuminuria and macroalbuminuria and to derive the most suitable cutoff point with considerable sensitivity and specificity.

P values equal to or less than 0.05 were considered statistically significant.

Results

Our patients have type 2 diabetes of 8–10 years' duration. The demographic and laboratory data of our diabetic patients are shown in Table 1. Diabetic patients with a higher UAE had significantly higher serum creatinine and lower creatinine clearance levels. The serum creatinine level and the 24-h UAE were significantly higher in patients with macroalbuminuria compared with the other groups (Table 1).

In Table 2, PAI-1 was statistically significantly different between all groups ($P < 0.001$), and with the post-hoc Tukey's test for pairwise comparisons, it was significantly higher in diabetic patients in comparison with the control group and significantly higher in diabetic patients with macroalbuminuria in comparison with the other groups. Patients with macroalbuminuria showed significant elevation in the serum level of t-PA when compared with individuals without albuminuria ($P < 0.001$) and it was higher but of no statistical significance when compared with diabetic patients with microalbuminuria. The ratio of PAI-1/t-PA was statistically significantly higher in diabetic patients, with a higher UAE when compared with the control group ($P = 0.01$).

PAI-1 was strongly correlated with serum creatinine and UAE ($P < 0.001$) and negatively correlated with creatinine clearance ($P = 0.001$); t-PA also showed a significantly moderate positive correlation with UAE (Table 3 and Figs 1–3).

Table 1 Demographic and laboratory data of type 2 diabetic patients

ANOVA	Group 2 (mean ± SD)	Group 3 (mean ± SD)	Group 4 (mean ± SD)	*P* value
Age (years)	60.80 ± 5.83A	63.60 ± 7.84A	61.15 ± 6.71A	0.5
Weight (kg)	77.70 ± 5.79A	78.25 ± 6.63A	80.75 ± 6.19A	0.3
FBS (mg/dl)	173.00 ± 54.42A	171.30 ± 55.57A	158.05 ± 55.06A	0.7
2-h PPBS (mg/dl)	254.00 ± 53.49A	245.60 ± 113.56A	219.70 ± 43.71A	0.5
HbA1c %	8.25 ± 1.46A	9.45 ± 9.45A	8.57 ± 1.79A	0.2
Cholesterol (mg/dl)	186.30 ± 50.33A	178.00 ± 55.60A	155.30 ± 49.15A	0.2
TAG (mg/dl)	123.30 ± 47.74A	152.75 ± 112.97A	106.25 ± 33.50A	0.2
Creatinine (mg/dl)	0.73 ± 0.15A	1.49 ± 0.66A	2.50 ± 1.50B	<0.001*
Creatinine clearance (mg/dl)	109.34 ± 28.75A	59.65 ± 24.47B	45.08 ± 26.06B	<0.001*
24-h urinary albumin (mg/24 h)	16.30 ± 4.03A	107.05 ± 34.80A	1544.70 ± 769.36B	<0.001*

Values are expressed as mean ± SD; ANOVA, analysis of variance; FBS, fasting blood sugar; HbA1c, hemoglobin A_{1C}; 2-h PPBS, 2-h postprandial blood sugar; TAG, triglycerides; The post-hoc Tukey's test: groups having different letters are statistically significantly different at a *P* value of 0.05; *The *P* value is highly significant.

Table 2 Comparison of PAI-1, t-PA, and PAI/t-PA I between the studied groups

ANOVA	Group 1 (mean ± SD)	Group 2 (mean ± SD)	Group 3 (mean ± SD)	Group 4 (mean ± SD)	*P* value
PAI-1 (IU/ml)	28.00 ± 6.98A	46.50 ± 7.99B	64.10 ± 18.26C	87.40 ± 17.03D	<0.001*
t-PA (IU/ml)	7.95 ± 1.91A	10.45 ± 2.63AB	13.75 ± 3.04BC	16.85 ± 5.63C	<0.001*
PAI-1/t-PA	3.54 ± 0.43A	4.62 ± 1.01AB	4.75 ± 1.42AB	5.94 ± 2.81B	0.01*

Values are expressed as mean ± SD; ANOVA, analysis of variance; PAI-1, plasminogen activator inhibitor-l; t-PA, tissue-type plasminogen activator; The post-hoc Tukey's test: groups having different letters are statistically significantly different at a *P* value of 0.05; *The *P* value is statistically significant at $P < 0.05$ and highly significant with $P < 0.001$.

ROC curve analysis revealed that only PAI-1 and t-PA were significant discriminators for macroalbuminuria and microalbuminuria ($P < 0.001$) (Tables 4 and 5, Figs 4 and 5).

Discussion

Our study showed that the serum level of PAI-1 was statistically significantly higher in diabetic patient subgroups compared with the control group and there was a significant increase in the serum level of PAI-1 in the diabetic patient subgroup in comparison with each other according to the increased UAE, that is, the four groups were statistically significantly different from each other. Also, we found that the cutoff point of 73.5 IU/ml was the level of PAI-1 to discriminate macroalbuminuria, and the cutoff point of 43.0 IU/ml was the level of PAI-1 to discriminate microalbuminuria using ROC curve analysis.

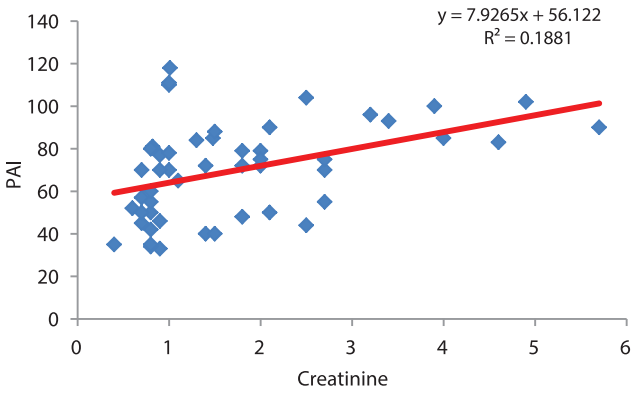
These findings were supported by Chudy P *et al.* [18], who found that PAI-1 levels were elevated in both diabetic patients with normoalbuminuria and diabetic patients with macroalbuminuria in comparison with the control group. Collins *et al.* [19] found that an elevated plasma level of PAI-1 mediates diabetic

Table 3 Correlation between PAI, t-PA, and the PAI/t-PA ratio with other variables of all patients

Parameter	PAI	t–PA	PAI/t–PA
Age			
r	−0.184	0.225	−0.333
P	0.201	0.116	0.018*
Weight			
r	0.048	0.143	−0.102
P	0.742	0.321	0.481
Fasting blood sugar			
r	−0.131	−0.236	0.11
P	0.365	0.099	0.446
2-h postprandial blood sugar			
r	−0.117	−0.152	0.085
P	0.418	0.291	0.557
HbA1c			
r	−0.078	0.117	−0.134
P	0.591	0.418	0.355
Cholesterol			
r	−0.243	−0.318	0.02
P	0.089	0.024*	0.893
Triglycerides			
r	−0.177	−0.23	0.012
P	0.219	0.108	0.936
Creatinine			
r	0.512	0.247	0.248
P	<0.001*	0.084	0.082
Creatinine clearance			
r	−0.448	−0.245	−0.193
P	0.001*	0.086	0.18
24-h urinary albumin			
r	0.638	0.382	0.18
P	<0.001*	0.006*	0.21

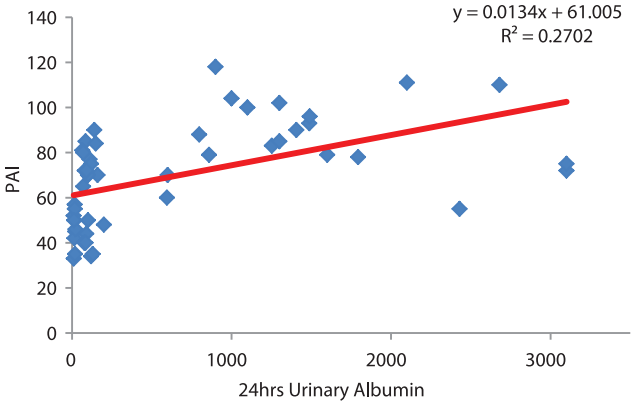
r, Spearman correlation coefficient; PAI-1, plasminogen activator inhibitor-I; t-PA, tissue-type plasminogen activator; *The *P* value is statistically significant.

Figure 1



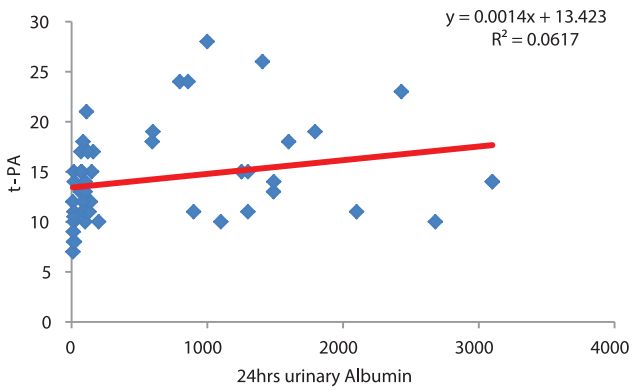
Correlation between plasminogen activator inhibitor-1 (PAI-1) and creatinine in the patient groups.

Figure 2



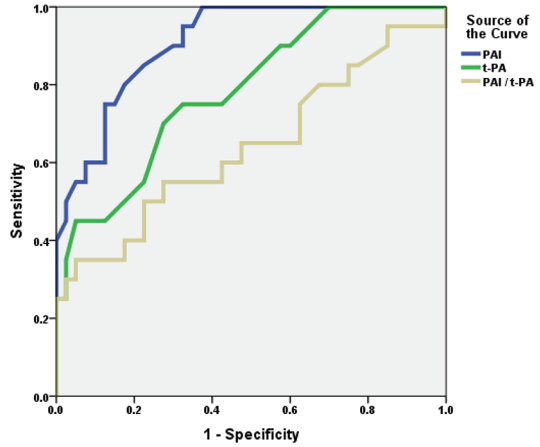
Correlation between plasminogen activator inhibitor-1 (PAI-1) and the 24-h urinary albumin excretion in the patient groups.

Figure 3



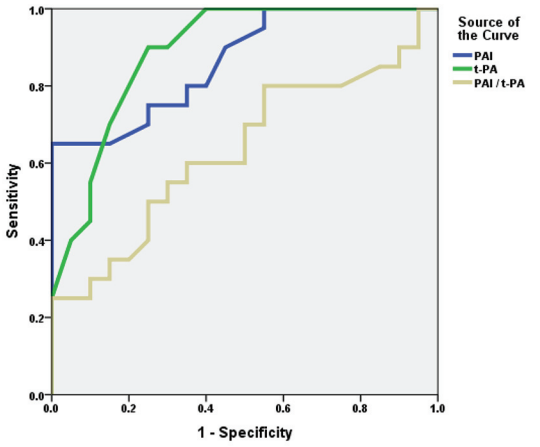
Correlation between tissue-type plasminogen activator (t-PA) and the 24-h urinary albumin excretion in the patient groups.

Figure 4



Receiver operating characteristic (ROC) curves for plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator (t-PA), and PAI-1/t-PA for discriminating macroalbuminuria.

Figure 5



Receiver operating characteristic (ROC) curves for plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator (t-PA), and PAI-1/t-PA for discriminating microalbuminuria.

Table 4 Discriminated ability of PAI, t-PA, and the PAI/t-PA ratio to predict macroalbuminuria

Parameters	AUC	95% CI	*P* value	Cutoff	Sensitivity (%)	Specificity (%)
PAI-1	0.908	0.837–0.979	<0.001	73.5	80.0	82.5
t-PA	0.784	0.663–0.904	<0.001	12.5	75.0	67.5
PAI-1/t-PA	0.641	0.480–0.801	0.078	3.7	70.0	37.5

AUC, area under the curve; CI, confidence interval; PAI-1, plasminogen activator inhibitor-I; t-PA, tissue-type plasminogen activator.

Table 5 Discriminated ability of PAI, t-PA, and the PAI/t-PA ratio to predict microalbuminuria

Parameters	AUC	95% CI	*P* value	Cutoff	Sensitivity (%)	Specificity (%)
PAI	0.865	0.756–0.974	<0.001	43.0	80.0	65.0
t-PA	0.889	0.787–0.990	<0.001	10.25	90.0	70.0
PAI/t-PA	0.634	0.459–0.809	0.148	3.5	80.0	40.0

AUC, area under the curve; CI, confidence interval; PAI-1, plasminogen activator inhibitor-I; t-PA, tissue-type plasminogen activator.

vascular complications and suggested that diabetic nephropathy was the major implication of high PAI-1 levels. Also, Szelag *et al.* [20] reported that the elevated PAI-1 was associated with diabetic complications, such as microalbuminuria, neuropathy, retinopathy, and lower-extremity arterial diseases, suggesting that circulating PAI-1 may correlate with increased vascular injury.

Bastard *et al.* [21] had shown that the plasma level of PAI-1 increased in diabetics. Also, Yener *et al.* [22] observed that the PAI-1 level was significantly elevated in 61 normotensive, normoalbuminuric type 2 diabetic patients without diabetes-related complications. Festa *et al.* [23] showed that the progression of PAI-1 over time, in addition to high baseline PAI-1 levels, was associated with incident diabetes.

However, Nagi *et al.* [24] found that the PAI-1 activity was similar between nondiabetic and diabetic patients.

Our study showed a significant increase in the serum level of t-PA in diabetic patients with albuminuria (either microalbuminuria or macroalbuminuria) and that t-PA was a significant discriminator for macroalbuminuria and microalbuminuria and the minimum values of t-PA to discriminate microalbuminuria and macroalbuminuria were 10.25 and 12.5 IU/ml, respectively.

This was supported by Beer *et al.* [25], who documented increased t-PA and PAI-1 in diabetic patients with and without vascular complications. Also, the prospective study conducted by Wannamethee *et al.* [26] documented higher levels of t-PA as a risk factor for the development of diabetes in the healthy individuals.

However, Chudy P *et al.* [18] found that plasma levels of t-PA were not significantly increased in either normoalbuminuric or microalbuminuric diabetic groups compared with controls.

Umpaichitra *et al.* [27] studied hemostatic factors in 12 type 2 diabetic patients and 17 nondiabetic obese adolescents; they found that PAI-1 activities were significantly greater in diabetic patients than in control individuals, whereas t-PA activities were significantly lower. They concluded that the elevated PAI-1 and lower t-PA activities suppress fibrinolysis in adolescents with type 2 diabetes, adding a risk factor for cardiovascular disease.

These contradictory results concerning t-PA were explained by Sobel *et al.* [28], who found that both PAI-1 and total t-PA antigen (free t-PA plus t-PA complexed with PAI-1) concentrations were increased in association with diabetes, but the concentration of free t-PA and hence the t-PA activity were not increased. Hence, the increased total t-PA is masking the impaired fibrinolytic system activity associated with type 2 diabetes.

We found that the ratio of PAI-1/t-PA in diabetic patients with macroalbuminuria was statistically significantly higher when compared with the control group, that is, diabetic patients with macroalbuminuria had significant elevation in PAI-1/t-PA than the control individuals. Thus, impaired fibrinolysis was higher in diabetic patients with a higher UAE.

Impaired fibrinolysis was related to the activated renin–angiotensin–aldosterone system in type 2 diabetic patients as angiotensin two directly induces the expression of PAI-1 and indirectly through the secretion of transforming growth factor-b, a potent profibrotic molecule that also induces the expression of PAI-1 and stimulates ECM synthesis and fibrosis [12]. Previous studies found that high doses of angiotensin 2 receptor inhibitor decrease glomerulosclerosis through the inhibition of PAI-1 [10]. The combination between decreased ECM degradation due to impaired fibrinolysis and proteolysis with the increased ECM synthesis and fibrosis induced by transforming growth factor-b predispose one to tissue fibrosis, and so increase renal nephropathy and renal mortality [12].

Our study showed a significantly higher serum creatinine concentration in patients with a higher UAE and impaired fibrinolysis. This was supported by the study conducted by Kamgar *et al.* [12], who examined the changes that occur in the serum creatinine concentration as an index of kidney function over a 7-year follow-up period in relationship to the fibrinolytic ratio. A marked increase in serum creatinine was observed in patients with a greater UAE and higher PAI-1/t-PA, and they related the association between high PAI-1 and UAE to the endothelial dysfunction that occurs in these diabetic patients.

We found a significant positive correlation between PAI-1 with serum creatinine and the 24-h urinary albumin and a significant negative correlation with creatinine clearance. Regarding t-PA, it showed a significant positive correlation with the 24-h urinary albumin. Hence, the deterioration in renal function was associated with elevations in PAI-1 and t-PA.

We did not find any correlations between PAI-1 and fasting serum lipids. This was supported by Ho and Jap [29] In contrast, Lira *et al.* [30] found a positive correlation between PAI-1 and serum lipids, suggesting that an increased risk of cardiovascular disease associated with a sedentary lifestyle is linked to an elevated PAI-1, and Peter *et al.* [18] found a positive correlation between PAI-1 levels and triglycerides, which proved that dyslipidemia and hypofibrinolysis are closely linked.

Also, we did not find any correlation between PAI-1 and fasting blood sugar; this was also supported by Ho and Jap [29] who did not find any correlation between PAI-1 activity and the increase in glucose and fasting insulin, but Heldgaard *et al.* [31] found increased levels of PAI-1 and t-PA to be associated with increased fasting blood sugar.

From this study, we concluded that serum levels of PAI-1 and t-PA and the PAI/t-PA ratio were significantly elevated in type 2 diabetic patients with higher UAE. PAI-1 and t-PA were significantly positively correlated with deteriorations in renal functions. Hence, our study suggested that impaired fibrinolytic activity in type 2 diabetic patients and increased UAE are correlated with the progression of renal disease through deteriorations in renal functions. These results may indicate that in addition to hyperglycemia, hypertension, and dyslipidemia that occur in association with type 2 diabetes, impaired fibrinolysis may be one of the important contributors that predispose one to renal complications.

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Conflicts of interest
There are no conflicts of interest.

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