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Original article

Circulating markers of endothelial dysfunction in type 2 diabetic patients with microalbuminuria

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Background: Progressive nephropathy represents a substantial source of morbidity and mortality in type 2 diabetes. Increasing albuminuria is a strong predictor of progressive renal dysfunction and heightened cardiovascular risk. Endothelial damage is associated with albuminuria. VonWillebrand factor (vWf) and Thrombomodulin (TM) are possible markers of endothelial dysfunction and damage.
Objectives: We studied the relationship between plasma vWf, TM, and urinary albumin excretion.
Methods: We conducted on 90 diabetic patients fulfilled the WHO criteria for type 2 diabetes. Diabetic patients were placed into three groups according to urinary albumin concentration (UAC) in a 24-hour urine collection. Group I: included 30 patients with normal urinary albumin concentration (without nephropathy); microalbumin/24 hour urine less than 30 mg. Group II: included 30 patients with microalbuminuria (incipient diabetic nephropathy), microalbumin/24 hour urine from 30 to 299 mg. Group III: included 30 patients with macroalbuminuria (overt diabetic nephropathy); microalbumin/24 hour urine greater than 300 mg. We measured plasma levels of vonWillebrand factor (vWf) and Thrombomodulin (TM) as markers of endothelial dysfunction, to evaluate their relationship to urinary albumin excretion in patients with type 2 diabetes mellitus.
Results: There were significant increase in the levels of cholesterol and triglyceride, and significant decrease in HDL in group III compared to the control group, while it was highly significant with group II and III. TM levels showed no significant difference between control group and group I while it was highly significant with group II and III (p <0.01), also there was highly significant difference between group I and III (p <0.01). TM index showed no significant difference between control group and group I and II, while there was highly significant difference between control group and group III (p <0.01) and highly significant difference between group II and III (p <0.01). Albumin concentration in 24 hours urine showed no significant difference between control group and group I, while there were highly significant difference between control group and group II and III (p <0.01), also there was significant difference between group II and III (p <0.01). There were highly significant positive correlation between vWF, TM levels and all studied variables. Multivariate analysis which showed that total cholesterol, urinary albumin and age retained significant influence on the plasma vWF and TM.
Conclusions: These results suggested that generalized vascular endothelial damage occurs in diabetic nephropathy including the microalbuminuric stage. Hence, plasma vWF and TM represents valuable markers of endothelial dysfunction that could be used for early detection of diabetic microvascular complications.

Keywords: Endothelial dysfunction, microalbuminuria, thrombomodulin, type 2 diabetes, von-Willebrand factor

Diabetes mellitus is a complex disease characterized by chronic hyperglycaemia responsible for complications affecting the kidneys, eyes, peripheral nerves and micro- and macrovascular systems. In individuals with type 1 or type 2 diabetes, microalbuminuria predicts not only the progression of nephropathy but also cardiovascular morbidity and mortality [1]. In addition, it has been shown that microalbuminuria is also a predictor of vascular disease in non-diabetic subjects [2]. The mechanisms underlying this association are unclear. Traditional risk factors, such as smoking, hypertension, and dyslipidaemia, only explain a small proportion of this increased cardiac risk. Identification of new risk markers may lead to earlier and more effective treatment to patients at risk. It has been proposed that the presence of microalbuminuria may reflect a

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generalized defect in vascular permeability and a concomitant atherogenic diathesis (i.e., endothelial dysfunction) [3].

The vascular endothelial cells produce several substances which are involved in the regulation of haemostasis, the soluble forms of some of these have been proposed as markers of endothelial damage. VonWillebrand factor (vWf) is a multimeric glycoprotein which plays a critical role in homeostasis as a cofactor in platelet adhesion to the vascular subendothelium and as the carrier of coagulation factor VIII in plasma. This molecule, synthesized mainly by endothelial cells, is present in plasma and platelets and also in endothelial cells and the subendothelial matrix of the vessel wall [4]. The plasma concentration of vWF has been used as an indicator of endothelial dysfunction and has been shown to increase in subjects with type 2 diabetes concomitant with the development of microalbuminuria. In contrast, vWF levels did not rise in those individuals with type 2 diabetes who remained normoalbuminuric throughout the period of follow-up [5]. These results suggest that endothelial dysfunction occurs in parallel with the development of microalbuminuria. On the other hand, in another study that included type 1 diabetic patients, the rise of vWF preceded the development of microalbuminuria [6].

Thrombomodulin (TM), a thrombin-binding glycoprotein expressed on the endothelial cell surfaces in various tissues, is involved in negative regulation of coagulation through the activation of protein C [7]. The soluble form of TM, which arises by proteolytic cleavage from membrane TM on endothelial cells, can be detected in human plasma and urine [8]. Since the plasma TM concentration is elevated in a variety of diseases accompanied by endothelial injury, soluble TM is believed to be a marker for endothelial damage. Several studies have reported that soluble TM is increased in plasma from patients with diabetes mellitus, particularly those with diabetic nephropathy [9].

In the present study, we tested the hypothesis that dysfunction of vascular endothelium, indicated by an increase in plasma level of von Willebrand factor (vWf), and TM are present in patients with type 2 diabetes mellitus who develop diabetic nephropathy (DN), therefore, we measured plasma levels of VonWillebrand factor (vWf) and Thrombomodulin (TM) as markers of endothelial dysfunction and evaluated their relationship to urinary albumin excretion (UAE) to elucidate the hypothesis that there may be a concomitant variation between urinary albumin concentrations and the proposed markers of endothelial function and that albuminuria in diabetic subjects reflects widespread vascular damage.

Patients and methods

Study population

This study was conducted on 90 diabetic patients (37 female and 53 male) who fulfilled the WHO criteria for type 2 diabetes, attending the outpatient clinic of Ain Shams University. Their average age was 59±8.7 years and their average body mass index was 24.1±2.1. Diabetic patients were placed into three groups according to urinary albumin concentration (UAC) in a 24-hour urine collection. Group I: included 30 patients with normal urinary albumin concentration (without nephropathy); microalbumin/24 hour urine less than 30 mg. Group II: included 30 patients with microalbuminuria (incipient diabetic nephropathy); microalbumin/24 hour urine from 30 to 299 mg. Group III: included 30 patients with macroalbuminuria (overt diabetic nephropathy); microalbumin/24 hour urine greater than 300 mg.

As a control group, 60 non-diabetic subjects (26 female and 34 male) were selected to match the overall age and gender distribution of the diabetic group. Their average age was 58.1±11.3 years, and their average body mass index was 23.8±3.9. The study was approved by the Ethical and Medical committee and all subjects studied gave informed consent for their participation.

Exclusion criteria for type 2 patients

Abnormal liver or thyroid function, advanced renal diseases (serum creatinine ≥1.4 mg/dl), diabetic patients with nephropathy who had albuminuria but did not have clinical or laboratory evidence of kidney disease other than diabetic glomerulosclerosis.

Other cardiovascular risk factors; including blood pressure ≥140/90 mmHg, smokers, cholesterol ≥250 mg/dl and triglyceride ≥180 mg/dl, BMI ≥30 kg/m2, family history of myocardial infarction, patients who need drugs with an effect on haemostatic function used during study, and patients with creatinine clearance less than 60 ml/min.

Sampling

Ten milliliters of venous blood was collected from patients and controls. The blood samples were divided

into two tubes. The first one containing sodium citrate, mixed and then centrifuged at 3000 rpm for 10 minutes, plasma were separated into small aliquots and kept at -70°C till assay of thrombomodulin and VonWillebrand factor. The other tube containing EDTA; blood was mixed with EDTA and used for assay of glycated hemoglobin (HbA1c).

Twenty four hour urine was collected from all patients and controls for assay of micoalbuminuria. The urinary samples contaminated with bacteria, white blood cells or red blood cells were excluded.

Methods

Body mass index (BMI) was calculated as body weight in kilogram divided by the square of height in meter (kg/m^2). Determination of plasma thrombomodulin (TM) was measured as indicator of endothelial dysfunction and/or damage with enzyme linked immunosorbent assay using a diagnostic kit supplied by Diagnostica Stago (Ashieres-sur seine, France) [10]**.** Determination of plasma VonWillebrand factor (vWf) was measured by an enzyme-linked immunosorbent assay using kit supplied by Dako (Glostrup, Denmark). Determination of glycated hemoglobin (HbA1c) in whole blood by quantitative colorimetric determination using kit supplied by Stanbio Laboratory (Boerne, Texas) [12]. Determination of blood creatinine by using the autoanalyzer Synchron CX-5 delta (Beckman Inst. Inc., California, USA).

Determination of 24 hours urine albumin by immunoturbidimetric method using kit (Pointe Scientific Inc., Michigan, USA) [13]. Calculation of thrombomodulin index which equals (TM (IU/ml)/ serum creatinine (mg %). Total cholesterol was determined by colorimetric method using Bio-Merieux test kit [14]. High density lipoprotein cholesterol was measured after precipitation of LDL and VDL using phosphotungastate [15].

Triglyceride level was determined by enzymatic colorimetric test with lipid clearing factor [16]. Determination of serum aspartate transaminase (AST) and serum alanine transaminase (ALT) levels by using the method recommended by the Committee on enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology [17].

Statistical analysis

Data are summarized as the mean±SD. Differences between groups were analyzed by an unpaired t test or a one-way analysis of variance (ANOVA) and post hock test was employed to identify the least significant difference (LSD) among groups. The correlation coefficients between two variable parameters were determined by Pearson correlation test. Multivariate analysis was performed to determine the relationship between plasma TM and vWF concentration and age, diabetes duration, glycemic control, renal function. P value less than .05 was accepted as indicating statistical significance.

Results

Table 1 summarizes characteristics of diabetic patients without and with microalbuminuria and the control group. All groups were well matched for age and sex. There were a significant increase (p <0.05) in the serum levels of cholesterol and triglyceride, and a significant decrease in HDL in group III compared to the control group. There were no significant differences (p >0.05) between the different studied groups with regards to the liver functions as compared to the control group as can be seen in **Figures 1 and 2**.

Table 2 showed highly significant statistical differences among all groups of diabetic patients and control (p <0.01) with regard to all laboratory findings. By doing post hock test to detect the least significant difference (LSD) we found that regarding vWf levels; there was no significant difference between control group and group I, while the difference it was highly significant (p <0.01) with group II and III. Also, there was highly significant difference between group I and III (p <0.01). Second, TM levels showed no significant difference between control group and group I while it was highly significant (p <0.01) with group II and III, also there was highly significant difference between group I and III (p <0.01). Third, serum creatinine showed significant showed significant difference between control group and group I (p <0.05) and highly significant with group II and III (p <0.01).Also, there was highly significant difference between group I and group III (p <0.01). Fourth, TM index showed no significant difference between control group and group I and II, while there was highly significant difference between control group and group III (p <0.01) and highly significant difference between group II and III (p <0.01). Fifth, albumin concentration in 24 hours urine showed no significant difference between control group and group I, while there were highly significant difference between control group and group II and III (p <0.01), also there was significant difference

between group II and III (p <0.01). Finally, HbA1c showed highly significant difference between control and all diabetic groups (I, II, III) (p <0.01).

Table 3 shows highly significant positive correlation among vWf levels, TM levels, and all variables as well as the correlation between TM index and vWF, TM, 24 hours albumin and duration of diabetes. To determine independent factors affecting plasma vWF and TM concentrations, which reflect endothelial damage, we performed multivariate analysis which showed that total cholesterol, urinary albumin and age retained significant influence on the plasma vWF and TM.

Table 1. General Characteristics of studied groups.

Parameters	Controls	Group I	Group II	Group III
Number	60	30	30	30
Sex (Female/Male)	26/34	12/18	13/17	12/18
Age (years)	48.3±6.4	48.2±5.1	51.4±4.2	51.6±2.1
Duration of diabetes (years)	-	7.2±2.6	10.2±2.5	12.9±2.2
Cholesterol (mg/dl)	188±14	192±15	198±20	220±29*
Triglyceride (mg/dl)	90±23	98±21	99±16	147± 37*
HDL (mg/dl)	55±12	52±8	52±9	37±8*
ALT(U/L)	15.7±5.6	16.9±4.6	18.7±4.3	17.9±6.4
AST(U/L)	17.5±5.4	19.6± 3.2	19.9±2.8	20.5±4.2
vWF (IU/mL)	1.22±0.74	1.33±0.76	1.67±0.68*	2.99±0.54*
TM (IU/mL)	2.13±0.14	2.49±0.07	3.4±0.18*	6.47±1.65*
Creatinine (mg/dL)	0.48±0.09	0.64±0.11	0.78±0.14	0.92±0.22*
TM index	3.98±0.6	4.5±0.67	4.7±0.8	7.2±1.89*
Albumin in urine (mg/dL)	13±2.26	20±1.5	110.5±15.8*	2100±243.5*
HbA1c%	4.89±0.55	8.1±0.39*	8.6±0.56*	8.8±0.22*

Group (I) patients with normo-albuminuria, Group (II) patients with micro-albuminuria, Group (III) patients with macro-albuminuria, *Significant if p <0.05 when compared to control.

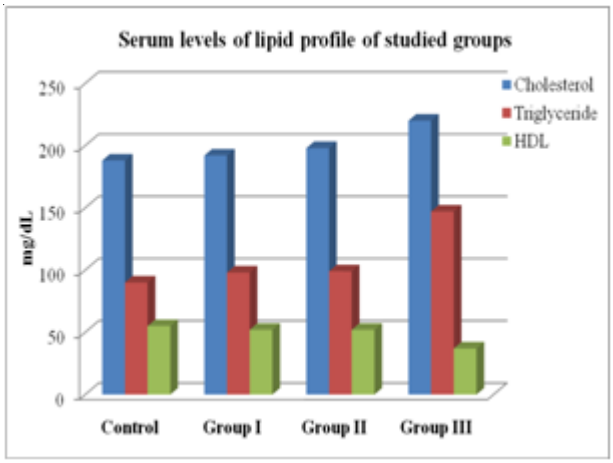

Figure 1. Lipid profile of studied groups.

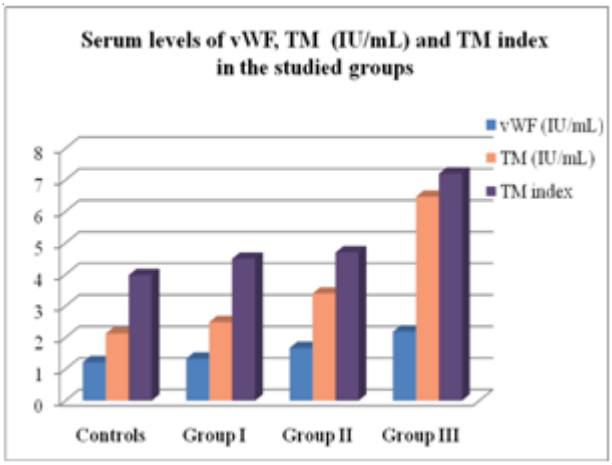

Figure 2. vWF, TM, and TM index in three studied groups.

Table 2. Comparisons between all groups of patients and controls as regard variable data

Data	Controls	Group I	Group II	Group III	f	*p*
vWF(IU/mL)	1.22±0.74	1.33±0.76	1.67±0.68	2.19±0.54	67.324	<0.01
TM (IU/mL)	2.13±0.14	2.49±0.07	3.4±0.18	6.47±1.65	58.169	<0.01
Creatinine(mg/dL)	0.48±0.09	0.64±0.11	0.78±0.14	0.92±0.22	14.837	<0.01
TM index	3.98±0.6	4.5±0.67	4.7±0.8	7.2±1.89	15.499	<0.01
Albumin in urine (mg/dL)	13±2.26	20±1.5	110.5±15.8	2100±243.5	674.987	<0.01
HbA1c %	4.89±0.55	8.1±0.39	8.6±0.56	8.8±0.22	186.456	<0.01

Significant if $p < 0.05$

Table 3. Correlation among vWF (IU/ml), TM (IU/ml), TM index, and different variables in all diabetic patients

Variables	r	*p* value
TM (IU/mL)	0.567	<0.001
Serum creatinine (mg/dL)	0.043	0.067
TM index	0.422	<0.01
24hrs albumin in urine (mg/dL)	0.734	<0.001
HbA1c %	0.521	<0.001
Duration of diabetes (years)	0.566	<0.001
vWF (IU/mL)	0.567	<0.001
Serum creatinine (mg/dL)	0.692	<0.001
TM index	0.806	<0.001
24hrs albumin in urine (mg/dL)	0.855	<0.001
HbA1c %	0.456	<0.01
Duration of diabetes (years)	0.765	<0.001
vWF (IU/mL)	0.422	<0.01
TM (IU/mL)	0.806	<0.001
Serum creatinine (mg/dL)	0.146	0.435
24hrs albumin in urine (mg/dL)	0.702	<0.001
HbA1c %	0.353	0.055
Duration of diabetes (years)	0.643	<0.001

Significant if $p < 0.05$

Discussion

Endothelial dysfunction is regarded as an early step in the development of atherosclerosis. It is characterized by an increased permeability of endothelium, a tendency to vasospasm and thrombosis [18]. Endothelial cells synthesize and release molecules with haemostatic impact: von Willebrand factor (vWF), soluble thrombomodulin (TM), plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA). Although none of these molecules are specific for endothelial cells, their plasma levels have been shown to increase with endothelial damage and some authors suggest them as plasma markers of endothelial dysfunction [19].

This study showed that, the plasma vWF and TM showed highly significant elevation in diabetic patients with microalbuminuria (group II) and macroalbuminuria (group III) as compared to controls and diabetic patients with normoalbuminuria. Also, group III showed highly significant elevation in both markers compared to group II. These results were in agreement with Hirano et al. [20] and Viswanathan et al. [21] who found that vWF and TM are elevated in diabetic patients with albuminuria, they stated that several substances synthesized by endothelial cells have been proposed as a marker for systemic vascular damage, but especially vWF and TM have been recognized as representative markers for endothelial cell damage and that albuminuria in diabetes is not only an indication of renal disease, but also reflects a more generalized vascular injury.

Several previous studies have already shown that plasma vWF [5, 22] and TM levels [23, 24] increased in diabetic subjects with albuminuria. In a cross-sectional study in patients with diabetes showed a progressive rise in plasma concentration of vWF were demonstrated with increasing urinary albumin excretion, other independent of risk factors and increasing prevalence of atherosclerosis [25]**.** More supportive data revealed that elevated vWF levels were shown to be related to the incidence and progression of microalbuminuria in type 2 diabetic subjects. These data underscore the concept that endothelial dysfunction precedes the onset of microvascular disease and nephropathy [26]**.** This has been suggested by Seligman et al. [27] who found that increased vWF levels preceded the onset of microalbuminuria in type 1 diabetic subjects. The development of hypertension may be an important mediator of this process in type 1 diabetes.

Hyperglycemia may lead to intracellular changes in the redox state resulting in depletion of the cellular NADPH pool. Overexpression of growth factors has also been implicated in diabetes with proliferation of both endothelial cells and vascular smooth muscle, possibly promoting neovascularization. This can also lead to increased blood levels of von Willebrand factor (vWF), thrombomodulin, selectin, PAI-1, type IV collagen, and t-PA The diabetic state is typified by an increased tendency for oxidative stress and high levels of oxidized lipoproteins, especially the so-called small, dense low-density lipoprotein (LDL) [28, 29].

Takanashi and Inukai [30] stated that the plasma concentrations of TM and tPA in diabetic patients with microalbuminuria were significantly higher than those in healthy subjects. This suggested that diabetic patients with microalbuminuria had more marked endothelial dysfunction which can create conditions favorable to the evolution of unstable atherosclerotic arteries plaques, thereby rendering diabetes patients with microalbuminuria highly susceptible to rupture of vulnerable plaques and acute cardiovascular events. The mechanisms of endothelial dysfunction in insulin-resistant states are not well established, although increased transvascular LDL transport, dyslipideamia, oxidative stress and chronic inflammation may be important factors. Other investigators reported that the plasma concentrations of soluble TM increased in patients with diabetes mellitus, particularly those with diabetic nephropathy [31]. Although the precise mechanism of increase in plasma concentrations of soluble TM in type 2 diabetes is not fully clear, activation of neutrophilic elastase, enhancement of free radicals and/or advanced glycation endproducts can give rise to endothelial dysfunction /damage and induce proteolytic cleavage of membrane TM on the vascular endothelium, leading to the release of soluble TM into the blood [32, 33].

Since endothelial damage and activation have been proposed as the initial, pivotal factor in the early stage of atherogenesis [34]**,** the soluble TM in plasma also could be a predictor of atherosclerosis. A study demonstrated a positive relationship between the concentrations of soluble TM and coronary artery disease in individuals with atherosclerotic diseases, suggesting that soluble TM could predict coronary heart disease [35]**.**

The results of this study also showed that there was significant correlation between vWF, TM and the duration of the disease, albumin concentration in

24 hours urine and HbA1c. In accordance with our findings, Hayakawa et al. [36] stated that there was a significant elevation in serum TM levels in diabetics over time (one year to one year and eight months). They suggested that TM could be used as index to assess the development of clinical complications in diabetes. Baggs et al. [37] also, found a correlation between the duration of diabetes and the elevation of vWF. Thus, the dysfunction of the vascular endothelium would appear to develop with time, leading to vascular complications in diabetic patients. Furthermore, a significant correlation between vWf levels and rheological determinants, such as plasma viscosity was found. They suggested that endothelial cell dysfunction over years causes proteinuria. Renal endothelium partly determines the intrinsic biochemical and biophysical properties of glomerular basement membrane. As this membrane is thickened and functionally abnormal in patients with incipient nephropathy, and since these abnormalities progress as clinical nephropathy develops, endothelial dysfunction would seem to be involved in the pathogenesis of the renal abnormalities of diabetes, thereby contributing to proteinuria [38].

This study did not show a significant correlation between plasma vWF level and serum creatinine, which is in good agreement with the report by Mezzano et al., [39] who stated that renal dysfunction is not directly associated with increased circulating vWF. Nevertheless, various metabolic abnormalities related to chronic renal failure, may deteriorate vascular function. For TM, there was a close correlation with creatinine suggesting that plasma TM level was strongly affected by kidney function. Thus, TM index was used as an endothelial marker. This TM index was substantially increased in diabetic patients with microalbuminuria and macroalbuminuria. This result was supported by Hirano et al. [40] who stated that plasma TM index is a more specific marker for generalized vascular injury than plasma TM even though renal function is only mildly impaired, as it has no correlation with serum creatinine and was markedly increased in diabetic nephropathy. Taken together, it was suggested that increases in vWF and TM index found in diabetic nephropathy is less associated with kidney damage per se, but mainly attributable to systemic vascular damage. Also, TM index showed significant correlation with duration of diabetes while it showed no significant correlation with HbA1c and this was in agreement with findings of Hirano et al. [40].

Conclusions

Our results suggested that generalized vascular endothelial damage occurs in diabetic nephropathy including the microalbuminuric stage. Hence, plasma vWF and TM represents valuable markers of endothelial dysfunction that could be used for early detection of diabetic microvascular complications. If the present findings can be confirmed in other populations, endothelial dysfunction/ hemostatic dysfunction might represent a novel therapeutic target for prevention of diabetic microvascular complications (nephropathy) in people with type 2 diabetes mellitus.

The authors had no conflict of interest to report.

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