

## The association of TGF- $\beta_1$ , angiotensin II and oxidative stress with diabetic nephropathy in type 2 diabetic patients

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### Abstract

Diabetic nephropathy (DN) is a severe complication of diabetes which may progress to end-stage renal disease (ESRD). Chronic hyperglycemia is considered as the major initiator of DN, either by creation of oxidative stress or by induction of growth factors and cytokines. Moreover, dyslipidemia plays a role in DN progression. The aim of our study was to examine the changes in lipid profile, malondialdehyde (MDA), transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) and angiotensin II (Ang II) levels in type 2 diabetic patients associated with kidney disease. Diabetic microalbuminuric (n=25) and macroalbuminuric (n=15) patients showed significantly higher levels of blood glucose, glycated hemoglobin (HbA1c), triglycerides (TG), total cholesterol (TC), MDA, TGF- $\beta_1$  and Ang II than either diabetic normoalbuminuric (n=14) or control (n=16) subjects. In the microalbuminuric and macroalbuminuric diabetic groups, albumin excretion rate (AER) was positively correlated with MDA (r=0.448, p<0.01), TGF- $\beta_1$  (r=0.81, p<0.01) and Ang II (r=0.772, p<0.01). Additionally, MDA correlated with TGF- $\beta_1$  (r=0.625, p<0.01) and Ang II (r=0.428, p<0.01). In conclusion, dyslipidemia, oxidative stress, and increased TGF- $\beta_1$  and Ang II are associated with DN in type 2 diabetic patients.

**Keywords:** angiotensin II, oxidative Stress, diabetic patients

### Introduction

The incidence of diabetes mellitus (DM) is rapidly increasing and already affects a large number of subjects all over the world, with an incidence expected to increase to over 200 million by 2010.<sup>1</sup> Diabetes is a leading cause of mortality and morbidity largely due to its micro and macrovascular complications. Microvascular complications include retinopathy and nephropathy while macrovascular complications include coronary artery disease and cerebrovascular disease.<sup>2</sup>

Diabetic nephropathy (DN) is one of the most severe complications of DM. Although the progression of DN is very slow, many diabetic patients develop end-stage renal disease (ESRD) and require hemodialysis therapy.<sup>3</sup> Clinically incipient nephropathy is first manifested as persistent microalbuminuria. Subsequently, the onset of overt DN is heralded by the appearance of persistent proteinuria.<sup>4</sup>

Chronic hyperglycemia is considered as the major initiator of diabetic kidney disease.<sup>5</sup> One proposed mechanism is that

hyperglycemia creates a state of oxidative stress both by generating free radicals and attenuating antioxidant mechanisms.<sup>6</sup> This oxidative stress causes cellular injury and tissue damage by promoting several reactions such as lipid peroxidation.<sup>7</sup> Malondialdehyde (MDA), a product of lipid peroxidation is increased in the serum of DM patients.<sup>8</sup>

Another postulated mechanism is that hyperglycemia induces a number of growth factors and cytokines in the kidney.<sup>9</sup> Of these, transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) represents the central player in the fibrogenic process because it causes extracellular matrix (ECM) production and inhibits its degradation. Moreover, the experimental use of TGF- $\beta_1$  neutralizing antibodies prevented glomerular enlargement and increase in renal cell mass.<sup>10</sup> ECM accumulation and renal cell hypertrophy are the most prominent hallmarks of diabetic kidney disease.<sup>11</sup>

Angiotensin II (Ang II), a vasoactive peptide, is a potent stimulus for TGF- $\beta_1$  production by the kidney. It acts in synergy with elevated glucose concentration to stimulate matrix production.<sup>12</sup>

Besides hyperglycemia, diabetic dyslipidemia plays an important role in the progression of DN. Diabetic nephropathy per se, in addition to diabetes, impairs lipid metabolism.<sup>13</sup> The aim of the present study was to examine the changes in lipid profile, MDA, TGF- $\beta_1$  and Ang II levels in type 2 diabetic patients associated with kidney disease.

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**Table 1:** Clinical characteristics of control subjects (group I), diabetic patients with normoalbuminuria (group II), diabetic patients with microalbuminuria (group III), and diabetic patients with macroalbuminuria (group IV) (mean  $\pm$  S.D.)

	Group I	Group II	Group III	Group IV
N	16	14	25	15
Sex (M/F)	7/9	6/8	12/13	7/8
Age (years)	51.9 $\pm$ 4.4	52.5 $\pm$ 5.2	53 $\pm$ 4.5	51 $\pm$ 5.7
BMI (kg/m <sup>2</sup> )	25.7 $\pm$ 1.8	29.9 $\pm$ 4 <sup>a</sup>	30 $\pm$ 3.8 <sup>a</sup>	29 $\pm$ 3.7 <sup>a</sup>
DM duration (years)	-	6.8 $\pm$ 1.8	7.7 $\pm$ 2	11.3 $\pm$ 2.3 <sup>b,c</sup>
SBP (mm Hg)	114.4 $\pm$ 5.2	120 $\pm$ 7.8 <sup>a</sup>	131 $\pm$ 7.6 <sup>a,b</sup>	141.7 $\pm$ 8.2 <sup>a,b,c</sup>
DBP (mm Hg)	74.4 $\pm$ 5.1	80.7 $\pm$ 7.3 <sup>a</sup>	83.8 $\pm$ 16.6 <sup>a,b</sup>	94.3 $\pm$ 7.3 <sup>a,b,c</sup>
AER (mg/day)	11.6 $\pm$ 6.6	17.2 $\pm$ 5.9	128.9 $\pm$ 52.5	519.3 $\pm$ 83.2

SBP (systolic blood pressure); DBP (diastolic blood pressure); AER (albumin excretion rate); a: Significantly different from control group at  $p < 0.05$ ; b: Significantly different from the diabetic patients with normoalbuminuria group at  $p < 0.05$ ; c: Significantly different from the diabetic patients with microalbuminuria group at  $p < 0.05$

**Table 2:** General biochemical parameters of control subjects (group I), diabetic patients with normoalbuminuria (group II), diabetic patients with microalbuminuria (group III) and diabetic patients with macroalbuminuria (group IV) (mean  $\pm$  S.D.)

	Group I	Group II	Group III	Group IV
Glucose (mg/dl)	87.6 $\pm$ 8.8	194.36 $\pm$ 33.6 <sup>a</sup>	274.6 $\pm$ 28.3 <sup>a,b</sup>	314.8 $\pm$ 24.7 <sup>a,b,c</sup>
HbA1c (%)	5.3 $\pm$ 0.5	6.3 $\pm$ 0.6 <sup>a</sup>	7.8 $\pm$ 1.4 <sup>a,b</sup>	8.1 $\pm$ 1.5 <sup>a,b</sup>
Creatinine (mg/dl)	0.81 $\pm$ 0.16	0.82 $\pm$ 0.14	0.91 $\pm$ 0.14	1.1 $\pm$ 0.36 <sup>a,b,c</sup>
GFR (ml/min)	106 $\pm$ 8.4	103.8 $\pm$ 10.4	99.9 $\pm$ 5.5	81.1 $\pm$ 15.8 <sup>a,b,c</sup>

a: Significantly different from control group at  $p < 0.05$ ; b: Significantly different from the diabetic patients with normoalbuminuria group at  $p < 0.05$ ; c: Significantly different from the diabetic patients with microalbuminuria group at  $p < 0.05$

**Table 3:** Lipid profile of control subjects (group I), diabetic patients with normoalbuminuria (group II), diabetic patients with microalbuminuria (group III) and diabetic patients with macroalbuminuria (group IV) (mean  $\pm$  S.D.)

	Group I	Group II	Group III	Group IV
TG (mg/dl)	125 $\pm$ 20	187 $\pm$ 24 <sup>a</sup>	225 $\pm$ 23 <sup>a,b</sup>	227 $\pm$ 18 <sup>a,b</sup>
TC (mg/dl)	161 $\pm$ 17	205 $\pm$ 36 <sup>a</sup>	313 $\pm$ 56 <sup>a,b</sup>	331 $\pm$ 66 <sup>a,b</sup>
HDL-C (mg/dl)	47 $\pm$ 5	36 $\pm$ 9 <sup>a</sup>	30 $\pm$ 6 <sup>a,b</sup>	26 $\pm$ 7 <sup>a,b</sup>
LDL-C (mg/dl)	89 $\pm$ 17	132 $\pm$ 33 <sup>a</sup>	239 $\pm$ 55 <sup>a,b</sup>	260 $\pm$ 65 <sup>a,b</sup>

TG (triglycerides); TC (total cholesterol); HDL-C (high density lipoprotein cholesterol); LDL-C (low density lipoprotein cholesterol). a: Significantly different from control group at  $p < 0.05$ ; b: Significantly different from the diabetic patients with normoalbuminuria group at  $p < 0.05$

## Materials and methods

### Subjects

This study was conducted on 70 subjects including males ( $n = 32$ ; age 45-55 years) and females ( $n = 38$ ; age 50-60 years) (Table 1). Subjects were divided into 16 healthy volunteers as control group (group I) and 54 patients having type 2 diabetes for 5-15 years. These patients were subdivided into three groups according to their AER. The first group comprised 14 diabetic patients with normoalbuminuria (group II) having AER  $< 25$  mg/day, the second group comprised 25 diabetic patients with microalbuminuria (group III) having AER 25-250 mg/day and the third group comprised 15 diabetic patients with macroalbuminuria (group IV) having AER  $> 250$  mg/day. Body mass index (BMI) was calculated as an index of the weight in Kg/m<sup>2</sup>. Diabetic patients were diagnosed according to the criteria established by the American

Diabetes Association.<sup>14</sup> These patients were attending the outpatient's clinic of Diabetes and Endocrinology Department, Ain Shams University Hospital, Cairo, Egypt. A detailed medical history and drug treatment(s) were collected for all subjects. They were treated with oral hypoglycemic agents and/or insulin. Exclusion criteria were: smoking, hepatic, thyroid or acute infectious diseases, pregnancy, and treatment with angiotensin converting enzyme inhibitors or hypolipidemic drugs.

### Study protocol and samples

The day before the test, subjects were instructed to follow an isocaloric diet. After a 12 h overnight fast, venous blood (5-10 ml) was drawn from the cubital vein. Samples were taken routinely before the daily medication time. These samples were divided into two parts; one part was added to Na<sub>2</sub>-EDTA containing tubes (final concentration 1 mg/ml)

and used for HbA1c determination in whole blood and for Ang II assay after plasma separation by centrifugation at 1500 rpm for 15 min. The other part was added to vacutainer clotted tubes and serum were obtained by centrifugation at 4000 rpm, for 15 min, at 4°C. The separated sera were used for the measurement of glucose, routine lipid profiles, and creatinine. Other aliquots were stored at -70°C for MDA and TGF- $\beta_1$  assays. Patients were instructed to collect first morning urine samples for urinary albumin determination.

#### Analytical procedures

Urinary albumin was assayed by microalbuminuria ELISA kit provided by Argentica, Germany and was used in the classification of diabetic patients into groups as mentioned before. Fasting blood glucose level was determined in the serum according to Trinder.<sup>15</sup> HbA1c% was measured by an ion exchange method according to Geiger and Binder<sup>16</sup> using a kit provided by Stanbio Diagnostics, USA. Serum creatinine was assayed according to the method of Henry.<sup>17</sup> Glomerular filtration rate (GFR) was calculated according to the Cockcroft and Gault equation.<sup>18</sup> Serum TG was measured by the glycerol oxidase method.<sup>19</sup> TC and high density lipoprotein cholesterol (HDL-C) were determined by the cholesterol oxidase method.<sup>20</sup> Low density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald equation.<sup>21</sup> All lipid profile measurements were done using kits provided by Centronic GmbH, Germany. All spectrophotometric measurements were performed with a Shimadzu, 1650 UV/Visible spectrophotometer.

#### Determination of MDA concentrations

MDA, as a lipid peroxidation product, was determined as thiobarbituric acid reactive substances (TBARS), according to the method of Uchiyama and Mihara.<sup>22</sup>

#### Determination of TGF- $\beta_1$ and Ang II concentrations

TGF- $\beta_1$  and Ang II were determined by using ELISA kits provided by DRG Diagnostics, USA. All ELISA procedures were done by Hyprep® automated ELISA system, USA according to the instructions of the manufacturer.

#### Data and statistical analysis

The data were collected, tabulated and recorded and all statistical calculations were made using Statistical Package for Social Science (SPSS) version 9. The results are expressed as means  $\pm$  standard deviation (Mean  $\pm$  SD). Analysis of Variance (ANOVA) was used to compare the four groups and post-hoc LSD was applied to compare individual groups. The mean difference is considered significant at  $p < 0.05$ . Pearson correlation coefficient was used to determine correlation between different parameters. Graphs were constructed using GraphPad Prism (ISI® software, USA) version 4 software.

#### Results

The clinical data of the groups studied are presented in Table 1. Diabetic patients were classified according to their AER. The subject groups were not different with respect to age and sex distribution. Type 2 DM patients (group II, III

**Table 4:** Correlation coefficients of albumin excretion rate with different parameters in diabetic patients (group II, III and IV).

	Albumin excretion rate (AER)	
	r	p
Glucose	0.695	<0.01
HbA1c	0.337	<0.05
TG	0.368	<0.01
TC	0.457	<0.01
HDL-C	-0.38	<0.01
LDL-C	0.475	<0.01
MDA	0.448	<0.01
TGF- $\beta_1$	0.81	<0.01
Ang II	0.722	<0.01

and IV) were overweight. Group III showed shorter duration of diabetes than subjects of group IV. Systolic and diastolic blood pressures were significantly increased in all DM patients when compared with the control group.

General biochemical parameters are shown in Table 2. With regard to glycemic status, serum glucose and HbA1c % were higher in diabetic patients as compared with control subjects. Furthermore, there was a significant difference in serum glucose among the three diabetic groups. However, when comparing group III and group IV patients, there was no significant difference in HbA1c %. As for kidney function, creatinine serum concentrations were markedly elevated in group IV associated with significant reduction in glomerular filtration rate. It is noteworthy that there was no statistically significant difference in those two parameters between both control and other diabetic groups.

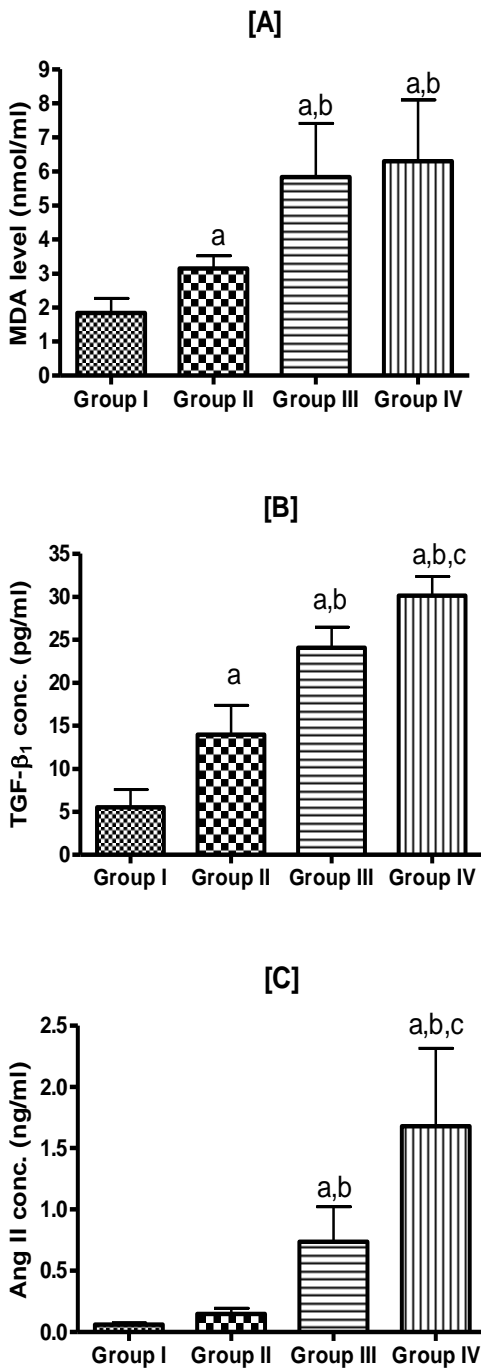
Lipid profile results in the different groups are shown in Table 3. The control group had lower serum TG values than those of diabetic patients. A dramatic elevation in TC as well as in LDL-C was also noticed. HDL-C values were decreased. Upon comparing diabetic groups, no statistically significant difference was detected in any lipid profile parameters between group III and group IV. However, there was an obvious difference between groups II and III.

Oxidative stress was evaluated by assessing lipid peroxidation (as indicated by MDA levels). Fig. 1 (A) demonstrates a significant increase in group II ( $3.1 \pm 0.4$  nmol/ml) and group III ( $5.8 \pm 1.6$  nmol/ml) as compared to group I ( $1.8 \pm 0.4$  nmol/ml). However, no significant difference was present between group IV ( $6.3 \pm 1.8$  nmol/ml) and III.

Regarding TGF- $\beta_1$  serum levels, Fig. 1 (B) shows a relatively similar pattern to MDA. A significant increase in TGF- $\beta_1$  levels in group II ( $14 \pm 3.4$  pg/ml) and group III ( $24.1 \pm 2.4$  pg/ml) when compared with group I ( $5.5 \pm 2$  pg/ml) is seen. In contrast to MDA, group IV ( $30.1 \pm 2.2$  pg/ml) has significantly higher TGF- $\beta_1$  level than group III.

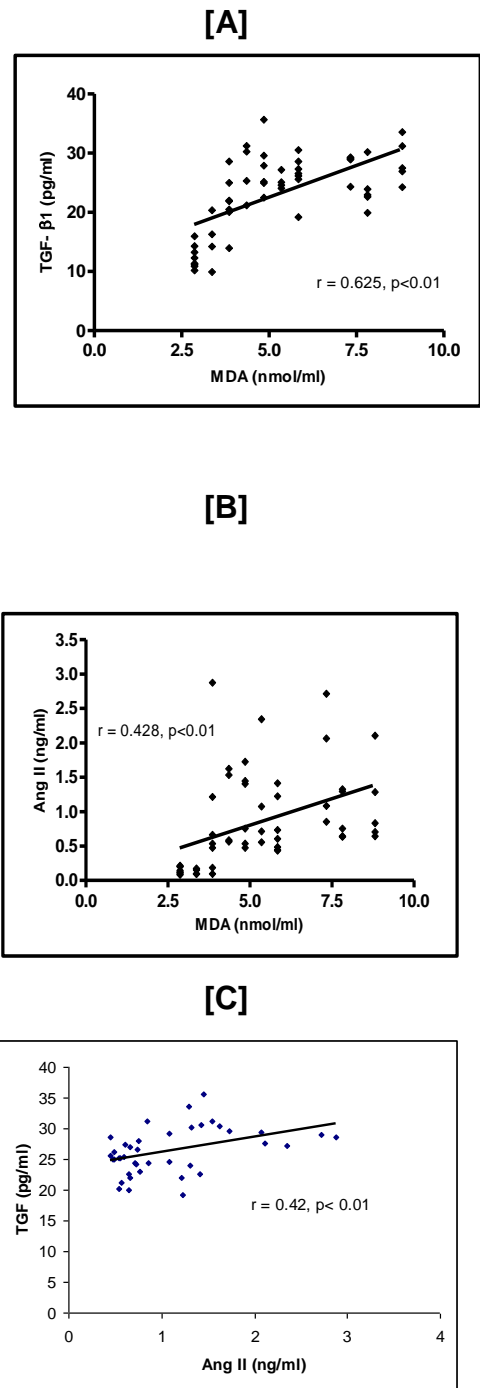
Fig. 1 (C) depicts plasma Ang II levels. A significant difference was observed among the three diabetic groups. Group III ( $0.74 \pm 0.28$  ng/ml) has lower plasma Ang II values than group IV ( $1.68 \pm 0.6$  ng/ml). On the other hand, group II ( $0.15 \pm 0.05$  ng/ml) did not differ from control subjects ( $0.06 \pm 0.01$  ng/ml).

**Figure 1**



**Figure 1:** MDA (Panel A), TGF-β (Panel B) and Ang II (Panel C) levels in control subjects (group I), diabetic patients with normoalbuminuria (group II), diabetic patients with microalbuminuria (group III) and diabetic patients with macroalbuminuria (group IV) (mean ± S.D.)  
a: Significantly different from control group at  $p < 0.05$ ; b: Significantly different from diabetic patients with normoalbuminuria group at  $p < 0.05$ ; c: Significantly different from the diabetic patients with microalbuminuria group at  $p < 0.05$ .

**Figure 2**



**Figure 2:** Correlation of MDA (nmol/ml) with TGF-β<sub>1</sub> (pg/ml) (Panel A), MDA (nmol/ml) with Ang II (ng/ml) (Panel B) and Ang II (ng/ml) with TGF-β<sub>1</sub> (pg/ml) (Panel C) in diabetic patients group II, III and IV (n=40). Each individual value is represented by a symbol (♦),  $r$  = Pearson correlation coefficients.

When the correlation coefficients were evaluated between AER and different parameters for the diabetic groups (II, III and IV) (Table 4), positive and significant moderate correlations were detected for glucose, TG, TC, LDL-C,

MDA, TGF- $\beta_1$  and Ang II, and a significant negative correlation for HDL-C serum levels. However, HbA1c % showed a weak but significant association with urinary albumin. While positive correlations of MDA with TGF- $\beta_1$ , MDA with Ang II and TGF- $\beta_1$  with Ang II are shown in Fig. 2 (A), (B) and (C) respectively.

### Discussion

The pathophysiology of DN is an interplay of metabolic and haemodynamic factors that underlie ECM accumulation and glomerular fibrosis. The extent of ECM accumulation correlates strongly with the degree of fibrosis, renal failure and proteinuria.<sup>23</sup> Factors responsible for these structural changes, such as hyperglycemia, growth factors and vasoactive hormones, are of considerable interest.<sup>12</sup> Thus, the present study was designed to assess whether dyslipidemia, serum MDA, TGF- $\beta_1$  and plasma Ang II levels in type 2 diabetic patients are associated with diabetic kidney disease as reflected by AER.

Results revealed that glucose and HbA1c % were significantly elevated in the diabetic groups (II, III and IV) as compared with the control group. However, the highest glucose and HbA1c % values were observed in group IV, which also shows a remarkable decline in GFR. These findings confirm that hyperglycemia is toxic to renal cells corroborating the reports of Wendt et al.<sup>24</sup> Moreover, Lappin et al.<sup>25</sup> considered hyperglycemia as the major driving force behind renal injury in DN, via altered cell growth and gene expression, increased ECM accumulation and stimulated growth factor production.<sup>24</sup>

According to the lipid profiles, all diabetic patients showed significant dyslipidemia compared with control subjects. Lipid profile parameters, except for HDL-C were positively associated with urinary albumin. Despite no statistically significant difference between groups III and IV, there was a tendency towards higher levels of TG, TC and LDL-C and lower HDL-C was observed in group IV. Moreover, our data were in agreement with the work of Appel et al.<sup>26</sup>, which showed that elevated TC and LDL are important predictors of ESRD development in patients with type 2 DN.

Diabetic dyslipidemia could be partially attributed to hyperglycemia, which was reported to induce non-enzymatic glycation and glycoxidation of lipoproteins and consequently reduces their receptor uptake and catabolism.<sup>27</sup> These chemical modifications may be involved in diabetic renal complications.<sup>27, 28</sup>

The elevated lipid peroxidation clearly observed in the diabetic groups were consistent with the previous study of Mahboob and his coworkers,<sup>8</sup> who found that diabetic patients, irrespective of sex, were exposed to an increased oxidative stress via lipid peroxidation. In our work, MDA levels were positively correlated with AER. Despite the fact that the difference between the two diabetic groups (III and IV) was not significant, a slight increase might be observed in the later group which shows definitive renal disease.

These findings suggest the involvement of oxidative stress in the development of DN. Oxidative stress could be due to the ability of glucose overload to generate excessive reactive oxygen species (ROS). These ROS initiate a chain reaction leading to the formation of oxidized lipoproteins as oxidized LDL.<sup>27</sup> Oxidized LDL can affect functions of renal cells by enhancing the expression of a number of cytokines including TGF- $\beta_1$ .<sup>27, 29</sup>

Diabetic groups (II, III and IV) had elevated serum values of TGF- $\beta_1$  compared to control subjects. These higher values are in concert with the increase in AER in these groups. This could be explained on the basis that hyperglycemia causes overexpression of TGF- $\beta$  in diabetes.<sup>9</sup> This profibrotic cytokine, in turn, promotes renal cell hypertrophy and ECM accumulation,<sup>11</sup> leading to decline in renal function as observed in this study. Meanwhile, the present results were in harmony with the work of Chen et al.,<sup>30</sup> who provided evidence that renal TGF- $\beta$  system is significantly up-regulated in diabetes. Positive association between MDA and TGF- $\beta_1$  levels suggests interaction between oxidative stress and TGF- $\beta_1$ . This is consistent with Lee et al.,<sup>31</sup> who reported that TGF- $\beta_1$  increases intracellular ROS which in turn upregulates TGF- $\beta_1$ .

To gain more insight into the conditions underlying renal complications of type 2 DM, the plasma levels of Ang II was examined. Diabetic groups III and IV showed marked elevation of Ang II which was greater in group IV. A positive correlation of Ang II levels and both AER and TGF- $\beta_1$  levels was found. Taken together, these findings provide an indication that Ang II plays a role in DN. This could be explained on the basis that high glucose levels stimulate the production of Ang II in mesangial cells in a concentration-dependent manner.<sup>32</sup> Veldman and Vervoort,<sup>23</sup> reported that Ang II causes haemodynamic changes in diabetes by increasing systemic and intrarenal blood pressures. Furthermore, it causes non-haemodynamic effects including glomerular cell hypertrophy and ECM accumulation. These non-haemodynamic effects are mediated by TGF- $\beta_1$ .<sup>23</sup> Moreover, glucose induced TGF- $\beta$  secretion was abrogated by blockade of type 1 Ang II receptors.<sup>32</sup> The positive correlation between MDA and Ang II supports previous studies which demonstrated Ang II involvement in glucose induced oxidative stress.<sup>31</sup>

In conclusion, dyslipidemia, MDA, TGF- $\beta_1$  and Ang II are associated with the development of diabetic renal complications. This provides further evidence to consider these alterations as targets for treatments to hinder the progression of diabetes to DN.

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