Urinary Transforming Growth Factor-β Excretion in Patients With Hypertension, Type 2 Diabetes, and Elevated Albumin Excretion Rate

Effects of angiotensin receptor blockade and sodium restriction

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OBJECTIVE — Transforming growth factor-β (TGF-β) is a prosclerotic growth factor implicated in the pathogenesis of diabetic nephropathy. In addition to high glucose, other factors implicated in renal fibrosis and increased TGF-β synthesis include angiotensin II and high dietary sodium intake. The aim of this study was to examine the effect of angiotensin receptor blockade (ARB) and dietary sodium restriction on the plasma concentration and urinary excretion of TGF-β in hypertensive patients with type 2 diabetes and elevated albumin excretion rate (AER).

RESEARCH DESIGN AND METHODS — Twenty-one subjects with hypertension and AER between 10 and 200 μg/min were randomized to receive either 50 mg losartan daily (n = 11) or placebo (n = 10). Drug therapy was given in two 4-week phases, separated by a 4-week washout period. In the last 2 weeks of each phase, patients were assigned to regular- or low-sodium diets in random order. Parameters measured at week 0 and 4 of each phase included plasma TGF-β concentration, TGF-β urinary excretion, AER, clinic mean arterial blood pressure, and urinary sodium excretion.

RESULTS — Plasma TGF-β was unaffected by losartan treatment or sodium intake. In the losartan group, urinary TGF-β excretion decreased by 23.2% (−39.2 and 13.6) [median (interquartile range)] and 38.5% (−46.8 and −6.1) in the regular- and low-sodium phases, respectively ($P < 0.05$ for drug effect). In the placebo group, median changes of 0.0% (−12.1 and 44.4) and 0.0% (−29.2 and 110.7) occurred in the regular- and low-sodium phases, respectively. Sodium restriction did not affect urinary TGF-β excretion in either losartan- or placebo-treated patients (P = 0.54 for overall dietary effect), and there was no evidence of interaction between drug and diet (P = 0.29).

CONCLUSIONS — In hypertensive type 2 diabetic patients with elevated AER, the ARB losartan, but not sodium restriction, reduced urinary TGF-β excretion. These data suggest that the renoprotective effects of losartan in patients with type 2 diabetes and nephropathy may include a reduction in renal TGF-β production.

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Abbreviations: AER, albumin excretion rate; ARB, angiotensin receptor blocker; MAP, mean arterial blood pressure; PRA, plasma renin activity; TGF-β, transforming growth factor-β.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Blockade of the renin angiotensin system reduces the rate of progression of renal dysfunction in patients with diabetic nephropathy (1–3). Although the renoprotective effects of ACE inhibitors and angiotensin receptor blockers (ARBs) have been attributed to their hemodynamic effects, emerging evidence suggests that these agents may also exert beneficial effects by reducing the production of locally active growth factors. In particular, angiotensin II, acting at its type 1 receptor, potently induces synthesis of transforming growth factor-β (TGF-β) (4), a profibrotic growth factor that has been consistently implicated in the pathogenesis of diabetic nephropathy (5).

Increased renal production of TGF-β is a feature of diabetes (6), with increased urinary TGF-β excretion noted in patients with type 1 diabetes and nephropathy (7). Indeed, urinary TGF-β excretion may not only be a marker of evolving nephropathy but has also been implicated in the pathogenesis of tubulointerstitial fibrosis in diabetic kidney disease (8). In addition to diabetes and angiotensin II, high dietary salt may also induce renal fibrosis via TGF-β–dependent pathways (9), suggesting that it may also contribute to the pathogenesis of diabetic nephropathy.

The aims of the present study were twofold. First, we sought to examine the effects of angiotensin II receptor blockade on the plasma concentration and urinary excretion of TGF-β in subjects with hypertension, elevated albumin excretion rates (AER), and type 2 diabetes. Second, the effects of dietary sodium restriction on serum and urinary TGF-β were investigated.

RESEARCH DESIGN AND METHODS

— Twenty-one patients with type 2 diabetes, hypertension, and elevated urinary AER were studied on an

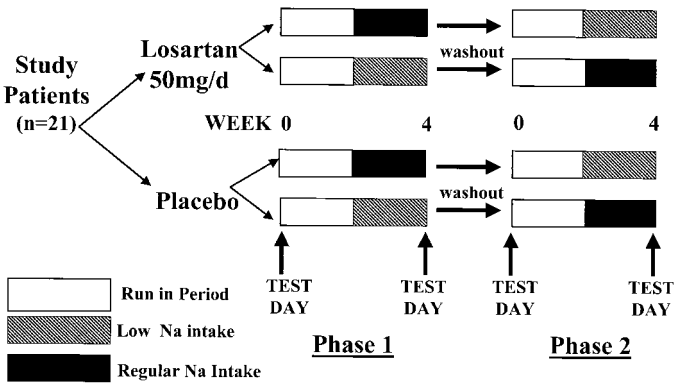

Figure 1—*Study protocol.*

ambulatory basis. A previous study has reported the changes in albuminuria, systemic, and renal hemodynamics in this population (10). Patients were included if seated systolic blood pressure was between 130 and 165 mmHg, AER was between 10 and 200 μg/min, HbA_{1c} was <11.0%, and 24-h urinary sodium excretion on patients usual diet was >100 mmol/24 h on at least two consecutive measurements 1 month apart. Exclusion criteria were hyperkalemia >5.5 mmol/l, plasma creatinine >200 μmol/l, cardiac failure, or concomitant nitrate therapy. All antihypertensive or diuretic treatments were stopped at least 2 weeks before study entry.

Study protocol

The study protocol has been previously described (10). Patients were randomly assigned in a double blind fashion to receive either the ARB losartan 50 mg daily (n = 11) or matching placebo (n = 10). Medication was taken daily for two 4-week phases with a 4-week washout in between. Patients remained on their usual diets during the first 2 weeks of each phase (run-in) and were then randomly assigned to a 2-week dietary period of either sodium restriction (low-sodium phase) or continuation of regular-sodium diet (regular-sodium phase). In the second phase, there was a crossover in dietary assignment (Fig. 1).

Low-sodium diets were conducted on an ambulatory outpatient basis, with patients having received dietary advice from a clinical nutritionist and subsequently preparing their own food. The dietary sodium target during the 2 weeks of low-sodium diet was 50–70 mmol/day.

Clinical and laboratory parameters were assessed at week 0 and 4 of each phase and included urinary TGF-β excretion, urinary electrolytes, urine volume, plasma TGF-β concentration, clinic blood pressure, albuminuria, and plasma renin activity (PRA).

Blood pressure was measured by an automatic oscillometric digital blood pressure monitor (Omron model HEM-705CP). The average of three measurements taken in the sitting position, 5 min apart and after 5 min of rest, were recorded. The study was approved by the Human Research and Ethics Committee at the Austin and Repatriation Medical Center, and all patients gave informed consent before commencement of the study.

Laboratory methods

Urine electrolytes were measured on an Hitachi 911 automatic analyzer (Roche Diagnostics, Mannheim, Germany). AER was measured on a 24-h urine collection. Radioimmunoassay for albumin was performed by a double antibody method with intra- and interassay coefficients of variation of 1.8 and 4.8%, respectively, for a concentration of 27 mg/l (11). Specimens for PRA were collected in EDTA tubes on ice, spun, and plasma frozen within 1 h for analysis at a later date. PRA was determined by measuring the rate of generation of angiotensin I by radioimmunoassay after incubating plasma at 37°C for 1 h (12).

For plasma TGF-β, blood was drawn and placed in EDTA-containing tubes and placed on ice. Samples were then sequentially centrifuged to provide platelet-free plasma as previously described (13). In brief, specimens were centrifuged at 800*g* for 10 min at 4°C, then recentrifuged for 15 min at 2,500*g*, and then again at 3,600*g* for 20 min to remove platelets. Complete removal of platelets was confirmed by an automatic cell counter. Aliquots were stored at −80°C.

For urinary TGF-β excretion, a 2.0-ml aliquot from each 24-h urine collection was obtained and stored at −80°C. Before assay, specimens were thawed, placed in a filter unit (Centricon-10 filter; Amicon, Danver, MA), and concentrated 25-fold by centrifugation for 60 min at 6,500 rpm, as previously described (7). Plasma and urinary TGF-β1 were assayed by solid-phase ELISA (enzyme-linked immunsorbent assay) (Quantikine; R&D Systems, Abingdon, U.K.) according to the manufacturer's instructions. The intra- and interassay coefficients of variation were 7.5 and 12.2%, respectively.

Table 1—*Baseline characteristics of study patients*

Baseline characteristics	Placebo	Losartan	*P*
n	10	11	—
Sex (M/F)	9/1	11/0	—
Age (years)	63.1 (3.9)	60.5 (3.3)	0.62
Duration of diabetes (years)	4.0 (1–10)	7.0 (1–38)	0.14
BMI (kg/m^2)	28.1 (1.6)	29.9 (2.0)	0.50
HbA_{1c} (%)	7.4 (0.4)	7.8 (0.5)	0.48
24-h urine sodium (mmol/day)	210 (26)	229 (33)	0.64
Clinic MAP (mmHg)	110.6 (2.6)	114.5 (2.4)	0.29
AER (μg/min)	32.6 [1.3]	21.3 [1.5]	0.36
Plasma TGF-β (ng/ml)	2.3 [1.1]	2.0 [1.1]	0.31
Urine TGF-β excretion (pg/min)	20.9 [1.1]	25.0 [1.2]	0.39

Data are expressed as means (SE); AER and plasma and urinary TGF-β as geometric mean [TF]; and diabetes duration as median (range).

Table 2—*Urinary sodium excretion, urine volume, plasma renin activity, and plasma TGF-β levels in placebo and losartan groups*

	Placebo				Losartan			
	Regular sodium		Low sodium		Regular sodium		Low sodium	
	Week 0	Week 4	Week 0	Week 4	Week 0	Week 4	Week 0	Week 4
Urinary Na (mmol/day)	208 ± 15	204 ± 27	205 ± 29	80 ± 22*	225 ± 30	204 ± 19	225 ± 22	91 ± 14*
Urine volume (ml)	2319 ± 265	2156 ± 204	1967 ± 183	2032 ± 172	2167 ± 288	1986 ± 218	1966 ± 224	1535 ± 121
PRA (ng · ml^{-1} · h^{-1})	0.49 [1.3]	0.43 [1.3]	0.57 [1.3]	0.92 [1.3]†	0.41 [1.3]	0.99 [1.5]†	0.48 [1.3]	2.68 [1.6]*
Plasma TGF-β (ng/ml)	2.2 [1.1]	2.0 [1.1]	2.1 [1.1]	2.3 [1.1]	2.0 [1.1]	1.9 [1.1]	2.0 [1.1]	1.9 [1.0]

Urinary Na and urine volume are presented as means ± SE; PRA and plasma TGF-β as geometric mean [TF]. *P* values refer to change from week 0 within each subgroup; *$P < 0.001$, †$P < 0.01$ vs. week 0.

Statistical analysis

Because of their skewed distribution, plasma TGF-β concentration, urinary TGF-β excretion, albuminuria, and PRA were logarithmically transformed before statistical analysis and expressed as the geometric mean x/÷ tolerance factor. Other results were expressed as means ± SE, unless stated otherwise. Changes in urinary TGF-β excretion and albuminuria are expressed as median percent change (interquartile range). Repeated measures, two-way ANOVA was used to examine the effects of drugs, diet and their interaction on the urinary excretion of TGF-β. Paired *t* tests were used for comparisons of the two phases within losartan and placebo groups and unpaired *t* tests for comparisons between treatment groups. The first measurement in each patient on study entry was considered as baseline. Simple linear regression analysis (using log-transformed values where appropriate) was used to determine the relationship between variables using Excel 97. Other analyses were performed using Stata and Statview (SAS Institute, Cary, NC). A *P* value <0.05 was considered statistically significant.

RESULTS

Clinical parameters

There were no significant baseline differences in plasma or urinary TGF-β, blood pressure, urinary sodium excretion, AER, BMI, or diabetes duration between placebo- and losartan-treated groups (Table 1).

Equivalent levels of sodium restriction were achieved with urinary sodium excretion of 80 ± 22 and 91 ± 14 mmol/day in placebo and losartan groups, respectively (both $P < 0.001$ vs. regular-sodium diet). Urine volume did not differ significantly between regular- and low-sodium dietary phases in either group (Table 2). Both losartan treatment and low-sodium diet (both groups) were accompanied by renin angiotensin system activation, as demonstrated by a rise in PRA (Table 2).

Losartan treatment was associated with similar decreases in mean arterial blood pressure (MAP) during the regular- and low-sodium phases (Table 3), and in the placebo group, the changes in MAP were also not significantly different between the regular- and low-sodium phases. However, the changes in MAP in the losartan group were significantly greater than in the placebo group for both the regular- and low-sodium phases (Table 3). In the losartan group, a significant reduction in albuminuria was observed during the low-sodium phase only (Table 3). In the placebo group, a trend toward a reduction in albuminuria during the low-sodium phase was noted ($P = 0.06$) (Table 3).

TGF-β

At baseline, significant correlations were noted between urinary TGF-β and sodium excretion, fasting plasma glucose and HbA_{1c} (Fig. 2*A*–*C*). No significant baseline correlation was found between urinary TGF-β and MAP or AER.

In the losartan group, urinary TGF-β excretion decreased by 23.2% (−39.2 and 13.6) [median (interquartile range)] and 38.5%(−46.8 and −6.1) in the regular- and low-sodium phases, respectively. In the placebo group, median changes of 0.0% (−12.1 and 44.4) and 0.0% (−29.2 and 110.7) occurred in the regular- and low-sodium phases, respectively. When the groups were analyzed using two-way ANOVA (repeated measures), there was a significant difference for an overall drug effect ($P = 0.042$). However, there was no significant difference for an overall diet effect ($P = 0.54$), and no evidence of a significant interaction between drug and diet ($P = 0.29$) (Fig. 3).

Change in urinary TGF-β excretion in the losartan group did not correlate with change in MAP ($r = 0.02$, $P = 0.65$) or AER (0.39, $P = 0.23$). No significant changes in plasma TGF-β were noted in losartan- or placebo-treated subjects during either the regular- or low-sodium phases (Table 2).

Table 3—*Changes in blood pressure and albuminuria over dietary phases in placebo and losartan groups*

	Placebo		Losartan	
	Regular sodium	Low sodium	Regular sodium	Low sodium
MAP (mmHg)	−1.3 ± 2.2	−5.9 ± 2.2	−9.9 ± 2.0*	−12.1 ± 1.7†
AER (%)	−4.4 (−19.9 and 7.3)	−30.1 (−35.9 and −7.1)	14.1 (−44.7 and 55.7)	−53.3 (−60.0 and −32.9)‡

Data are means ± SE or median (interquartile range). *$P = 0.01$ vs. placebo regular-sodium phase; †$P = 0.04$ vs. placebo low-sodium phase; and ‡$P = 0.02$ vs. losartan regular-sodium phase.

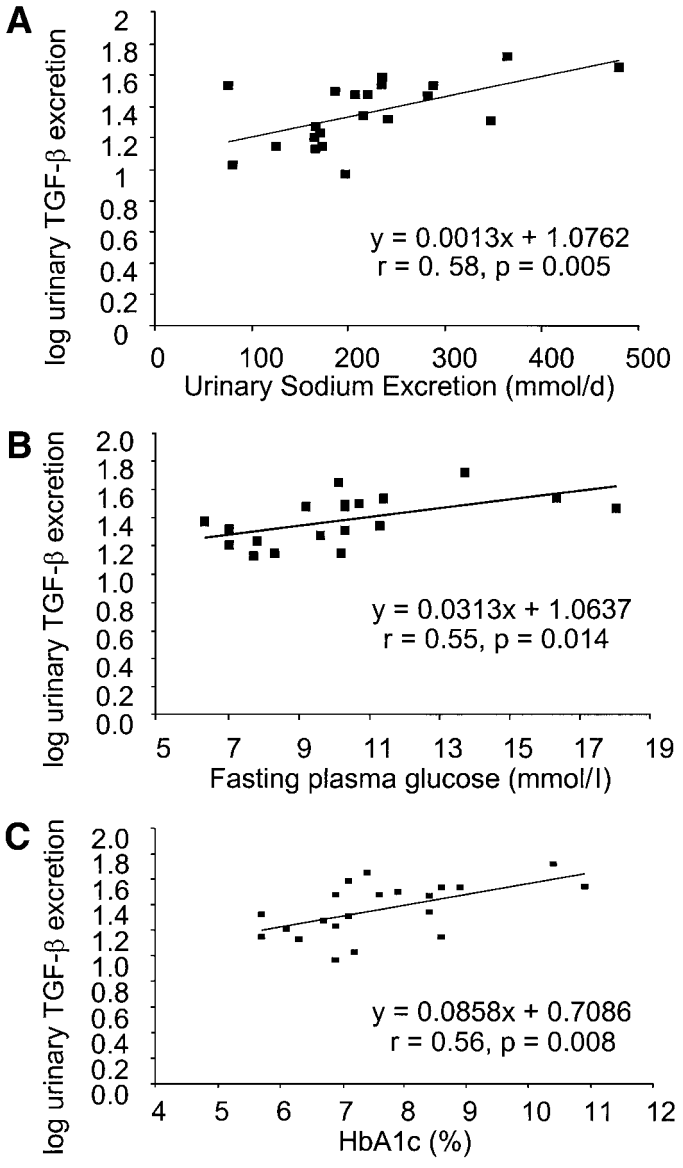

Figure 2—*Baseline correlations between urinary TGF-β and sodium excretion* (A)*, fasting plasma glucose* (B)*, and* HbA_{1c} (C).

CONCLUSIONS — The present study demonstrates several findings in relation to urinary TGF-β and incipient nephropathy in subjects with type 2 diabetes. First, urinary TGF-β excretion was reduced by the ARB losartan but was unaffected by dietary sodium restriction, suggesting that reduction in renal TGF-β production may contribute to the renoprotective effects of this class of drug. Second, urinary TGF-β excretion at baseline correlated closely with indexes of glycemic control, consistent with the role of hyperglycemia in the pathogenesis of diabetic kidney disease.

Over the past several years, experimental evidence has suggested a key role for TGF-β in the pathogenesis of the extracellular matrix accumulation that characterizes diabetic nephropathy (5) and correlates closely with declining renal function (14). The mechanisms for this fibrogenic or prosclerotic action of TGF-β are multiple and include both stimulation of extracellular matrix synthesis and inhibition of its degradation (15). Several studies have documented that not only is TGF-β expression increased in the diabetic kidney but that it is also present in a biologically active form (16). Moreover, inhibition of TGF-β action with neutralizing antibodies has recently been shown to attenuate mesangial expansion and declining GFR in experimental diabetic nephropathy (17).

In patients with diabetes, renal production accounts for the majority of the TGF-β that appears in the urine (6). Blockade of the renin angiotensin system by either ACE inhibition (1) or ARBs (2,3) has been shown to attenuate the rate of progression of renal dysfunction in patients with type 1 and type 2 diabetes, respectively. These important clinical trials have been accompanied by experimental data that angiotensin II, the effector molecule of RAS, stimulates extracellular matrix protein synthesis through induction of TGF-β in mesangial cells (18), renal interstitial fibroblasts (19), and proximal tubular epithelial cells (20). Thus, the reduction in urinary TGF-β excretion with losartan, demonstrated in the present study, may reflect attenuated TGF-β synthesis by all of these renal cell types.

In addition to being a manifestation of renal disease, increased urinary TGF-β has also been implicated in the pathogenesis of tubulointerstitial fibrosis in diabetic nephropathy. Indeed, as recently reviewed (21), there are several mechanisms whereby increased urinary TGF-β may lead to progressive renal disease. First, TGF-β in tubular fluid may act directly on tubular epithelial cells to induce the expression of extracellular matrix proteins by receptor-mediated mechanisms. Second, TGF-β–mediated activation of tubular epithelial cells induces these cells to elaborate platelet-derived growth factor, another prosclerotic growth factor that leads to matrix production by neighboring interstitial fibroblasts. Third, TGF-β may induce chemokine expression leading to macrophage infiltration and acceleration of tissue injury. Thus, the reduction in urinary TGF-β excretion with losartan, as demonstrated in the present study, may explain, at least in part, the beneficial effects of RAS blockade on tubulointerstitial disease in experimental diabetic nephropathy (22).

In the current study, the decrease in urinary TGF-β excretion in the losartan group was linked to a reduction in MAP. Despite no significant correlation between reductions in MAP and urinary excretion of TGF-β in the losartan group, the relatively small sample size in the present study does not preclude an underlying relationship between these two

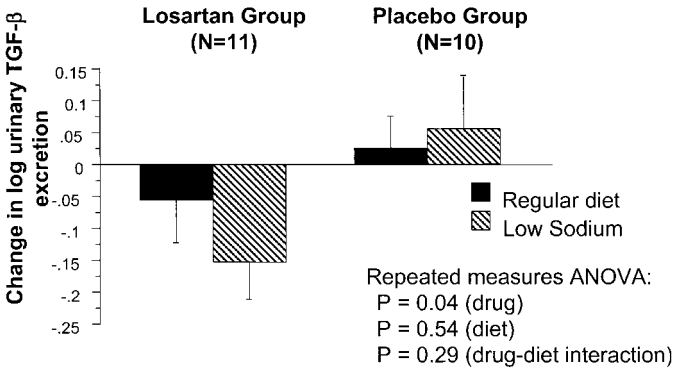

Figure 3—*Change in urinary TGF-β excretion during the regular- and low-sodium diet phases in the losartan and placebo groups.*

parameters. Previous in vitro studies have linked pressure and TGF-β, demonstrating that pulsatile mechanical stress applied to mesangial cells (23) and vascular smooth muscle cells (24) enhances TGF-β expression. Although it is likely that the decreases in MAP and urinary TGF-β are related, the relative importance of the local renin-angiotensin system versus systemic blood pressure is difficult to determine. In a study using a model of progressive diabetic nephropathy, despite equivalent blood pressure reduction by angiotensin and endothelin receptor antagonists, only the ARB was associated with a reduction in renal TGF-β mRNA levels and severity of diabetic renal structural changes (25).

Although AER and urinary TGF-β both declined with losartan treatment during the low-sodium phase, there was no significant correlation between these changes in individual patients. Although there may be specific regulating mechanisms for urinary TGF-β excretion over and above the excretion of albumin, they were not determined in this study.

In the present study, while losartan treatment reduced urinary TGF-β excretion, plasma levels remained unchanged. In contrast, in a previous study, captopril was reported to reduce serum TGF-β in patients with type 1 diabetes and nephropathy who participated in the Collaborative Study Group Trial (26). There are several reasons that may account for these differences. In the latter study, serum rather than plasma TGF-β was measured. Platelets contain abundant TGF-β, stored within their α-granules (27). A low-force step-wise centrifugation process was therefore used in the present study to avoid platelet contamination and degranulation (12). Other differences include study duration, diabetes type, and drug treatment used.

Previous experimental studies have suggested that high dietary sodium intake may induce cardiac and renal fibrosis via TGF-β–dependent pathways (9) and have also demonstrated high dietary sodium to increase urinary TGF-β excretion within a 2-week period (28). In the present study, although a close correlation between urinary sodium and TGF-β excretion was noted at baseline, a low-sodium diet over 2 weeks did not significantly influence urinary TGF-β in either losartan- or placebo-treated patients. For the effect of a low-sodium diet on the change in urinary TGF-β excretion, this study had 80% power (assuming $P = 0.05$) to detect an overall mean difference of ~31%. The findings in the present study raise the possibility that the beneficial effects of reducing dietary sodium intake in diabetic humans may develop over prolonged periods, as seen, for instance, with the effects of improved glycemic control (29).

In the present study, no significant changes in urine flow rate were found between the regular- and low-sodium phases, and it is therefore unlikely that urinary flow rates impacted on the urinary excretion of TGF-β. In addition, a previous experimental study demonstrated that increasing urinary flow rate with diuretics did not increase the urinary excretion of TGF-β (28).

Baseline urinary TGF-β excretion was found to correlate with HbA_{1c} and fasting plasma glucose in the current study. In type 2 diabetes, glycemic control has been shown to be a risk factor for development of microvascular complications (30). The association of urinary TGF-β with these markers of glycemic control supports the concept that urinary TGF-β may be a pathogenetic risk factor for diabetic nephropathy.

In summary, losartan treatment reduced urinary TGF-β in patients with hypertension, type 2 diabetes, and incipient nephropathy. Further longitudinal intervention studies will be required to determine whether serial measurements of urinary TGF-β may serve as a surrogate marker for the development of diabetic nephropathy and its response to therapeutic interventions.

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