Advanced glycation end-products associate with podocytopathy in type II diabetic patients

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Original Article

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Abstract

Background The prevalence of diabetes reaches epidemic proportions, affecting the incidence of diabetic nephropathy (DN) and associated end-stage kidney disease (ESKD). Diabetes is the leading cause of ESKD since 30–40% of diabetic patients develop DN. Albuminuria and eGFR have been considered a surrogate outcome of chronic kidney disease, and the search for a biomarker that predicts progression to diabetic kidney disease is intense.

Methods We analyzed the association of serum advanced glycation end-products (AGEs) index (AGI) with impaired kidney function in uncontrolled diabetic patients (type II, n = 130) with albuminuria ranging from (150 to 450 mg/day). The kidney biopsy specimens were also examined for the association of AGEs, particularly carboxymethyl lysine (CML) with kidney function. Further, we also assessed the effect of carboxymethyl lysine on glomerular injury and podocytopathy in experimental animals.

Results We observed a strong correlation between AGI and impaired kidney function in miroalbuminuric patients with hyperglycemia. A significant association between CML levels and impaired kidney function was noticed. Administration of CML in mice showed heavy proteinuria and glomerular abnormalities. Reduced podocyte number observed in mice administered with CML could be attributed to the epithelial-mesenchymal transition (EMT) of podocytes.

Conclusion Serum AGEs could be independently related to the podocyte injury vis-a-vis the risk of DN progression to ESKD in patients with microalbuminuria. AGEs or CML could be considered a prognostic marker to assess microalbuminuria progression to ESKD in diabetic patients.

Introduction

Diabetes mellitus (type II) has long been a growing epidemic. Asia accounts for 60% of the world's diabetic population[1,2]. The increased prevalence of diabetes led to a surge in the incidence of macro-and microvascular complications such as visual impairment, coronary heart disease, stroke, neuropathy, and diabetic nephropathy (DN). DN is a chronic disease that accounts for 44% of new end-stage kidney disease (ESKD) cases, with 6% attributed to type I and 38% attributed to type II diabetes [3]. It was projected that an increase of 3 million DN cases over the course of 20 years [3]. DN clinical manifestations include glomerular transient hyperfiltration, proteinuria, kidney hypertrophy, fibrosis, and decreased glomerular filtration rate (GFR) [4]. During the early DN stage, a patient shows hyperfiltration, represented by a rise in GFR and occasional microalbuminuria (ratio of urine albumin to creatinine ≥ 30 mg/g) [4]. The DN's progressive stage is represented by a gradual decline of the GFR, persistence of microalbuminuria, and subsequent macroalbuminuria (ratio of urine albumin to creatinine ≥ 300 mg/g). The advanced DN stage is characterized by severe proteinuria and chronic kidney insufficiency that ultimately manifest in ESKD. Both albuminuria and impaired GFR are the strongest predictors of progression to ESKD in patients with diabetes.

Biomarkers play an important role in the early detection of DN and its progression to ESKD, whereas microalbuminuria is one of the predominant marker [5]. Microalbuminuria also indicates generalized endothelial dysfunction and suggest kidney involvement with cardiovascular and cerebral impairment. Microalbuminuria is considered an early stage of, rather than a predictor of, DN and subsequent kidney impairment. Furthermore, microalbuminuria reflects not only glomerular injury but also tubular lesions [5]. Among the myriad of hemodynamic, metabolic, and inflammatory factors that participate in DN's pathogenesis, persistent hyperglycemia is predominant. It is noteworthy that a strong relationship between poor glycemic control and DN exists [6,7]. Prolonged hyperglycemia ensures the formation of advanced glycation end-products (AGEs) in the kidney and other sites of diabetic complications [8]. AGEs comprise heterogeneous compounds formed during a series of non-enzymatic (Maillard) glycation (NEG) reactions between the amino group of proteins, lipids, and nucleotides with reducing sugars [9-11]. DN patients with macroalbuminuria and patients on hemodialysis had significantly higher serum AGEs than those with microalbuminuria [12]. One of the most widely studied AGEs is carboxymethyl-lysine (CML) and is being used as markers for *in vivo* formation of AGEs [11,13,14]. CML has been used as a biomarker for long-term protein damage. Elevated tissue CML concentrations are associated with the kidney and retinal complications in patients with diabetes [15,16].

In the case of DN, early screening and evaluation of the kidney injury may help assess the risk of ESKD and strategizing the therapeutic regimen. Although glycated hemoglobin (HbA1c) has proven to be a reliable prognostic marker in the general diabetic population, it may not be valid in patients with diabetes and chronic kidney disease [17]. It is debated whether HbA1c corresponds to the same mean glucose concentrations in people with ESKD [18,19]. Further, HbA1c is influenced by several factors, including the RBCs' lifespan, administration of erythropoietin, uremic environment, and blood transfusions [17,20,21]. In contrast, glycated albumin (GA) is suggested as a preferred marker for assessing glycemic control in advanced chronic kidney disease only [17]. According to UK prospective diabetes study (UKPDS), intensive blood-glucose control in patients with type II diabetes reduces microvascular complications, particularly in patients with a diabetic kidney disease whose cardiovascular risk increases with worsening proteinuria [20,22]. Therefore, a biomarker that could predict impaired kidney function in patients with poor glycemic control and microalbuminuria would help manage DN effectively. Accumulation of serum AGEs in DN not only due to increased accumulation but decreased elimination by the kidney [12]. We examined serum and glomerular AGEs association with glomerular injury and macroalbuminuria in patients with DN. Our study identified glomerular CML levels correlate significantly with epithelial-mesenchymal transition (EMT) of glomerular podocytes and glomerulosclerosis in patients with DN.

Materials And Methods

Materials: The primary antibodies are as follows: anti-E-cadherin (#3195), anti-N-cadherin (#13116), anti-vimentin (#PAB040Hu01), anti-colIV (#PAA180Hu01), and anti-fibronectin (#PAA037Hu01) were

purchased from Cloud-clone (Houston, TX). We obtained glutaraldehyde solution (#G5882) from Sigma-Aldrich (Bangalore, India).

Study population: Study subjects were enrolled from outpatients attending several diabetes specialities centers in Vijayawada and Guntur in the state of Andhra Pradesh, India. We recruited 130 subjects with albuminuria ranging from 150 to 450 mg/day. Inclusion criteria are diabetes with more than 5 years, persistently inadequate glycemic control, and proteinuria above 150 mg/day. These subjects are devoid of other diabetic complications such as diabetic retinopathy, diabetic neuropathy, and cardiovascular complications at recruitment. Exclusion criteria were hematuria, clinical and laboratory findings suggestive of non-diabetic glomerulopathy, and secondary kidney damage due to hypertension. This protocol was by the latest revision of the Declaration of Helsinki involving clinical research on human subjects and approved by the Institutional Review Board of Guntur General Hospital, Guntur, Andhra Pradesh, India.

Clinical Examination: Anthropometric measurements, including weight, height, and waist measurements were recorded for the patients. Body mass index (BMI) was calculated using the formula: weight (kg)/ height (m^2). Blood pressure (BP) was monitored thrice by a digital oscillometer (Omron Healthcare Co. Ltd. #HEM-7120). Fasting blood glucose (FBG) was estimated in the whole blood using a glucometer (Accu-Chek Aviva, Roche Diagnostics GmbH, Germany). Blood samples (12h overnight fasting and post-prandial) were collected in heparin tubes and were centrifuged at 3500 rpm, 4^0C for 20 min to separate plasma and RBC. HbA1c was estimated in whole blood using a D-10 analyzer (Bio-Rad#12010405) based on the principle of fully automated boronate affinity assay. We collected 24 h urine and early morning spot urine, and albumin content was determined by kit from BioSystems (Barcelona, Spain).

AGE index: Plasma AGE index (AGI) was estimated as described earlier by Sampathkumar et al. [23]. Briefly, patients' plasma was diluted serially into PBS and recorded the AGE-specific fluorescence (Ex:370 nm and Em:440 nm; JASCO-FP-4500). AGE fluorescence values were curve fitted to linear regression, and the slope of the regression termed as AGI and presented as arbitrary units.

Biopsy Specimens: The DN patients archived kidney biopsies were collected from the pathology lab. Patients who underwent nephrectomy for a localized kidney tumor was selected for the control group. The non-affected part of the kidney tissue was utilized for histological examinations. The control group's mean age was subsidized to match the mean age of the DN patients included in the present study.

Immunohistochemical analysis: For histological analysis, the kidney cortical samples were fixed with 4% neutral buffered paraformaldehyde before embedding in paraffin. Paraffin-embedded tissues were sliced longitudinally into 4 μm thick sections, subjected to staining with hematoxylin and eosin (H&E) for general evaluation of the cellular structure, periodic acid–Schiff (PAS) staining to observe morphological changes in the glomerular basement membrane, tubular basement membrane, and mesangium. Masson's trichrome staining is used to observe the extracellular mesangial volume, interstitial fibrosis percentage, and tubular atrophy (IFTA). At least 6 glomeruli were captured for each biopsy sample and

quantified for histological changes. We took images with a BX51 light microscope (Olympus, Tokyo) with appropriate filters. Histological positive staining intensity was quantified using Image J analysis software (NIH, USA).

Transmission electron microscopy (TEM) : For the analysis using TEM, the kidney cortex tissue were fixed in 2.5% glutaraldehyde for 24 h followed by washing with1× phosphate-buffered saline (PBS) for four times, postfixed in osmium tetroxide for 2 h and ultra-thin sections (60 nm) were cut and mounted on 200-mesh copper grids. These copper grids were stained with 3% aqueous uranyl acetate and 3% lead citrate solution. Images were acquired on a JEM-1400 TEM (Jeol, Peabody, MA) using a Gatanultrascan CCD camera (Gatan Inc, Pleasanton, CA) 2K×2K resolution and 120kV.

Preparation of glucose-derived AGEs: Glucose derived AGEs were prepared as reported earlier [24]. Briefly, sterile preparations of BSA (100 mg/mL) were mixed with D-glucose (90 mg/mL) and 1mM sodium azide in 0.4M phosphate buffer, pH 7.6 and incubated for 2 weeks at 37°C. Formation of glucose-derived AGEs was confirmed using non-tryptophan AGE fluorescence (λex:370nm and λem:400-500nm) and by Western blotting with AGE-specific antibody.

Human podocyte culture : Human podocytes were maintained and differentiated essentially as detailed earlier [25] Differentiated podocytes were treated with AGEs in the presence or absence of inhibitor (FPS-ZM1). Protein lysate and RNA were prepared from these podocytes and used for Western blotting and qRT-PCR. A wound-healing assay was also performed with podocytes essentially as described earlier [25].

Animals and tissues: The Animal experimental procedures were performed in adherence with the Institutional Animal Ethics Committee of the University of Hyderabad. C57black/6J male mice (6-8 weeks old, 31±3g) were used in this study. These mice were randomly distributed into 3 groups viz. Control, AGEs, and AGEs+FPS-ZM1 (n=6, each group). FPS-ZM1 is an inhibitor for a receptor for AGEs (RAGE). Mice in the control group received an equal volume of phosphate buffer as a vehicle, whereas the experimental group received i.p. injections of in vitro prepared AGEs (10mg/kg b.w); AGEs and FPS-ZM1 (1mg/kg b.w) on the daily basis for 4 weeks. At the end of the experimental period, 24h urine was collected to measure GFR, albumin, and creatinine levels. Additionally, urine was subjected to SDS-PAGE and stained with silver nitrate to visualize the proteins in urine. Animals were perfused and kidneys were harvested. Kidney sections from paraffin-embedded tissues were used for immunostaining and glomerular lysate was used for immunoblotting and mRNA expression analysis.

Statistics: Data are represented as a mean with SD. Statistical analysis between groups was performed by t-test using GraphPad prism 6. Relationships between parameters were analyzed using Pearson's correlation coefficient with R version 4.0.3. Stepwise linear regression was performed using excretory albumin or eGFR as the outcome variable.

Results

3.1 Advanced glycation index (AGI) is associated with impaired kidney function in type II diabetic patients: The clinical characteristics of non-diabetic (controls) and diabetic patients are provided in **Table 1.** Mean age of 130 patients were 56±4.4 years, fasting blood glucose 159±30, post-prandial blood glucose 203±35, BMI 28.2±4.6, and HbA1c 9.75±1.8% (Table 1). The mean urinary albumin (242.1 vs. 24.68 mg/24h), serum creatinine (1.59 vs. 0.94 mg/dL), eGFR (57.3 vs. 82.93ml/min/1.73m^2), and AGI were significantly different between the controls and diabetic patients (Fig.1A-D). Protein content in the urine normalized for creatinine was significantly high in diabetic patients than age-matched non-diabetic subjects as analyzed on SDS-PAGE and visualized by Coomassie staining (Fig.1E). The correlations of AGI with urinary albumin and eGFR was examined by linear regression analysis. Interestingly, AGI was correlated significantly and positively with both albuminuria & eGFR (Fig.1F&G). The data suggest that poor glycemic control in type II diabetic patients associate with adverse kidney function.

3.2 Both serum and glomerular AGEs correlate with decreased podocin expression: Poor glycemic control in diabetics is presented with excess advanced glycation end-products (AGEs) [9]. Therefore, we assessed the advanced glycation index (AGI) in serum and urine to determine the status of AGEs empirically (Fig. 2A&B). AGI was significantly high in type II diabetic patients and correlated with declined kidney function (Fig. 2A&B vs. Fig. 1A&B). Carboxymethyl lysine (CML) is one of the well-characterized AGEs, and elevated CML levels were found in diabetic kidneys and glomeruli [2]. Thus, we determined the extent of AGEs by immunoblotting and immunostaining using an anti-CML antibody. Interestingly, we found elevated CML in both serum (Fig. 2C) and glomerulus (Fig. 2D) of diabetic patients. Accumulation of CML in diabetic rat glomeruli was proportional with decreased podocyte number[10]. Therefore, we stained for podocin, a podocyte-specific marker, and found that decreased podocin expression in glomerular sections from DN patients (Fig. 2E). Further, we noticed a significant correlation between the extent of CML staining in the glomerulus and decreased podocin expression (Fig. 2F). Decreased expression of WT1 (a podocyte-specific protein) also suggests decreased podocyte number in DN subjects (Fig. 2G). We next examined the morphology of podocytes using TEM. Our analysis revealed foot-processes of podocytes significantly effaced (Fig. 2H). Together the data suggest that excess AGEs particularly CML associated with decreased podocin expression, foot-process effacement of podocytes from type II patients with nephropathy.

3.3 Association of excess glomerular CML with epithelial-mesenchymal transition (EMT) of podocytes: Since excess glomerular CML correlates with reduced podocin number, we next ascertained the mechanisms of podocyte depletion in diabetic patients. An earlier study from our group reported that podocytes undergo EMT in mice administered with CML[2]. Therefore, we investigated whether EMT occurs or not in glomeruli from DN patients. E-cadherin (a bonafide marker of epithelial phenotype) expression significantly decreased in DN patients (Fig. 3A). A strong correlation was observed between decreased E-cadherin expression and the accumulation of glomerular AGEs (Fig. 3B). Nephroseq data also corroborated with our observation that in DN, decreased expression of epithelial markers (E-cadherin/CDH1) and increased expression of mesenchymal markers (N-cadherin/CDH2) and transcription factors that ensure EMT such as SNAI1 and TWST1 (Fig. 3C). Nephroseq data also revealed

upregulation of receptor for AGE (RAGE) in DN patients (Fig. 3C). H&E staining and TEM imaging revealed detached podocytes in glomerular space (arrow mark) of DN patients (Fig. 3D&E). Together the data suggest podocytes in DN patients undergo EMT, which might be responsible for the observed detached phenotype.

3.4 AGE index and decreased podocyte count are associated with glomerulosclerosis: It demonstrated the correlation of decreased podocyte count with the onset of proteinuria and glomerulosclerosis [2]. Since these podocytes counteract the outward forces of glomerular pressure and help to maintain the capillary loop's shape, depletion of podocytes leads to bulging of the GBM [26]. Additionally, the denuded GBM form a synechia attachment with the parietal epithelial cells and Bowman's capsule, which is thought to ensure focal segmental glomerular sclerosis (FSGS). Since we observed decreased podocyte count in diabetic patients, we assessed the extent of fibrotic changes in the kidney sections. As anticipated, PAS and MT staining revealed significant fibrotic changes in the glomerular region (Fig. 4A), concomitant with a high glomerular injury score (Fig. 4B). Expression of fibrotic markers such as SMA, Col IV, and fibronectin was up-regulated in these injured glomeruli as evidenced by immunostaining (Left panel and quantification (Right panel) (Fig. 4C). Nephroseq analysis of DN patients also revealed elevated expression of fibrotic markers (Fig.4D). Both our experimental and Nephroseq data suggest that AGE/RAGE activation associated with glomerular fibrosis.

3.5 Administration of AGEs manifested in impaired kidney function and EMT of podocytes both *in vivo* and *in vitro*: As we observed increased AGI and accumulation of AGEs associated with glomerular injury in patients with type II diabetes, next, we ascertained whether administration of AGEs to mice induces similar pathological features. Administration of AGEs to mice manifested in the GFR decline and albuminuria (Fig. 5A-C). PAS and MT staining of paraffin-embedded sections from mice administered with AGEs revealed glomerulosclerosis (Fig. 5D). Histological analysis of AGE-treated mice showed that a high glomerular injury (Fig. 5E). Further, decreased expression of podocin, nephrin, and E-cadherin was also noticed in these mice administered with AGEs (Fig. 5F). Decreased number of podocytes per glomerulus was observed in these mice administered with AGEs as assessed in WT1 staining (Fig. 5G). Human podocytes exposed to AGEs showed enhanced migratory property with decreased epithelial markers (Fig. 5H), corroborating our *in vivo* observation that AGEs elicit podocyte injury. RAGE inhibitor protected the mice from podocyte depletion and glomerulosclerosis (Fig. 5D&G). Together, our data suggest AGEs adversely affect kidney function by eliciting podocyte injury and depletion, possibly by promoting podocyte EMT.

Discussion

The incidence of ESKD is increasing globally, and DN is one of the leading causes. Although eGFR and albuminuria reflect kidney function, these parameters are part of the diagnosis. Declined eGFR and albuminuria may not seldom predict the DN's extent when the serum creatinine levels have risen already. Therefore, a more effective indicator that can predict DN's progression is greatly warranted to deal with DN and consequently ESKD. In the present study, we found the HbA1c, GA, AGI index significantly

associated with decreased kidney function in patients with DN as evidenced by altered eGFR and albuminuria. Both serum and urinary AGEs are significantly associated with adverse kidney function in these patients with DN. Elevated CML proportionate with decreased expression of podocyte-specific markers such as nephrin and podocin. Our study suggests that AGEs associate with EMT of podocytes and glomerulosclerosis in DN patients. Similarly, *in vivo* administration of AGEs resulted in podocyte EMT, glomerulosclerosis, and proteinuria. RAGE inhibition prevented AGEs induced adverse kidney effects both *in vivo* and *in vitro* such as podocyte depletion, sclerosis, and proteinuria. Together, the data presented in our study demonstrate that AGEs may predict DN progression, particularly podocyte injury.

Chronic elevation of blood glucose levels is an exacerbating factor that ensures the non-enzymatic glycation and formation of AGEs, which deposit irreversibly in several organs and blood vessels [27]. Serum levels of AGEs not only associate with the severity of diabetic complications, including retinopathy and nephropathy [28] but also predict mortality [29-32]. In addition to predicting the risk of diabetic complications, Luft et al. reported that circulating CML levels predict the risk of developing diabetes [33]. Each 100 ng/ml increment in CML the risk of developing diabetes increases by 35% in individuals with impaired fasting glucose [33]. While in American cohort circulating CML levels were associated with insulin resistance (HOMA-IR), in Japanese cohort, no association was found for CML despite AGEs were association with HOMA-IR index [34,35]. AGEs elicit intracellular signaling events via interaction with transmembrane receptor for AGEs (RAGE) localized to endothelial cells, macrophages, and vascular smooth muscle cells. The dominant AGE epitope for binding to the RAGE is CML [36]. At the same time CML modifications of proteins are predominant AGEs that accumulate in vivo [37]. Elevated serum CML levels were observed in patients with kidney failure [38]. Enhanced CML accumulation was observed in glomerular nodular lesions from patients with DN [39]. AGEs-RAGE interaction elicits cellular injury by producing reactive oxygen species, activating profibrotic and proinflammatory cascades [10,40]. A recent report suggests that it may be necessary to evaluate glycemic control in patients with diabetes undergoing hemodialysis by combining several glycemic control indicators, including GA, HbA1c, and pre-dialysis blood glucose levels [41].

Infusion of AGEs into rats induced albuminuria and histological changes like that occurs during DN. Contrastingly, preventing AGEs formation improved proteinuria and preserved kidney function. DN is presented with reduced podocyte density. The specific effect of AGEs on podocyte biology is being investigated recently. AGEs, particularly CML, induce epithelial-mesenchymal transition (EMT) of podocytes by inducing transcription factor Zeb2, a transcription factor that regulates E-cadherin expression [2]. A recent study showed that CML induced Notch signaling in podocytes contributing to their EMT [24]. Administration of AGEs elicits decreased podocyte count in mice [24]. AGEs accumulate in glomeruli and elicit the expression of ECM components such as type IV collagen and laminin. AGEs provoke premature senescence of the kidney cells, particularly cells in the proximal tubule. These novel actions of AGEs in eliciting podocytopathy vis-a-vis the pathogenesis of proteinuria and DN could be

adapted as prognostic markers to assess the glomeruli's irreversible damage during the progression of DN.

HbA1c is the most used marker for glycemic control, and it is also used to predict the morbidity of vascular complications. HbA1c reflects plasma glucose levels for the past 2-3 months due to erythrocytes' long lifespan. However, certain clinical conditions such as kidney anaemia and hemolytic anaemia during which lifespan of erythrocytes vis-a-vis HbA1c measurements are affected and underestimate glycemic control. Furthermore, low hemoglobin levels may result in falsely low HbA1c values underestimate glycemic control in dialysis patients. Increased hemoglobin turnover might contribute to lower glycated hemoglobin in advanced CKD and may mislead the clinical judgment. On the other hand, erythropoietin treatment in anaemic patients with kidney disease significantly alters the HbA1c levels [20]. Therefore, it is considered that over-reliance on HbA1c as the sole marker of glycemic control could lead to errors in assessing true changes in glycemia [20]. In this setting, an additional assessment of glycemic control is required.

Studies reported that AGI might represent a better glycemic control marker than HbA1c in diabetic patients with the kidney insufficiency. Therefore, markers that provide an index of long-term glycemic control are essential tools in DN patients' care, considering the increased incidence of DM and progression towards ESKD. In this study, we measured only one AGEs-CML. The association of other AGEs with podocyte injury may be similar as we observed or may be different, which needs to be investigated. Other limitation a relatively small sample size. Our subjects were abnormally hyperglycemic, and the data with diabetic patients with a good glycemic index may be different. A prolonged follow-up study with more patient numbers would make the present observation stronger. However, given the supportive findings from animal studies and biopsy samples, AGI's potential and measurement of individual AGEs could give a better index of progression of DN to ESKD. We are currently pursuing a study with an extended patient number for a longer duration. In conclusion, serum and urinary AGI and CML might be considered a potential surrogate prognostic marker for DN.

Declarations

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Disclosure:

The authors declare no conflict of interest.

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Table

Table 1: Clinical characteristics of the study subjects with DN and non-diabetics.

Parameter	Diabetic (n=130)	Non-diabetic (n=130)
Age (yrs)	56±4.4	57±2.3
BMI	28.2±4.6	N/A
Known duration of diabetes (yrs)	10±3.4	N/A
Known duration of proteinuria (yrs)	2.5±2.8	N/A
Fasting Glucose (mg/dL)	159±30	98 ±15
PP Glucose (mg/dL)	203±35	110±35
Systolic blood pressure (mm Hg)	156±37	115±18
Diastolic blood pressure (mm Hg)	98±6	79±8
Creatinine (mg/dL)	1.59±6.31	0.94±0.36
Albumin (mg/24h)	242.1±207.9	24.68±7.32
eGFR (ml/min/1.73m^2)	57.3±36.3	82.93±37.07
HbA1c (%)	9.75±1.8	4.5±0.94
Glycated Albumin	28.8±3.2	11.45±2.9
AGI (AU)	0.57±0.12	0.13±0.08

Data presented as mean±SD. $p < 0.001$

Abbreviations: PP, post-prandial; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, hemoglobin, A1c; GA, glycated albumin; UACR, urinary albumin to creatinine ratio; eGFR, estimated glomerular filtration rate.

Figures

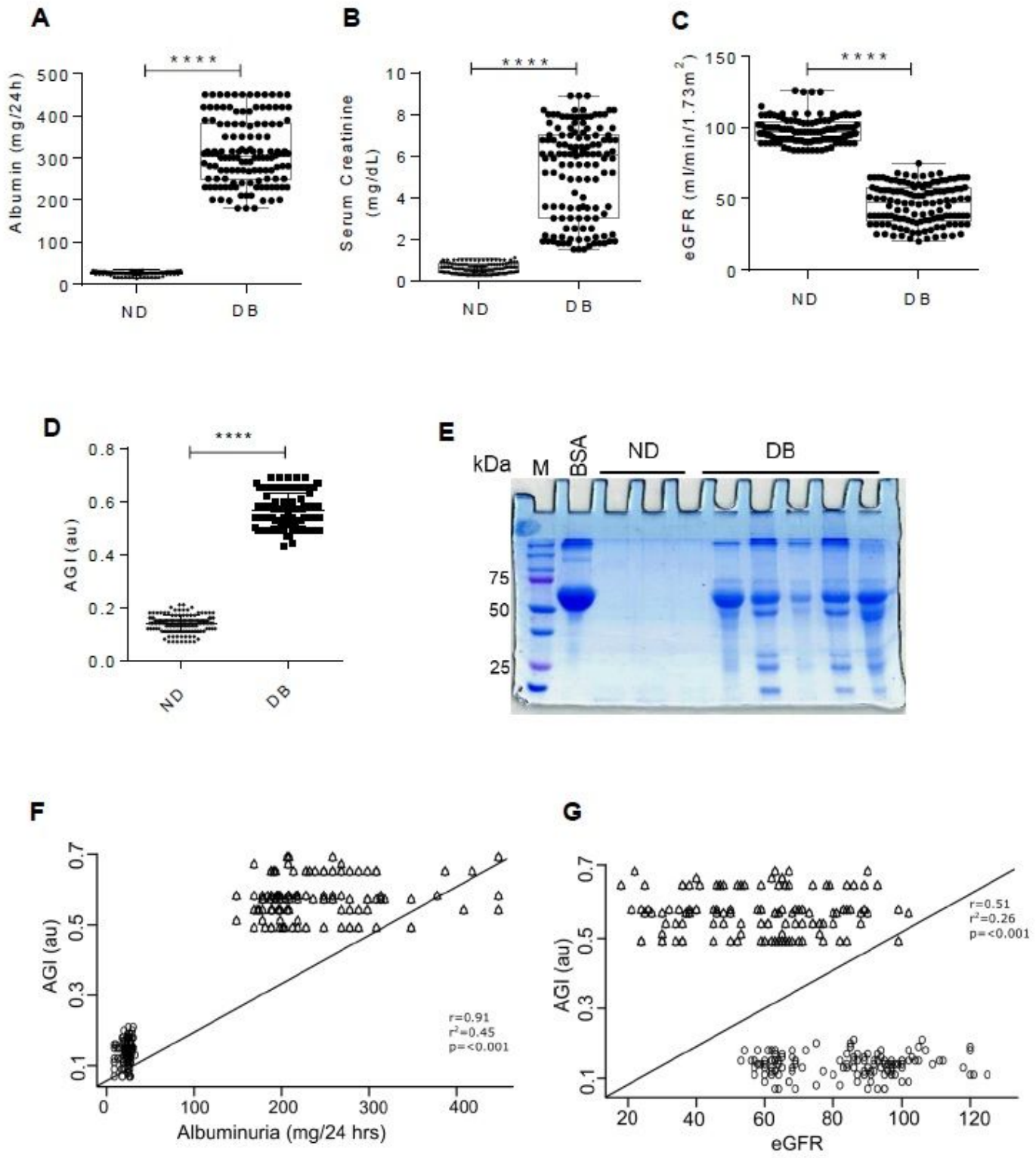

Figure 1

Urinary albumin (A) and serum creatinine (B) was quantified by ELISA in non-diabetic (ND) and diabetic (DB) groups. ****p<0.0001. (C) The estimated glomerular filtration rate (eGFR) was measured in ND and DB groups. ****p<0.001. Non-diabetic (n=130) and diabetic (n=130). (D) Advanced glycation index (AGI) was measured in non-diabetic and diabetic groups as described in methods. ****p<0.001. n-130 in each group. (E) Urine samples from ND and DB groups were resolved on SDS-PAGE and stained with

Coomassie Blue. Bovine serum albumin (BSA) is used as a standard control. M= standard protein marker. Correlation analysis between AGI vs. albuminuria (F) and AGI vs. eGFR (G) in the ND and DB groups. **Correlation is significant at the 0.01 level (2-tailed). Data are the mean± SD, and statistical significance is calculated by using the student t-test.

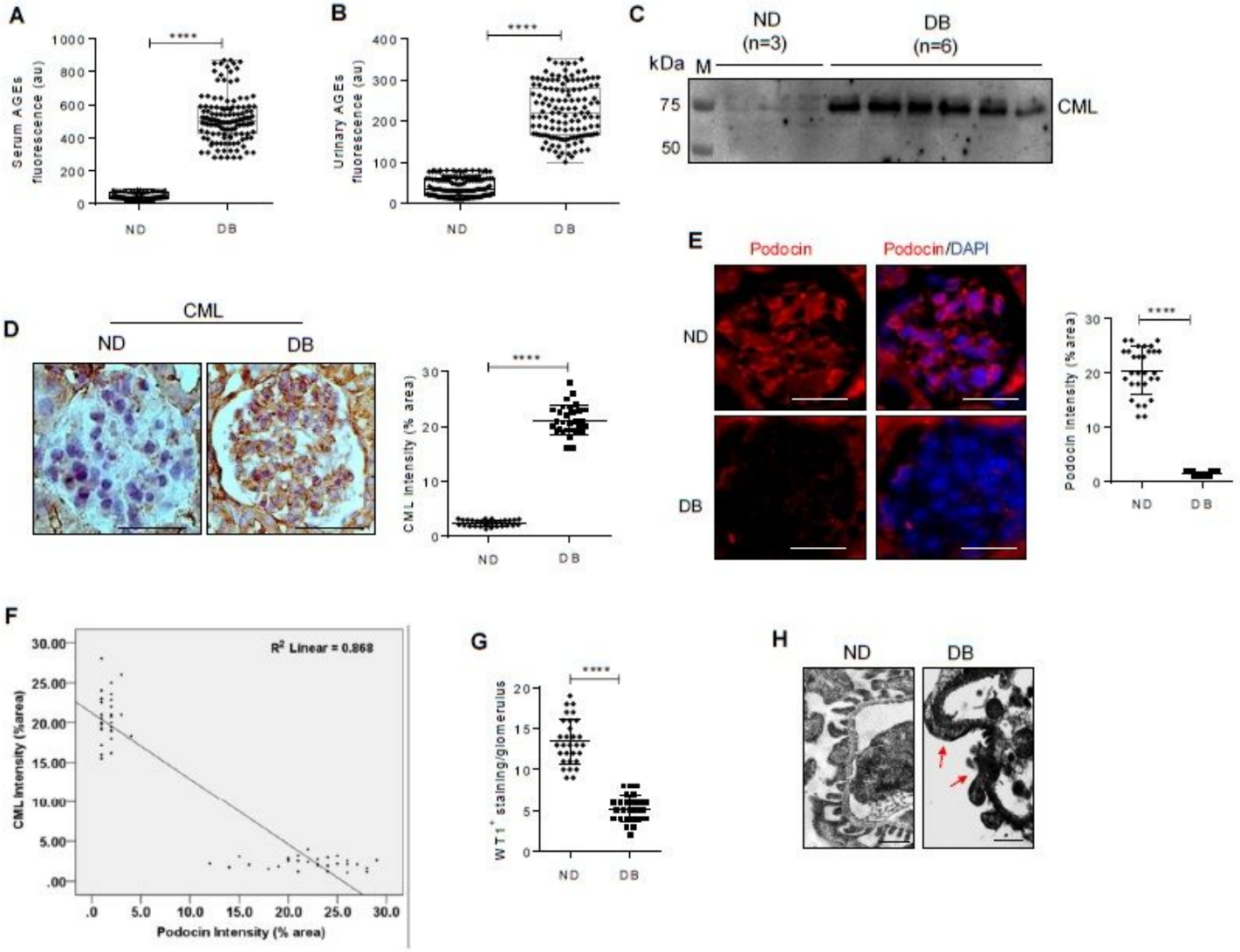

Figure 2

Fluorometric assessment of serum (A) and urinary (B) AGEs in non-diabetic (ND) and diabetic (DB) groups (Ex:370 nm; Em:440 nm). (C) Immunoblotting analysis of CML in serum samples from ND and DB groups. M= standard protein marker. (D) Immunohistochemical analysis of CML in the glomerulus of kidney biopsy samples from ND and DB groups (Left panel). Magnification at x630. Scale bar=20 µm. The percentage of stained area per field from glomerulus were quantified and represented as a dot plot (Right panel). Each data point represents an average value of 6 glomeruli. ****P<0.0001. (E) Immunohistochemical staining for podocin in the glomerulus of ND and DB kidney biopsy samples (Left panel). Magnification at x630. Scale bar=20 µm. The percentage of stained area per field from glomerulus were quantified and represented as a dot plot (Right panel). Each data point represents the

average value of 6 glomeruli from ND and DB groups. ****P<0.0001. The intensity of glomerular expression of CML and podocin were quantified using ImageJ. (F) Correlation analysis of CML vs. podocin staining intensity in the glomerulus of ND and DB groups. Correlation is significant at the 0.01 level (2-tailed). (G) WT1 positive cells were counted from each glomerulus, and each data point represents the average value of 40 glomeruli from ND and DB groups. ****P<0.0001. (H) TEM images of podocytes from ND and DB kidney biopsy samples. The red arrow indicates the loss of foot processes. Scale bars indicate 0.5 μm. Data presented in this figure are the mean± SD, and statistical significance is calculated by using the student t-test.

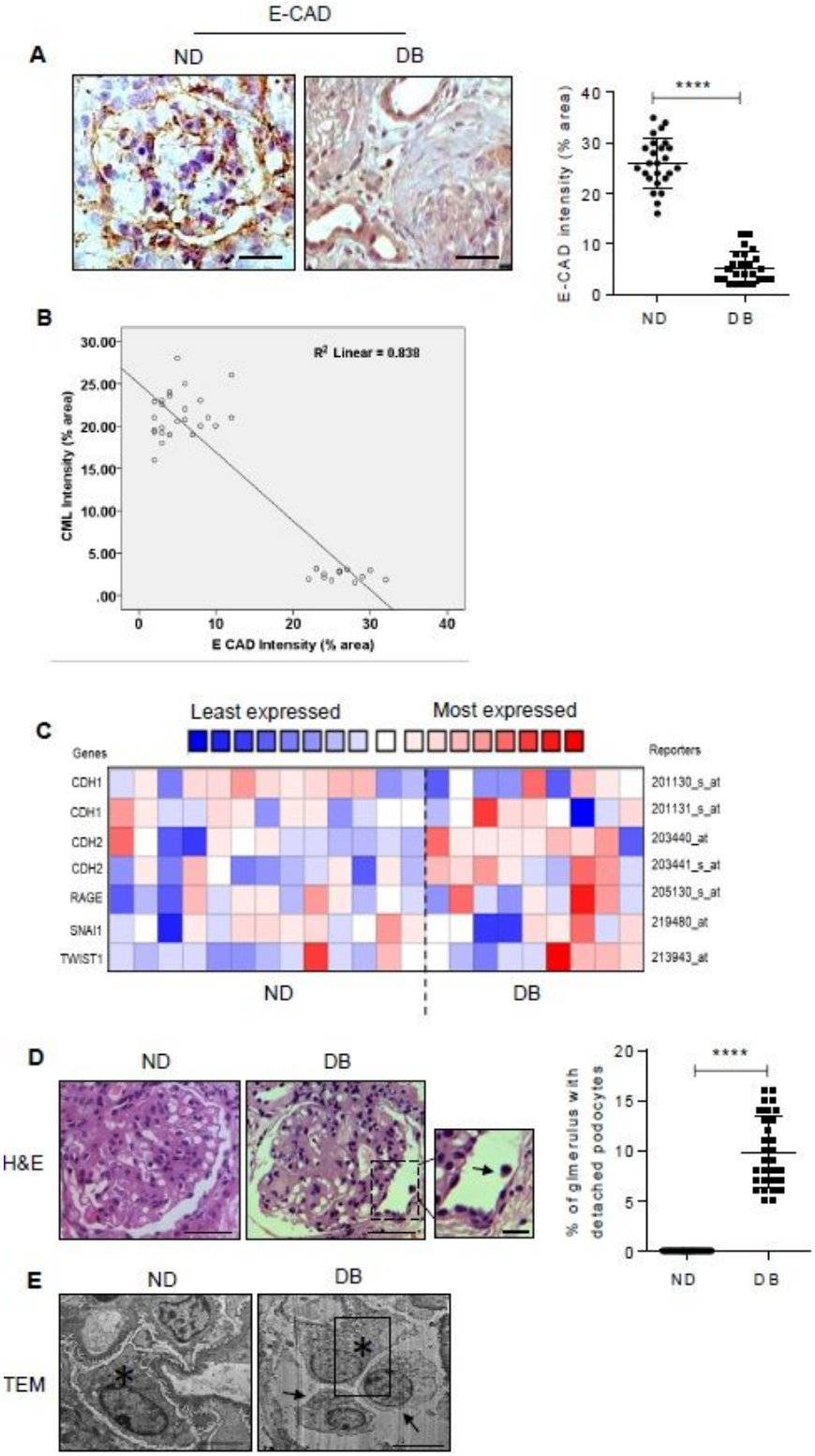

Figure 3

(A) Immunohistochemical analysis of E-cadherin in the kidney biopsies sections from non-diabetic (ND) and diabetic (DB) patients. Magnification at x630. Scale bar=20 µm. The stained area (%) per field from glomerulus was quantified, and each data point represents the average value of 6 glomeruli from ND and DB groups. ****P<0.0001. The intensity of glomerular expression of E-cadherin was quantified using Image J. (B) Correlation between CML vs. E-cadherin expression in the glomeruli from ND and DB biopsy

samples. Correlation is significant at the 0.01 level (2-tailed). (C) Nephroseq (University of Mich-igan O'Brien Renal Center, Michigan, Ann Arbor, MI) analysis comparing CDH1 (E-cadherin), CDH2 (N-cadherin), RAGE, and TWIST expression levels in non-diabetic (n=13) vs. diabetic individuals (n=9) data set from Woroniecka diabetic glomerulus dataset. (D) H&E staining in glomeruli from non-diabetic and diabetic kidney biopsy samples. A zoomed image representing the detached podocyte (black arrow). Magnification at x630. Scale bar=20 µm. Non-diabetic (n=12) and Diabetic (n=25). Detached podocytes were quantified (%), and each data point represents the average value of 60 glomeruli from ND and DB groups. ****P<0.0001. (E) TEM images of podocyte from non-diabetic vs. diabetic kidney biopsy samples. The asterisks indicate attached podocytes with the glomerular basement membrane, and the black arrow indicates detached podocytes into the urinary space. Scale bars indicate 2µm. Data are the mean± SD, and statistical significance is calculated by using the student t-test.

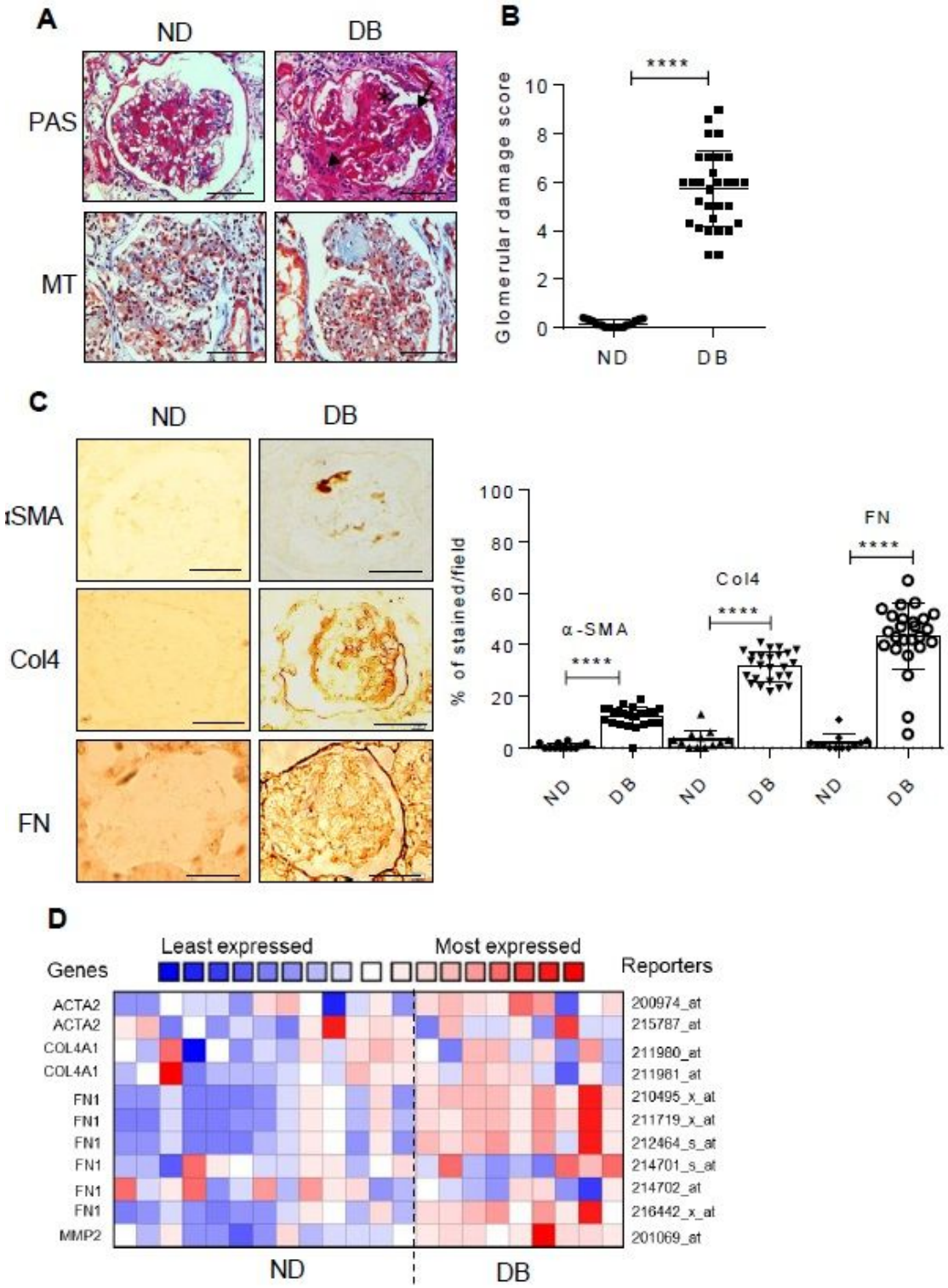

Figure 4

(A) Representative images of PAS and MT staining in glomeruli from non-diabetic (ND) and diabetic (DB) patients. Magnification= x630. Scale bar=20 μm. (B) Glomerular damage score was derived from PAS-stained images by summing the glomerular capillary blockage (black asterisk), adhesion of glo-merular tuft to Bowman's capsule (black arrowhead), and focal segmentation of glomerular tuft (black arrow). Each data point represents the average value of 6 glomeruli from the ND and DB groups. ****p<0.0001.

(C) Representative images of immunohistochemical analysis for Collagen IV, αSMA, and fibronectin in glomerular sections from ND and DB biopsies. Magnification x400. Scale bar=50 μm. The percentage of stained area per field from glomerulus were quantified and represented, and each data point represents the average value of 6 glomeruli from ND and DB groups. ****P<0.0001. The intensity of glomerular expression of each marker was quantified using ImageJ. (D) Nephroseq (Uni-versity of Michigan O'Brien Renal Center, Michigan, Ann Arbor, MI) analysis compar-ing ACTA2(encodes α-SMA), COL4A1 (encodes ColIV) FN1 (encodes FN), and MMP2 (encodes Matrix Metalloprotease2) expression in ND (n=13) vs. DB individuals (n=9). Nephroseq data is acquired from Woroniecka diabetic glomerulus dataset.

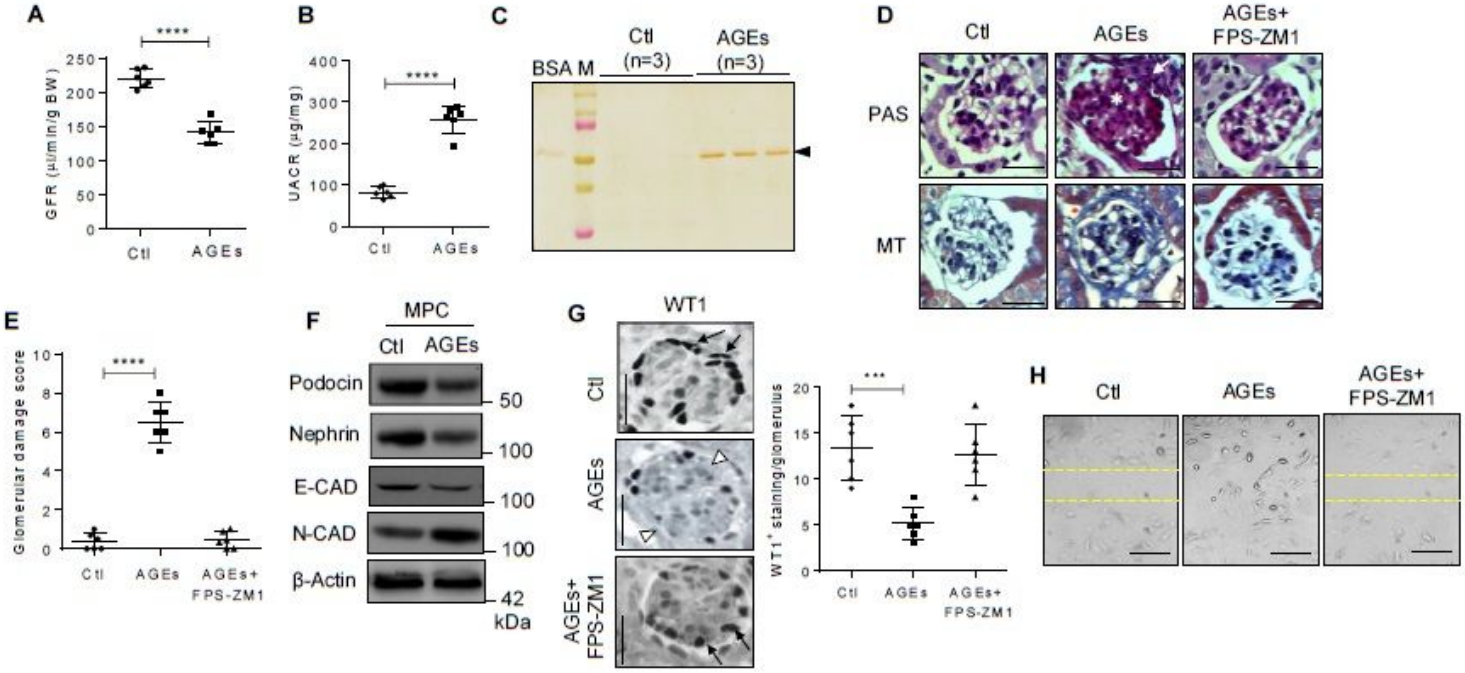

Figure 5

(A) GFR and (B) UACR in control and AGEs administered mice (n=6). ****, p<0.0001. (C) Urinary sam-ples from control and AGEs administered mice were subjected to SDS-PAGE and silver-stained as de-scribed under "Experimental Procedures." BSA was used as a standard. M= standard protein mark-er. (D) Expression of podocin, nephrin, E-cadherin and N-cadherin, and β-Actin in mice podocytes (MPC) from control and AGEs administered mice (n=6). (E) Staining for WT1 (podocyte) in glomerulus from mice from each group (Control, AGEs, and AGEs+FPS-ZM1 (n=6). Scale bars indicate 20 μm. Mag-nification (x630). The red arrow indicates the podocytes, and the white arrowhead indicates the loss of podocytes in the glomerulus (Left panel). An average number of WT1 positive (+) cells from 20 glo-meruli from each group (Right panel). ****p<0.004. (F) PAS and MT staining in glomeruli from control, AGEs, and AGEs+FPS-ZM1 treated mice (n=6). Scale bars indicate 20 μm. Magnification (x630). (G) Glomerular damage score was derived from PAS-stained images by summing the glomeru-lar capillary blockage (white asterisk) and adhesion of glomerular tuft to Bowman's capsule (white ar-row). Each data point represents the average value of 20 glomeruli from a single animal from each group (n=6). ****p<0.0001. (H) A wound-healing assay was performed to determine the extent of human podocytes' motility from

CTL, AGEs, and AGEs+FPS-ZM1 treatment. Images were captured af-ter 0-12 h treatment using an Olympus inverted microscope. Scale bar indicates 100 μm. Magnification=x100.