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

Rajkishor Nishad  
University of Hyderabad School of Life Sciences

Tahaseen V Syed  
Acharya Nagarjuna University

Manga Motrapu  
University of Hyderabad School of Life Sciences

Rajesh Kavvuri  
University of Hyderabad School of Life Sciences

Kiranmayi Kodali  
Acharya Nagarjuna University

Anil Kumar Pasupulati (pasupulati.anilkumar@gmail.com)  
University of Hyderabad School of Life Sciences  https://orcid.org/0000-0001-9467-7650

Original Article

Keywords: Advanced glycation end-products (AGEs), carboxymethyl lysine (CML), podocytes, EMT, proteinuria.

DOI: https://doi.org/10.21203/rs.3.rs-192615/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
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 microalbuminuric 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. 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. It was projected that an increase of 3 million DN cases over the course of 20 years. DN clinical manifestations include glomerular transient hyperfiltration, proteinuria, kidney hypertrophy, fibrosis, and decreased glomerular filtration rate (GFR). 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). 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 markers. Microalbuminuria also indicates generalized endothelial dysfunction and suggests 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.

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. Prolonged hyperglycemia ensures the formation of advanced glycation end-products (AGEs) in the kidney and other sites of diabetic complications. 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. DN patients with macroalbuminuria and patients on hemodialysis had significantly higher serum AGEs than those with microalbuminuria. One of the most widely studied AGEs is carboxymethyl-lysine (CML) and is being used as markers for in vivo formation of AGEs. 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.

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. It is debated whether HbA1c corresponds to the same mean glucose concentrations in people with ESKD. Further, HbA1c is influenced by several factors, including the RBCs' lifespan, administration of erythropoietin, uremic environment, and blood transfusions. In contrast, glycated albumin (GA) is suggested as a preferred marker for assessing glycemic control in advanced chronic kidney disease only. 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. 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. 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), antivimentin (#PAB040Hu01), anti-collIV (#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 (m²). 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°C 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. 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 with 1× 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 Gatan ultrascans CCD camera (Gatan Inc, Pleasanton, CA) 2K×2K resolution and 120kV.

**Preparation of glucose-derived AGEs:** Glucose derived AGEs were prepared as reported earlier. 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. 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.

**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 ßSMA, 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. Serum levels of AGEs not only associate with the severity of diabetic complications, including retinopathy and nephropathy but also predict mortality. In addition to predicting the risk of diabetic complications, Luft et al. reported that circulating CML levels predict the risk of developing diabetes. Each 100 ng/ml increment in CML, the risk of developing diabetes increases by 35% in individuals with impaired fasting glucose. 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 associated with HOMA-IR index. 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. At the same time CML modifications of proteins are predominant AGEs that accumulate in vivo. Elevated serum CML levels were observed in patients with kidney failure. Enhanced CML accumulation was observed in glomerular nodular lesions from patients with DN. AGEs-RAGE interaction elicits cellular injury by producing reactive oxygen species, activating profibrotic and proinflammatory cascades. 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.

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. A recent study showed that CML induced Notch signaling in podocytes contributing to their EMT. Administration of AGEs elicits decreased podocyte count in mice. 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. 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. 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**

**Acknowledgments:**

The authors thank the patients and their families for their kind support to conduct this study. The authors thank Dr. Bhanuprakash Reddy (NIN, ICMR) for donating Anti-AGEs and Anti-CML antibodies. The authors also thank the Indian Council of Medical Research and the Department of Health Research, India for funding AKP.

**Disclosure:**

The authors declare no conflict of interest.

**References**
1  Hu, F. B. Globalization of diabetes: the role of diet, lifestyle, and genes. *Diabetes Care* **34**, 1249-1257, doi:10.2337/dc11-0442 (2011).

2  Kumar, P. A. *et al.* Carboxymethyl lysine induces EMT in podocytes through transcription factor ZEB2: Implications for podocyte depletion and proteinuria in diabetes mellitus. *Archives of biochemistry and biophysics* **590**, 10-19, doi:10.1016/j.abb.2015.11.003 (2016).

3  de Boer, I. H. *et al.* Temporal trends in the prevalence of diabetic kidney disease in the United States. *JAMA* **305**, 2532-2539, doi:10.1001/jama.2011.861 (2011).

4  Kowalski, A., Krikorian, A. & Lerma, E. V. Diabetic nephropathy for the primary care provider: new understandings on early detection and treatment. *Ochsner J* **14**, 369-379 (2014).

5  Gluhovschi, C. *et al.* Urinary Biomarkers in the Assessment of Early Diabetic Nephropathy. *J Diabetes Res* **2016**, 4626125, doi:10.1155/2016/4626125 (2016).

6  Bash, L. D., Selvin, E., Steffes, M., Coresh, J. & Astor, B. C. Poor glycemic control in diabetes and the risk of incident chronic kidney disease even in the absence of albuminuria and retinopathy: Atherosclerosis Risk in Communities (ARIC) Study. *Archives of internal medicine* **168**, 2440-2447, doi:10.1001/archinte.168.22.2440 (2008).

7  Fioretto, P. *et al.* Renal protection in diabetes: role of glycemic control. *Journal of the American Society of Nephrology : JASN* **17**, S86-89, doi:10.1681/ASN.2005121343 (2006).

8  Vallon, V. & Komers, R. Pathophysiology of the diabetic kidney. *Compr Physiol* **1**, 1175-1232, doi:10.1002/cphy.c100049 (2011).

9  Vlassara, H. & Uribarri, J. Advanced glycation end products (AGE) and diabetes: cause, effect, or both? *Curr Diab Rep* **14**, 453, doi:10.1007/s11892-013-0453-1 (2014).

10 Kumar Pasupulati, A., Chitra, P. S. & Reddy, G. B. Advanced glycation end products mediated cellular and molecular events in the pathology of diabetic nephropathy. *Biomol Concepts* **7**, 293-309, doi:10.1515/bmc-2016-0021 (2016).

11 Luevano-Contreras, C. & Chapman-Novakofski, K. Dietary advanced glycation end products and aging. *Nutrients* **2**, 1247-1265, doi:10.3390/nu2121247 (2010).

12 Shimoike, T. *et al.* The meaning of serum levels of advanced glycosylation end products in diabetic nephropathy. *Metabolism* **49**, 1030-1035, doi:10.1053/meta.2000.7738 (2000).

13 Ahmed, N. Advanced glycation endproducts–role in pathology of diabetic complications. *Diabetes Res Clin Pract* **67**, 3-21, doi:10.1016/j.diabres.2004.09.004 (2005).
14 Ahmed, N. & Thornalley, P. J. Quantitative screening of protein biomarkers of early glycation, advanced glycation, oxidation and nitrosation in cellular and extracellular proteins by tandem mass spectrometry multiple reaction monitoring. *Biochem Soc Trans* **31**, 1417-1422, doi:10.1042/bst0311417 (2003).

15 Beisswenger, P. J. *et al.* Formation of immunochromatographic advanced glycosylation end products precedes and correlates with early manifestations of renal and retinal disease in diabetes. *Diabetes* **44**, 824-829, doi:10.2337/diab.44.7.824 (1995).

16 Genuth, S. *et al.* Glycation and carboxymethyllysine levels in skin collagen predict the risk of future 10-year progression of diabetic retinopathy and nephropathy in the diabetes control and complications trial and epidemiology of diabetes interventions and complications participants with type 1 diabetes. *Diabetes* **54**, 3103-3111, doi:10.2337/diabetes.54.11.3103 (2005).

17 Vos, F. E., Schollum, J. B. & Walker, R. J. Glycated albumin is the preferred marker for assessing glycaemic control in advanced chronic kidney disease. *NDT Plus* **4**, 368-375, doi:10.1093/ndtplus/sfr140 (2011).

18 Inaba, M. *et al.* Glycated albumin is a better glycemic indicator than glycated hemoglobin values in hemodialysis patients with diabetes: effect of anemia and erythropoietin injection. *J Am Soc Nephrol* **18**, 896-903, doi:10.1681/ASN.2006070772 (2007).

19 Peacock, T. P. *et al.* Comparison of glycated albumin and hemoglobin A(1c) levels in diabetic subjects on hemodialysis. *Kidney Int* **73**, 1062-1068, doi:10.1038/ki.2008.25 (2008).

20 Ng, J. M., Jennings, P. E., Laboi, P. & Jayagopal, V. Erythropoetin treatment significantly alters measured glycated haemoglobin (HbA1c). *Diabet Med* **25**, 239-240, doi:10.1111/j.1464-5491.2007.02336.x (2008).

21 Vos, F. E. *et al.* Red blood cell survival in long-term dialysis patients. *Am J Kidney Dis* **58**, 591-598, doi:10.1053/j.ajkd.2011.03.031 (2011).

22 Group, U. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes *Lancet* **352**, 837-853 (1998).

23 Sampathkumar, R., Balasubramanyam, M., Rema, M., Premanand, C. & Mohan, V. A novel advanced glycation index and its association with diabetes and microangiopathy. *Metabolism* **54**, 1002-1007, doi:10.1016/j.metabol.2005.02.017 (2005).

24 Nishad, R., Meshram, P., Singh, A. K., Reddy, G. B. & Pasupulati, A. K. Activation of Notch1 signaling in podocytes by glucose-derived AGEs contributes to proteinuria. *BMJ Open Diabetes Res Care* **8**, doi:10.1136/bmjdrd-2020-001203 (2020).
25 Nishad, R., Mukhi, D., Tahaseen, S. V., Mungamuri, S. K. & Pasupulati, A. K. Growth hormone induces Notch1 signaling in podocytes and contributes to proteinuria in diabetic nephropathy. *The Journal of biological chemistry* **294**, 16109-16122, doi:10.1074/jbc.RA119.008966 (2019).

26 Shankland, S. J. The podocyte's response to injury: role in proteinuria and glomerulosclerosis. *Kidney Int* **69**, 2131-2147, doi:10.1038/sj.ki.5000410 (2006).

27 Singh, R., Barden, A., Mori, T. & Beilin, L. Advanced glycation end-products: a review. *Diabetologia* **44**, 129-146, doi:10.1007/s001250051591 (2001).

28 Vlassara, H. & Striker, G. E. Advanced glycation endproducts in diabetes and diabetic complications. *Endocrinol Metab Clin North Am* **42**, 697-719, doi:10.1016/j.ecl.2013.07.005 (2013).

29 Kilhovd, B. K. *et al.* Increased serum levels of advanced glycation endproducts predict total, cardiovascular and coronary mortality in women with type 2 diabetes: a population-based 18 year follow-up study. *Diabetologia* **50**, 1409-1417, doi:10.1007/s00125-007-0687-z (2007).

30 Goh, S. Y. & Cooper, M. E. Clinical review: The role of advanced glycation end products in progression and complications of diabetes. *J Clin Endocrinol Metab* **93**, 1143-1152, doi:10.1210/jc.2007-1817 (2008).

31 Kizer, J. R. *et al.* Advanced glycation/glycoxidation endproduct carboxymethyl-lysine and incidence of coronary heart disease and stroke in older adults. *Atherosclerosis* **235**, 116-121, doi:10.1016/j.atherosclerosis.2014.04.013 (2014).

32 Semba, R. D. *et al.* Advanced glycation end products and their circulating receptors predict cardiovascular disease mortality in older community-dwelling women. *Aging Clin Exp Res* **21**, 182-190, doi:10.1007/BF03325227 (2009).

33 Luft, V. C. *et al.* Carboxymethyl lysine, an advanced glycation end product, and incident diabetes: a case-cohort analysis of the ARIC Study. *Diabet Med* **33**, 1392-1398, doi:10.1111/dme.12963 (2016).

34 Uribarri, J. *et al.* Circulating glycotoxins and dietary advanced glycation endproducts: two links to inflammatory response, oxidative stress, and aging. *J Gerontol A Biol Sci Med Sci* **62**, 427-433, doi:10.1093/gerona/62.4.427 (2007).

35 Tahara, N. *et al.* Serum levels of advanced glycation end products (AGEs) are independent correlates of insulin resistance in nondiabetic subjects. *Cardiovasc Ther* **30**, 42-48, doi:10.1111/j.1755-5922.2010.00177.x (2012).

36 Kislinger, T. *et al.* N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. *J Biol Chem* **274**, 31740-31749, doi:10.1074/jbc.274.44.31740 (1999).
37  Schleicher, E. D., Wagner, E. & Nerlich, A. G. Increased accumulation of the glycoxidation product N(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest* **99**, 457-468, doi:10.1172/JCI119180 (1997).

38  Degenhardt, T. P. *et al*. Technical note. The serum concentration of the advanced glycation end-product N epsilon-(carboxymethyl)lysine is increased in uremia. *Kidney Int* **52**, 1064-1067, doi:10.1038/ki.1997.429 (1997).

39  Horie, K. *et al*. Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions. Implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. *J Clin Invest* **100**, 2995-3004, doi:10.1172/JCI119853 (1997).

40  Yan, S. D. *et al*. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* **269**, 9889-9897 (1994).

41  Yukie Kitajima, S. U., Takashi Hosono, Satoshi Yoshikawa, Yuzuru Sato, Toru Hyodo Glycated hemoglobin and glycated albumin in patients with diabetes undergoing hemodiafiltration. *Renal Replacement Therapy* **6**, 10, doi:https://doi.org/10.1186/s41100-020-0260-5 (2020).

**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/1.73m²)                          | 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.