Advanced Glycation End-Products Predict Loss of Renal Function and Correlate with Diabetic Nephropathy Lesions in American Indians with Type 2 Diabetes

Running title: AGEs and Kidney Disease in Type 2 Diabetes

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We examined associations of advanced glycation end-products (AGEs) with renal function loss (RFL) and its structural determinants in American Indians with type 2 diabetes. Data were from a 6-year clinical trial which assessed renoprotective efficacy of losartan. Participants remained under observation after the trial concluded. Glomerular filtration rate (GFR) was measured annually. Kidney biopsies were performed at the end of the trial. Five AGEs were measured in serum collected at enrollment and at kidney biopsy. RFL was defined as ≥40% decline of measured GFR from baseline. Of 168 participants (mean baseline age 41 years, HbA_{1c} 9.2%, GFR 164 ml/min, and ACR 31 mg/g), 104 reached the RFL endpoint during median follow-up of 8.0 years. After multivariable adjustment, each doubling of carboxyethyl lysine (hazard ratio [HR]=1.60, 95% CI 1.08 to 2.37) or methylglyoxal hydroimidazolone (HR=1.30, 95% CI 1.02 to 1.65) concentration was associated with RFL. Carboxyethyl lysine, carboxymethyl lysine, and methylglyoxal hydroimidazolone correlated positively with cortical interstitial fractional volume (partial r=0.23, *P*=0.03, partial r=0.25, *P*=0.02, and partial r=0.31, *P*=0.003, respectively). Glyoxyl hydroimidazolone and methylglyoxal hydroimidazolone correlated negatively with total filtration surface per glomerulus (partial r=-0.26, *P*=0.01 and partial r=-0.21, *P*=0.046, respectively). AGEs improve prediction of RFL and its major structural correlates.

Keywords: Advanced Glycation End-Product, Biomarkers, Renal Function, Renal Structure, Type 2 Diabetes.

INTRODUCTION

Diabetic kidney disease (DKD) is a common complication of type 1 and type 2 diabetes, and the leading cause of end-stage renal disease (ESRD) worldwide. Early assessment of DKD is difficult because currently available biomarkers do not identify those at risk with sufficient accuracy. Persistently elevated urinary albumin/creatinine ratios (ACR) may return to normal and structural lesions within the kidneys may be present in the absence of currently available clinical indicators (1-3). Thus, better non-invasive biomarkers are needed which can identify individuals who are at increased risk for DKD even before irreversible structural changes occur.

The complications of diabetes are caused chiefly by persistent hyperglycemia, and intensive glycemic control slows development and progression of the microvascular complications (4,5) including the structural lesions of DKD (6). Hyperglycemia is thought to induce diabetes complications, in part, through non-enzymatic glycation of proteins and the production of advanced glycation end-products (AGEs) and related oxidative end-products (OPs) (7), with both glycation and oxidation playing roles in the genesis of these adducts. AGEs are produced by non-enzymatic glycation of amino groups on proteins by reducing sugars and dicarbonyl compounds. Glycoxidation adducts are formed by a combination of glycation and oxidation, are more chemically reactive, and are more likely to irreversibly cross-link proteins. AGEs and glycoxidation products are present in higher concentrations among people with diabetes compared with healthy individuals and are thought to increase oxidative stress *via* interaction with their receptor (RAGE), thereby promoting vascular complications (8).

AGEs are filtered through the glomerulus and reabsorbed by renal proximal tubules. Both their clearance and tubular reabsorption are complex and variable (9,10). AGEs accumulate in

glomeruli where they increase expression of type-IV collagen and laminin in the extra-cellular matricies (11) and induce irreversible cross-linked protein formations (12). In the proximal tubule they induce premature cell senescence (13). AGEs are signal-transducing ligands for RAGE, a transmembrane receptor. This interaction induces cellular injury through the production of reactive oxygen species and activation of proinflammatory and profibrotic cascades (14,15). Most of the existing RAGE data have used carboxymethyl lysine as the ligand, and it remains to be established which of the dicarbonyl-derived AGEs binds specifically to RAGE.

We reported previously that the highly reactive α-dicarbonyls methylglyoxal and 3-deoxyglucosone are associated with more rapid progression of DKD (16) and that blood levels of AGEs derived from methylglyoxal correlate positively with biopsy documented histological progression of DKD in type 1 diabetes (17). In the present study, we examined the clinical utility of glycation, glycoxidation, and oxidative end-products as predictors of renal function loss (RFL) and evaluated their association with the histological lesions of DKD.

RESEARCH DESIGN AND METHODS

Study Subjects and Design

From 1965-2007, Pima Indians from the Gila River Indian Community participated in a longitudinal study of diabetes and its complications. We invited 169 adults with type 2 diabetes from this population to participate in a randomized clinical trial testing the renoprotective efficacy of losartan in early DKD (NCT00340678) (18). Ninety-one participants had normal urinary albumin excretion (albumin/creatinine ratio [ACR] <30 mg/g) at baseline and 78 had

persistent microalbuminuria (ACR=30-299 mg/g). Glomerular filtration rate (GFR) was measured annually by the urinary clearance of iothalamate. At the end of the six-year clinical trial, 111 of the participants underwent percutaneous kidney biopsy (60 with normoalbuminuria and 51 with microalbuminuria at baseline) to determine whether treatment was associated with preservation of kidney structure. Among participants with microalbuminuria at baseline, those who received losartan had, on average, lower mesangial fractional volume than those who received placebo, suggesting that losartan preserved a key aspect of glomerular structure in DKD (19). However, losartan did not significantly affect the primary outcome, *i.e.*, decline in GFR, during the 6-year trial period (18). Upon completion of the clinical trial, participants were returned to the care of their primary physicians and annual research examinations that included measurement of GFR were continued for a median of 7.9 years (3.0 – 9.8 years).

In the present study, we measured AGEs/OPs in serum samples stored at -80°C since collection from the baseline visit of the clinical trial and in up to four subsequent research examinations, each two years apart. Samples were collected under standardized conditions and stored at -80°C, undergoing only one freeze-thaw cycle prior to assay. The fourth set of AGE/OP measurements obtained at the 6th year of the clinical trial was only available in subjects with a kidney biopsy. Of the 169 participants in the clinical trial, all but one had baseline samples available for measurement of AGEs/OPs. Thus, 168 participants had baseline AGE/OP measurements and were included in the present study; 95 of whom had a kidney biopsy and an AGE/OP measurement at the time of the biopsy (**Supplemental Figure 1**).

Vital status and development of ESRD were ascertained independently in all study participants through December 31, 2015. ESRD was defined by the initiation of renal

replacement therapy or death from diabetic kidney disease if the participant refused dialysis. Underlying causes of death for the participants who died before reaching the outcomes of interest were determined from death certificates. This study was approved by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases. Each subject signed an informed consent document.

Clinical and Anthropometric Measures

Blood pressure was measured while the subject was resting in the seated position; mean arterial pressure (MAP) was calculated as (2×diastolic blood pressure + systolic blood pressure)/3. Glycated hemoglobin (HbA_{1c}) was measured by high performance liquid chromatography. This method was also used to measure the concentration of non-radioactive iothalamate for GFR determination (11). Urinary albumin concentration was measured by nephelometric immunoassay and urinary creatinine by a modified Jaffé reaction (Siemens, Erlangen, Germany). Urinary albumin concentration below the detection limit of the assay (≤6.8 mg/L) was set to 6.8 mg/L in the analyses.

Biomarker Analytes

AGEs and OPs were measured in serum samples by liquid chromatography–mass spectrometry utilizing internal stable heavy isotope substituted standards. Analysis was performed in a blinded fashion on the serum filtrate following centrifugation through 10K cut-off Amicon™ filters. This fraction contains free AGEs and OPs, as well as peptides of varied sizes, and our analytical method measured the free products. An Agilent model 6490 Triple

Quadrupole MS System with a 1290 Rapid Resolution LC System was used. Concurrent quantitative measurement was performed using a single Waters X-select HSS T3 2.5 um x 2.1 x 150 mm column with a mobile phase of methanol/water gradient with 0.20% heptafluorobutyric acid and a total analysis time of 19 minutes. Seven biomarkers were measured: five dicarbonyl-derived AGE compounds—carboxymethyl lysine, carboxyethyl lysine, glyoxal hydroimidazolone, methylglyoxal hydroimidazolone, and 3-deoxyglucosone hydroimidazolone, and two OP compounds—methionine sulfoxide and 2-aminoadipic acid. Reproducibility of the biomarker assays was assessed by intra-class correlation of measurements from 20 duplicate samples blinded to the performance laboratory. Intra-class correlation for carboxymethyl lysine was 0.93, for carboxyethyl lysine was 0.86, for glyoxal hydroimidazolone was 0.76, for methylglyoxal hydroimidazolone was 0.80, for 3-deoxyglycosone hydroimidazolone was 0.76, for methionine sulfoxide was 0.60, and for 2-aminoadipic acid was 0.83, reflecting good-to-excellent agreement.

Morphometric Methods

Unbiased random sampling of tissue sections from the kidney biopsy provided digital images for study using quantitative morphometric methods to estimate renal structural parameters (18). Measurements were performed by an investigator (EJW) who was masked to the clinical data. Twelve predefined renal parameters were assessed: glomerular basement membrane (GBM) width, cortical interstitial fractional volume, mesangial fractional volume per glomerulus, glomerular filtration surface density, total filtration surface per glomerulus, number of non-podocyte cells (endothelial + mesangial cells) per glomerulus, number of podocytes per

glomerulus, podocyte foot process width, percent podocyte detachment, and percentage of normally fenestrated endothelium (18). An equation was used to calculate the percentage of globally sclerotic glomeruli that accounts for the difference in size and therefore in the probability of encountering a sclerotic or nonsclerotic glomerulus in a random cross-section (20). An average ± standard deviation (SD) of 14±6 glomeruli were examined in each participant by light microscopy and 3±1 by electron microscopy for the morphometric measurements. Morphometric variables for each individual were calculated as the mean of all measurements for that individual.

Statistical Analysis

Patient characteristics were expressed as mean ± SD, median (interquartile range [IQR]), or n (%). Pearson correlations were used to assess the relationship of biomarkers with each other and with clinical variables. For regression analyses, we log(2)-transformed all biomarker concentrations and morphometric variables with skewed distributions. The primary endpoint in the longitudinal analyses was RFL as defined by a decline in GFR during follow-up of ≥40% compared with the baseline value. This endpoint was recently recommended for use in clinical trials of kidney disease (21), and we reported previously that structural lesions strongly predicted this outcome (22). Follow-up continued until the last available research examination; 43 (41%) RFL endpoints occurred during the clinical trial and 61 (59%) during post-trial follow-up. Five of 26 participants who developed ESRD did not have their GFR drop sufficiently to meet the criteria for RFL by their last research examination, so they were included in the non-RFL group in the analyses. Cox proportional hazards regression was used to assess the effect of each

AGE/OP measured at baseline on the risk of RFL. A time-dependent Cox model was also examined to determine if serial measurement of AGEs/OPs (baseline, year two, and year four of the trial) improved the models ability to predict RFL. A univariate model and three multivariate models were considered. The first multivariate model (Model A) was adjusted for age, sex, treatment assignment during the clinical trial, diabetes duration, $HbA_{1c,}$ and MAP; model B was adjusted for model A + GFR; model C was adjusted for model B + ACR. We tested each model for log-linearity, and proportionality assumptions were met by each covariate when using cumulative sums of Martingale residuals. To assess the extent to which AGE/OPs enhanced prediction of RFL, generalized c-statistics were calculated for model C accounting for variable follow-up times (23). Comparisons between nested models that included or excluded the analyte of interest were assessed by likelihood ratios tests (24,25). In addition, the relative integrated discrimination improvement (rIDI) index was calculated to assess the improvement in 10-year RFL risk prediction of each biomarker in addition to traditional DKD risk factors (26); the 10-year risk was selected as it approximates the median follow-up time for the RFL outcome. The 95% CIs for the rIDIs were computed based on 10,000 bootstrap samples. To determine if treatment assignment during the clinical trial modified the relationship between AGEs/OPs and RFL, we tested the interaction of treatment assignment with each AGE/OP.

We performed several sensitivity analyses related to RFL to determine if our findings were robust to competing risk factors, different endpoint definitions, loss to follow-up, or to the effect of treatment with renin-angiotensin system (RAS) inhibitors, which are known to affect the rate of GFR loss. In the first analysis, we used the competing risk model of Fine and Gray to estimate the subdistribution hazard ratios for RFL, while accounting for the competing risk of

pre-RFL deaths (27). In the second analysis, we computed individual biomarker means and slopes as time-averaged rates of change by simple linear regression on all biomarker measurements in the 143 individuals who had AGE/OP measurements available at all baseline, year two, and year four examinations. We then assessed the effect of the mean and slope for each AGE/OP on RFL. Since the slope was computed from the AGE/OP measurements made at enrollment, year two, and year four, the considering year four examination was considered as the "baseline" examination, and these regression analyses were adjusted for age, sex, diabetes duration, MAP, HbA_{1c}, GFR and ACR measured atthis examination. In the third analysis, GFR measured at each research examination at which the participant was treated with a renin-angiotensin system (RAS) inhibitor was adjusted upward by 3.75% to account for the acute effects of initiating treatment with RAS inhibitors, as previously described (22). In the fourth analysis, we substituted the pre-specified primary endpoint from the clinical trial trial (decline in GFR to ≤60 ml/min or to half the baseline value in those who entered the study with a GFR <120 ml/min) for RFL defined by a ≥40% decline in GFR from baseline (18). During the post-trial follow-up, adherence to annual research examinations declined, and 5 participants progressed to ESRD without documentation of reaching the RFL endpoint at a research examination. To avoid the bias (informative censoring) that occurs when loss to follow-up is related to the study outcome, we performed the fifth and sixth analyses. In the fifth analysis, the five individuals who developed ESRD without reaching the RFL endpoint at a research examination were included as cases of RFL, with the last research examination considered as the onset of RFL. In the sixth analysis, we used linear imputation to estimate the date of onset of the study outcomes. To

estimate the date of onset of the RFL, a linear GFR slope was computed in each participant based on the last two GFR values with the last GFR value defined as follows:

- In participants who did not reach the RFL endpoint, the GFR measured at their last examination;
- In participants who reached the RFL endpoint at an examination, the GFR value measured at that examination;
- In participants who progressed to ESRD without a GFR measurement indicating that they had reached the RFL endpoint, a GFR of zero was assigned as of the date of onset of renal replacement therapy.

The estimated date of onset of the RFL endpoint was then imputed for all participants from the GFR slope, with follow-up continued for each participant for two years after the last measured GFR, or until the RFL endpoint, death, or December 31, 2015, whichever came first. This approach permitted us to determine whether a participant who missed scheduled visits and did not reach the RFL endpoint by their last examination would have done so if they had remained under observation. The 2-year follow-up interval was selected because it represented the median time interval between the last GFR measurement and the onset of ESRD in the study cohort.

Linear regression models were used to assess the relationships between biomarkers measured near the time of the kidney biopsy and renal structural variables. We did not include all previous measures of AGEs/OPs in this analysis, since we considered the measure proximate to the biopsy to be the best measure of lifetime exposure at the time of biopsy. Only the univariate model, and models A and B were considered for this analysis; Model C was not considered since albuminuria is highly correlated with the underlying structural lesions. We also tested the

interaction of treatment assignment with each AGE for the linear regression models. Model fit was assessed for normality and leverage by 'Studentized' residuals, and for multicollinearity using eigenvalues and the condition index (28). Associations between AGEs and morphometric measures were illustrated graphically by partial Pearson correlation coefficients and partial residual regression plots. Statistical analyses were performed with SAS version 9.3 (SAS Institute, Cary, NC). *P* values <0.05 were considered statistically significant.

RESULTS

The 168 participants had a mean age of 41.4±10.6 years, median diabetes duration 8.9 (6.2 to 14.9) years, mean HbA_{1c} 9.2±2.3%, mean GFR 164±42 ml/min, and median ACR 31 (13 to 76) mg/g. Clinical and biological characteristics of the participants at baseline are summarized in **Table 1**. AGEs correlated with one another (r=0.42 to 0.74, *P*<0.001 for all) whereas OPs correlated weakly with themselves (r=0.13, *P*=0.09) and in some cases with AGEs (r=0.03 to 0.29, *P*<0.001 to 0.70). AGEs correlated negatively with baseline GFR and positively with baseline age and diabetes duration (**Table 2**). 2-aminoadipic acid correlated positively with age and BMI, whereas methionine sulfoxide and glyoxal hydroimidazolone correlated negatively with HbA_{1c}. From baseline to year four, methionine sulfoxide, carboxymethyl lysine, methylglyoxal hydroimidazolone and 3-deoxyglucosone hydroimidazolone increased significantly in those with measurements at all three time points (*P*<0.0001, *P*=0.0001, *P*=0.004 and *P*=0.002, respectively). In the subset of 95 patients who underwent kidney biopsy, all serum AGE/OP concentrations except for 2-aminoadipic acid (*P*=0.10) had increased significantly by

year six ($P<0.001$). Losartan treatment assignment did not affect the rise in AGE/OP concentrations.

AGEs and Renal Function Loss

During a median follow-up of 8.0 (4.9 to 13.1) years, the primary endpoint of RFL (≥40% decline in GFR) occurred in 104 (62%) of the participants. In addition, 24 participants died before reaching the RFL endpoint; five from malignancy, four from cardiovascular disease, four from alcoholic liver disease, five from other natural causes, and three from external causes—death certificates were not yet available for the three remaining deaths). In univariate Cox models, a higher concentration of methylglyoxal hydroimidazolone at baseline was associated with a higher risk of RFL (**Table 3**). After multivariable adjustment (Model C), carboxyethyl lysine (HR per doubling=1.60, 95% CI 1.08 to 2.37) and methylglyoxal hydroimidazolone (HR=1.30, 95% CI 1.02 to 1.65) each predicted RFL. Adjustment for both GFR and ACR strengthened the association between these compounds and RFL, in comparison with models A and B. The c-statistic from model C for predicting the primary endpoint was 0.672, when neither an AGE nor OP was included in the model. The addition of methylglyoxal hydroimidazolone to the model C covariates alone increased the c-statistic to 0.680 (difference in c-statistic=0.008, P=0.02; rIDI=14.9%, 95% CI 1.7 to 51.8, $P = 0.04$) and the addition of carboxyethyl lysine increased the c-statistic to 0.682 (difference in c-statistic=0.010, P=0.02; rIDI=13.4%, 95% CI -1.1 to 47.8, $P = 0.10$). There was no interaction between losartan treatment assignment and AGEs/OPs in any of the models. None of the AGEs/OPs predicted RFL in the time-dependent Cox models that took all available measurements of AGEs/OPs into account.

Sensitivity Analyses for AGEs and Renal Function Loss

After accounting for the competing risk of mortality, the subdistribution hazard ratios for baseline methylglyoxal hydroimidazolone and carboxymethyl lysine were strengthened and for carboxyethyl lysine was attenuated (**Table 3**). In the subset of 143 participants with complete data for AGE/OP measurements at enrollment, year two, and year four examinations, 93 (65%) developed RFL. When the mean value of all 3 AGE/OP measurements and the slope of the three measurements were included in model C, mean methylglyoxal hydroimidazolone was significantly associated with the risk of RFL (HR=1.55, 95% CI 1.01 to 2.39) after accounting for the slope of methylglyoxal hydroimidazolone. Adjustment for the acute effects of RAS-inhibitor use on GFR had no effects on the results.

Supplemental Table 1 shows the results of the remaining sensitivity analyses. When the five participants who developed ESRD but did not meet the GFR criteria for RFL were included as cases of RFL, carboxymethyl lysine, in addition to carboxyethyl lysine and methylglyoxal hydroimidazolone, each predicted RFL in model C (HR=1.44, 95% CI=1.02 to 2.04; HR =1.68, 95% CI=1.14 to 2.46; and HR=1.32, 95% CI=1.05 to 1.67, respectively). When RFL was ascertained by linear imputation, the same 109 participants reached the RFL endpoint, and the findings were equivalent to the primary analyses. When we reanalyzed the data using the pre-specified primary GFR outcome from the clinical trial (decline in GFR to ≤60 ml/min or to half the baseline value in those who entered the study with a GFR <120 ml/min), 37 (22%) participants reached this endpoint, and 3-deoxyglucosone hydroimidazolone was predictive in

model C in addition to carboxyethyl lysine and methylglyoxal hydroimidazolone, which predicted the RFL outcome defined by a ≥40% decline in GFR.

AGEs and Renal Structure

Characteristics of the 95 participants who underwent a kidney biopsy and for whom AGE/OP concentrations were available near the time of biopsy are presented in **Table 4**. Forty-nine (52%) of those who underwent kidney biopsy had ACR<30 mg/g and 46 (48%) had ACR=30-299 mg/g at enrollment into the clinical trial; 54 (57%) were assigned to receive losartan (**Supplemental Figure 1**). AGE/OP concentrations were measured in serum samples obtained a median of 85 (IQR=43 to 87) days from the kidney biopsy. In univariate models (**Supplemental Table 2**), all dicarbonyl-derived compounds were significantly and positively correlated with the proportion of globally sclerotic glomeruli (r=0.25 to 0.33, *P*<0.001 to 0.02), mesangial fractional volume per glomerulus (r= 0.25 to 0.30, *P*<0.001 to 0.01), and cortical interstitial fractional volume (r=0.25 to 0.38, *P*=0.003 to 0.02). All AGEs, with the exception of 3-deoxyglucosone hydroimidazolone, were negatively associated with total filtration surface per glomerulus (r= -0.34 to -0.26; *P*<0.001 to 0.01). Glomerular volume was negatively associated with carboxyethyl lysine and methylglyoxal hydroimidazolone (r=-0.22, *P*=0.03 and r=-0.21, *P*=0.04, respectively). Filtration surface density was negatively associated with glyoxal hydroimidazolone (r=-0.24, *P*=0.02). Fenestrated endothelium was negatively associated with carboxymethyl lysine (r=-0.25, *P*= 0.02). Podocyte foot process width was positively associated with glyoxal hydroimidazolone (r=0.21, *P*= 0.04). One participant had a foot process width 6.5 standard deviations above the mean; removal of this outlier strengthened the relationship with

glyoxal hydroimidazolone (r=0.29, *P*= 0.004). Neither of the OPs was associated with any structural measures.

Although multivariate adjustment (Models A and B) attenuated many of the observed relationships, all dicarbonyl-derived AGEs remained associated with at least one morphometric parameter after full adjustment. In model B, carboxyethyl lysine and methylglyoxal hydroimidazolone were positively correlated with cortical interstitial fractional volume (partial r=0.27, *P*=0.01, and partial r=0.28, *P*=0.008, respectively) and carboxymethyl lysine, carboxyethyl lysine and methylglyoxal hydroimidazolone with mesangial fractional volume (partial r=0.25, *P*=0.02, partial r=0.23, *P*=0.03 and partial r=0.31, *P*=0.003, respectively). Glyoxyl hydroimidazolone and methylglyoxal hydroimidazolone were negatively correlated with total filtration surface per glomerulus (partial r=-0.26, *P*=0.01 and partial r=-0.21, *P*=0.05, respectively). 3-deoxyglucosone hydroimidazolone was positively correlated with percentage of globally sclerotic glomeruli (partial r=0.30, *P*=0.005). Finally, carboxymethyl lysine was negatively associated with the percentage of fenestrated endothelium. The partial correlations of each AGE/OP with relevant structural variables are shown in **Figure 1**. For illustration, partial residual regression plots of methylglyoxal hydroimidazolone with total filtration surface per glomerulus and cortical interstitial fractional volume are shown in **Figure 2**. We found no interactions between losartan treatment assignment and any of the AGEs/OPs, indicating that the relationship between these biomarkers and the morphometric variables was not modified by treatment.

DISCUSSION

In Pima Indians with type 2 diabetes and early-stage DKD, serum AGEs predicted RFL and correlated with the severity of DKD lesions associated with RFL. Both carboxyethyl lysine and methylglyoxal hydroimidazolone predicted RFL, and methylglyoxal hydroimidazolone significantly improved the accuracy of RFL prediction when considered in addition to traditional renal risk factors. These results were consistent across several definitions of renal function decline. In addition, several AGEs were associated with the severity of DKD lesions, including increased cortical interstitial fractional volume, mesangial fractional volume, decreased total filtration surface area per glomerulus, podocyte foot process width, and the percentage of endothelial cell surface with fenestrations. We reported previously that the early decline of GFR in type 1 diabetes is primarily related to classical DKD glomerular lesions (29,30). Tubulointerstitial lesions are critically important in the progression of DKD from moderately reduced GFR to end-stage renal disease (29,31). Hence, increased concentrations of the AGEs appear to be associated with both the initiation and progression of DKD. Measuring AGEs in renal tissue may more accurately reflect production and accumulation in the kidneys. Nevertheless, the serum measures used in the present study predicted RFL and its structural determinants and may provide a non-invasive means to more precisely determine risk of progressive DKD than traditional risk factors alone.

These results expand previous work showing a cross-sectional relationship between serum AGEs and renal function in dialyzed or renal transplanted diabetic patients by demonstrating a relationship with RFL in early DKD (32). They also accord with prior studies showing higher AGE concentrations associated with worsening renal structural lesions in patients with type 1 diabetes and either normal (17) or elevated urinary albumin excretion (16).

We previously showed that in a group of 45 Pima Indians, elevated methylglyoxal, the precursor of carboxyethyl lysine and methylglyoxal hydroimidazolone, was associated with increased GBM width and reduced podocyte number per glomerulus (16). In the same study we found that oxidative compounds were not associated with worsening of renal structural lesions, which is consistent with the present study, where neither methionine sulfoxide nor 2-aminioadipic acid was associated with RFL or any morphometric variables. While the dicarbonyl-derived AGE species we measured were individually associated with different structural variables, all AGEs were consistently associated with signs of greater structural damage commonly attributed to diabetes (33). The morphometric parameters associated with AGEs have previously been related to renal function loss in type 1 (34) and type 2 diabetes (22,35).

All AGE concentrations increased during follow up. This increase was expected, as AGEs accumulate with aging (36) and increased diabetes duration (37), and AGE clearance is reduced by renal impairment (38). In the present study, all AGEs correlated positively with age and diabetes duration and negatively with GFR. We believe that declining renal function with attendant decreases in the renal clearance of these AGEs (39) was predominantly responsible for their increasing concentrations seen during follow-up and is responsible for the time-dependent model not enhancing the prediction of RFL. The rising concentrations of AGEs/OPs attributable to decreased renal clearance may confound the biological processes that link AGEs with DKD in persons with normal renal function. Nevertheless, the progressive retention of AGEs that occurs with declining renal function may create a vicious cycle of kidney damage that accelerates the decline in renal function in the later stages of DKD. Negative correlations of methionine sulfoxide and glyoxal hydroimidazolone with HbA_{1c} are consistent with the observation that

AGE/OP formation is partly genetically determined and not entirely dependent on high ambient glycemia (40,41). Multiple glycation pathways in diabetes could produce discrepant levels of end-products, and adaptive mechanisms that alter processing of AGEs/OPs could also play a role (42).

Some drugs may affect AGE production or clearance. Among the angiotensin II receptor blockers, olmesartan (43) and valsartan (44,45) decrease AGE concentrations, whereas irbesartan does not (46,47). Despite losartan's known effect on AGE concentrations in an animal model (48), in the present study losartan treatment did not affect the relationship between AGE/OP concentrations and RFL or renal structural lesions, suggesting the absence of a class effect. Biguanides, especially metformin, may also decrease AGE concentrations via an indirect effect by lowering glucose and by reacting directly with dicarbonyl adducts (49). Seventy five percent of our study participants reported taking metformin, but 93% of those had only intermittent exposure during the study period.

The strengths of this study include an extended follow-up period, multiple measurements of AGE/OP concentrations over time, a detailed renal phenotype based on serial measures of GFR by the urinary clearance of iothalamate, and measures of kidney structure based on standardized unbiased random sampling morphometric methods. Samples were collected under standardized conditions and stored at -80°C, undergoing only one prior freeze-thaw cycle. We previously showed that AGE/OP concentrations were stable when stored under these conditions (17), and the assay used to measure these samples was robust and highly reproducible (50). In addition, a large proportion of the studied population reached the functional study endpoint, giving us good statistical power to examine the effects of AGEs/OPs on RFL. The conclusions of

the study were largely unchanged when different definitions of GFR decline were used in the analysis. Increased loss to follow-up as kidney disease progressed in the study participants was a potential limitation. This could lead to differential misclassification of the GFR outcome if participants with RFL were more likely to miss research examinations at which the GFR was measured. Sensitivity analyses suggest that bias attributable to this factor had little effect on the findings of the study. Another limitation is the single kidney biopsy at the end of the clinical trial, which did not allow us to assess structural changes over time. We recently completed a second kidney biopsy on a subset of the study participants and plan to explore the effects of AGEs/OPs on the structural changes of DKD when these data become available.

In conclusion, a number of serum AGEs in American Indians with type 2 diabetes and early DKD are associated with specific renal lesions of DKD and the loss of renal function that occurs in the presence of those lesions. These findings persisted after adjusting for traditional risk factors. Additionally, methylglyoxal hydroimidazolone improved the accuracy of predicting RFL over traditional renal risk factors, although the magnitude of improvement was modest. These results suggest that some serum AGEs may be useful biomarkers for progressive DKD and may play an active pathophysiological role in its development.

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No potential conflicts of interest relevant to this article were reported. S.H. is employed by PreventAGE Healthcare, where the assays of AGEs and OPs were performed and P.J.B. has a financial interest in the Company.

P.J.S. researched data and wrote the manuscript. K.W. researched data and reviewed and edited the manuscript. S.H. researched data and reviewed and edited the manuscript. E.J.W. researched data and reviewed and edited the manuscript. S.K.T. researched data, reviewed and edited the manuscript. W.C.K. researched data, reviewed and edited the manuscript, and contributed to the discussion. K.V.L. researched data, reviewed and edited the manuscript, and contributed to the discussion. M.M. researched data, reviewed and edited the manuscript, and contributed to the discussion. B.Y. researched data and reviewed and edited the manuscript. R.G.N. researched data and wrote the manuscript. P.J.B. researched data and wrote the manuscript. R.G.N. and P.J.B. are the guarantors of this work and, as such, had full access to all

the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. We thank all participants of the study. We express our gratitude to Dr. R. Hanson (NIDDK, Phoenix, AZ) for his statistical help and critical comments.

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TABLE 1. Clinical characteristics and concentrations of advanced glycation end-products and oxidative end-products at the onset of the clinical trial.

Variables	***n*=168**
Clinical Characteristics	
Age (years)	41.4 ± 10.6
Male: *n* (%)	46 (27%)
Losartan treatment group *n* (%)	83 (49%)
Body mass index (kg/m^2)	35.7 ± 8.4
Diabetes duration (years)	8.9 (6.2 – 14.9)
Systolic blood pressure (mm/Hg)	118 ± 13
Diastolic blood pressure (mm/Hg)	76 ± 8
HbA_{1c} (%) [mmol/mol]	9.2 ± 2.3 [77.0 ± 25.1]
Glomerular filtration rate (ml/min)	164 ± 42
Urinary albumin/creatinine ratio (mg/g)	30.6 (13.5 – 76.3)
Biomarker concentrations	
Methionine sulfoxide (nmol/L)	684 (581 – 803)
2-aminoadipic acid (nmol/L)	862 (659 – 1098)
Carboxymethyl lysine (nmol/L)	59 (46 – 74)
Glyoxal hydroimidazolone (nmol/L)	7.3 (6.5 – 8.6)
Carboxyethyl lysine (nmol/L)	45 (35 – 58)
Methylglyoxal hydroimidazolone (nmol/L)	71 (47 – 106)
3-deoxyglucosone hydroimidazolone (nmol/L)	194 (160 – 290)

Quantitative variables are described by mean ± standard deviation or median (interquartile range) and qualitative variables by number (%).

TABLE 2. Pearson correlations of advanced glycation end-products and oxidative end-products with clinical variables at enrollment in the clinical trial.

Clinical Variables	MetSO	2-AAA	CML	GH1	CEL	MGH1	3DGHI
Age	0.12 (0.13)	**0.21 (0.007)**	**0.33 (<0.001)**	**0.42 (<0.001)**	**0.31 (<0.001)**	**0.31 (<0.001)**	**0.35 (<0.001)**
Sex	0.08 (0.28)	0.11 (0.16)	0.05 (0.54)	0.17 (0.03)	0.11 (0.16)	0.09 (0.23)	0.23 (0.003)
Diabetes Duration	0.06 (0.44)	0.01 (0.94)	**0.22 (0.004)**	**0.25 (0.001)**	**0.17 (0.02)**	**0.22 (0.003)**	**0.20 (0.01)**
BMI	0.09 (0.24)	**0.21 (0.006)**	0.03 (0.68)	0.11 (0.15)	0.01 (0.90)	-0.07 (0.35)	**-0.16 (0.04)**
HbA_{1c}	**-0.24 (0.002)**	0.05 (0.53)	-0.02 (0.76)	**-0.24 (0.002)**	-0.10 (0.21)	-0.03 (0.70)	-0.08 (0.32)
MAP	0.03 (0.73)	-0.02 (0.81)	0.02 (0.84)	0.09 (0.25)	0.01 (0.94)	0.03 (0.71)	0.15 (0.05)
GFR	-0.04 (0.63)	0.09 (0.25)	**-0.22 (0.004)**	**-0.28 (<0.001)**	**-0.29 (<0.001)**	**-0.19 (0.01)**	**-0.24 (0.002)**
ACR	-0.07 (0.34)	-0.01 (0.95)	0.003 (0.96)	0.02 (0.79)	-0.0001 (0.999)	-0.0001 (0.98)	0.03 (0.65)

P-values <0.05 are shown in bold.

BMI, body mass index, MAP = mean arterial pressure; GFR = glomerular filtration rate; ACR = urinary albumin/creatinine ratio; MetSO = methionine sulfoxide; 2-AAA = 2-aminoadipic acid; CML = carboxymethyl lysine; GH1 = glyoxal hydroimidazolone; CEL = carboxyethyl lysine; MGH1 = methylglyoxal hydroimidazolone; 3DGHI = 3deoxyglucosone hydroimidazolone

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TABLE 3. Cox proportional hazards model for the risk of >40% decline in GFR from baseline associated with a doubling of the serum concentration of advanced glycation end-products and oxidative end-products.

Variable	Univariate	Model A	Model B	Model C
Cox models				
MetSO	0.77 (0.46-1.31) *P=0.34*	0.98 (0.57-1.70) *P=0.94*	0.93 (0.54-1.62) *P=0.80*	0.97 (0.56-1.69) *P=0.88*
2-AAA	0.95 (0.66-1.39) *P=0.80*	0.96 (0.63-1.46) *P=0.83*	0.82 (0.55-1.24) *P=0.35*	0.82 (0.55-1.22) *P=0.38*
CML	1.35 (0.99-1.85) *P=0.06*	1.30 (0.91-1.85) *P=0.15*	1.38 (0.97-1.96) *P=0.07*	1.39 (0.97-1.98) *P=0.06*
GH1	0.96 (0.59-1.58) *P=0.88*	1.00 (0.54-1.85) *P=0.998*	1.27 (0.69-2.32) *P=0.45*	1.29 (0.70-2.38) *P=0.40*
CEL	1.29 (0.93-1.80) *P=0.12*	1.35 (0.92-1.97) *P=0.13*	**1.59 (1.07-2.35)** ***P=0.02***	**1.60 (1.08-2.37)** ***P=0.01***
MGH1	**1.26 (1.03-1.56)** ***P=0.03***	**1.27 (1.00-1.61)** ***P= 0.046***	**1.28 (1.01-1.63)** ***P=0.04***	**1.30 (1.02-1.65)** ***P=0.03***
3DGHI	1.21 (0.93-1.58) *P=0.15*	1.16 (0.86-1.57) *P=0.32*	1.20 (0.88-1.64) *P=0.25*	1.21 (0.88-1.64) *P=0.21*
Fine and Gray competing risk models*				
MetSO	0.61 (0.37-1.01) *P=0.05*	0.82 (0.50-1.33) *P=0.41*	0.78 (0.48-1.28) *P=0.33*	0.78 (0.48-1.29) *P=0.33*
2-AAA	0.99 (0.68-1.43) *P=0.94*	1.03 (0.70-1.52) *P=0.89*	0.96 (0.64-1.44) *P=0.84*	0.96 (0.64-1.43) *P=0.83*
CML	1.33 (0.99-1.79) *P=0.06*	1.37 (0.97-1.92) *P=0.07*	**1.44 (1.02-2.03)** ***P=0.04***	**1.47 (1.05-2.06)** ***P=0.03***
GH1	0.92 (0.57-1.49) *P=0.73*	1.10 (0.59-2.04) *P=0.76*	1.33 (0.73-2.41) *P=0.35*	1.34 (0.73-2.43) *P=0.34*
CEL	1.24 (0.8-1.73) *P=0.22*	1.30 (0.88-1.92) *P=0.18*	1.40 (0.95-2.07) *P=0.09*	1.40 (0.95-2.08) *P=0.09*
MGH1	**1.29 (1.05-1.58)** ***P=0.01***	**1.34 (1.08-1.67)** ***P= 0.009***	**1.36 (1.10-1.69)** ***P=0.004***	**1.38 (1.11-1.71)** ***P=0.004***
3DGHI	1.13 (0.89-1.43) *P=0.31*	1.18 (0.93-1.49) *P=0.17*	1.24 (0.96-1.60) *P=0.11*	1.23 (0.95-1.64 *P=0.11*

Data are presented as hazard ratio (95% confidence interval). Hazard ratios are given per doubling of the AGE variable. Pvalues <0.05 are shown in bold.

*competing risk = all-cause death

Model A was adjusted for age, sex, treatment assignment, diabetes duration, HbA_{1c} and mean arterial

pressure.
Model B was adjusted for Model A covariates + glomerular filtration rate.
Model C was adjusted for Model B covariates + albumin/creatinine ratio.
MetSO = methionine sulfoxide; 2-AAA = 2-aminoadipic acid; CML = carboxymethyl lysine; GH1 = glyoxal hydroimidazolone; CEL = carboxyethyl lysine; MGHI = methylglyoxal hydroimidazolone; 3DGHI = 3deoxyglucosone hydroimidazolone
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TABLE 4. Clinical characteristics and concentrations of advanced glycation end-products and oxidative end-products at the time of biopsy. Morphometric characteristics from the biopsy are also shown.

Clinical Characteristics	***n*=95**
Age (years)	46.1 ± 9.9
Male: *n* (%)	26 (27%)
Losartan treatment group *n* (%)	54 (57%)
Body mass index (kg/m^2)	36.1 ± 8.2
Diabetes duration (years)	14.2 (11.3 – 19.9)
Systolic blood pressure (mm/Hg)	124 ± 17
Diastolic blood pressure (mm/Hg)	78 ± 10
HbA_{1c} (%) [mmol/mol]	9.3 ± 2.0 [78.0 ± 21.9]
Glomerular filtration rate (ml/min)	144 ± 60
Urine albumin/creatinine ratio (mg/g)	36.1 (12.1 – 110.7)
Biomarker Concentrations	
Methionine sulfoxide (nmol/L)	947 (812 – 1075)
2-aminoadipic acid (nmol/L)	776 (646 – 1018)
Carboxymethyl lysine (nmol/L)	74 (59 – 100)
Glyoxal hydroimidazolone (nmol/L)	8.2 (6.5 – 9.9)
Carboxyethyl lysine (nmol/L)	54 (43 – 76)
Methylglyoxal hydroimidazolone (nmol/L)	113 (65 – 190)
3-deoxyglucosone hydroimidazolone (nmol/L)	300 (209 – 430)
Morphometric Characteristics	
Mean glomerular volume (x10^{6}µm^3)	5.7 (4.7-6.9)
Global glomerular sclerosis (%)	5.6 (0.0-18.7)
Glomerular filtration surface density (µm^2/µm^3)	0.08 (0.06-0.09)
Total filtration surface per glomerulus (x10^5 µm^2)	4.0 (3.2-5.5)
Glomerular basement membrane width (nm)	500 (413-584)
Mesangial fractional volume (%)	18.0 (13.3-23.4)
Cortical interstitial fractional volume (%)	30.0 (24.4-33.4)
Podocyte number per glomerulus	620 (462-766)
Foot process width (nm)	458 (402-524)
Podocyte detachment (%)	0.4 (0.0-1.5)
Fenestrated endothelium (%)	27.4 (22.4-33.0)

Quantitative variables are described by mean ± standard deviation or median (interquartile range) and qualitative variables by number (%).

FIGURE LEGENDS

FIGURE 1. Partial correlations of advanced glycation end-products and oxidative end-products with renal structural variables. Partial Pearson correlation coefficients are shown on the y-axis for three different models: unadjusted (open bars), model A (hatched bars), and model B (black bars). Model A was adjusted for age, sex, losartan treatment assignment, diabetes duration, HbA_{1c}, and mean arterial pressure. Model B was adjusted for Model A covariates + glomerular filtration rate. * $P<0.05$.

FIGURE 2. Partial regression residual plots of methylglyoxal hydroimidazolone concentration and renal structural variables. The residuals were computed from regressing each of these variables on age, sex, diabetes duration, HbA_{1c}, treatment assignment, mean arterial pressure and GFR. Methylglyoxal hydroimidazolone, total filtration surface, and cortical fractional interstitial volume are shown on a log base 2 scale. Pearson's partial r and the corresponding *P*-value are shown. TFS = total filtration surface; V_VInt = cortical interstitial fractional volume.

Figure 1.

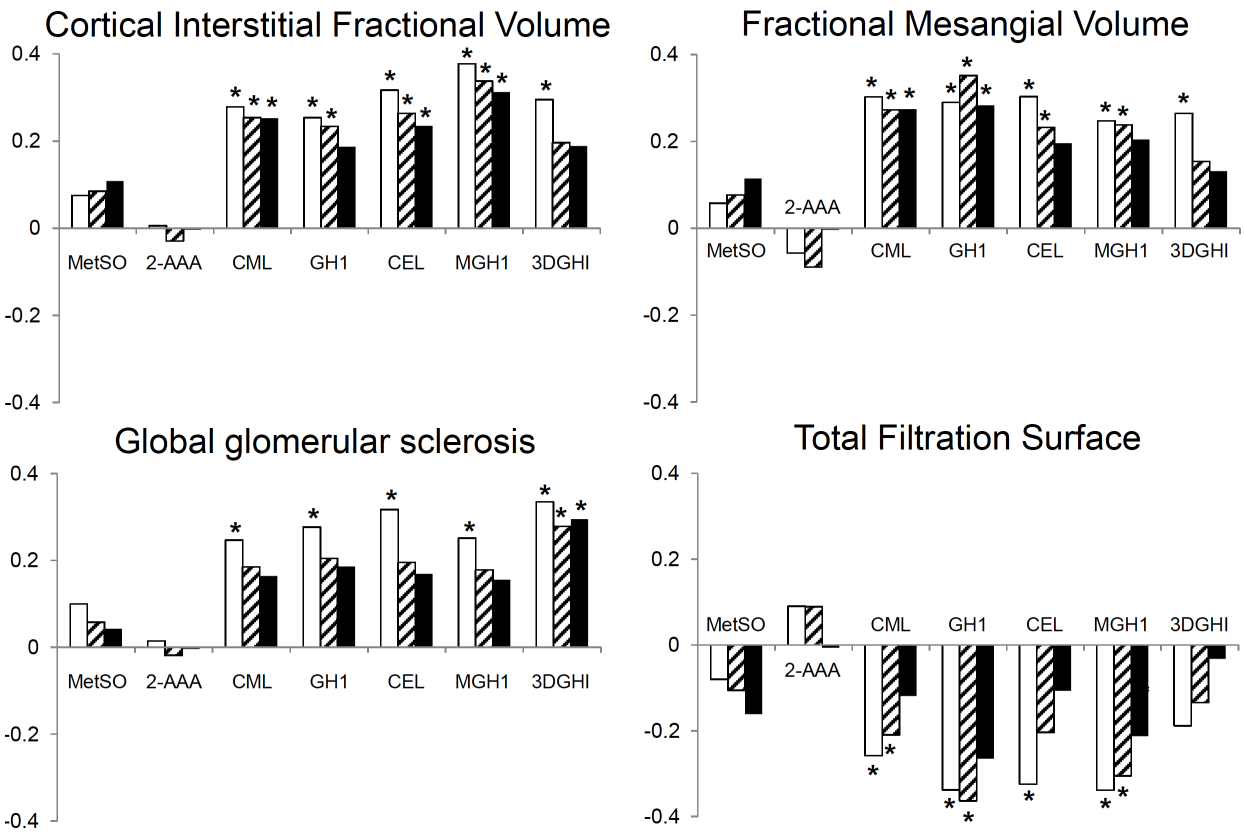

Cortical Interstitial Fractional Volume
Fractional Mesangial Volume
Global glomerular sclerosis
Total Filtration Surface
0.4
0.2
0
-0.2
-0.4
MetSO
2-AAA
CML
GH1
CEL
MGH1
3DGHI

Figure 2 A

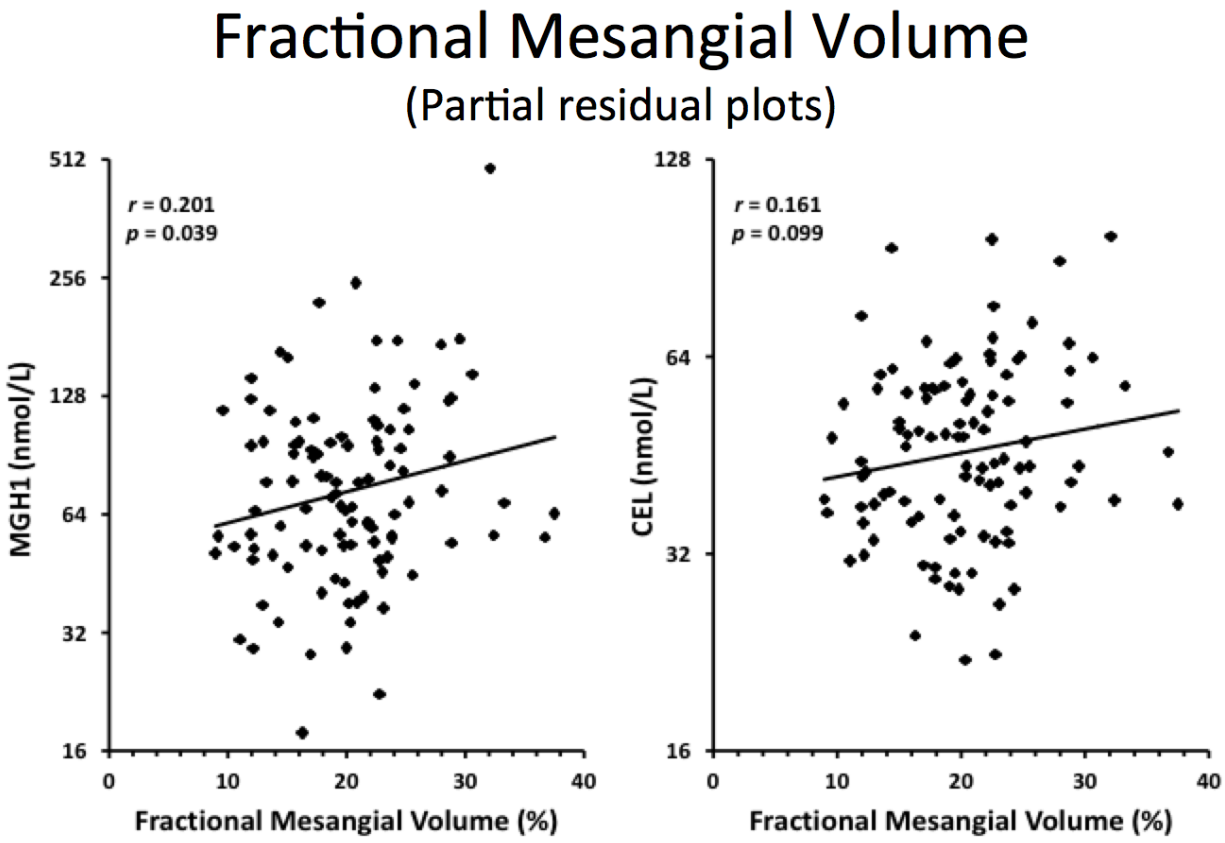
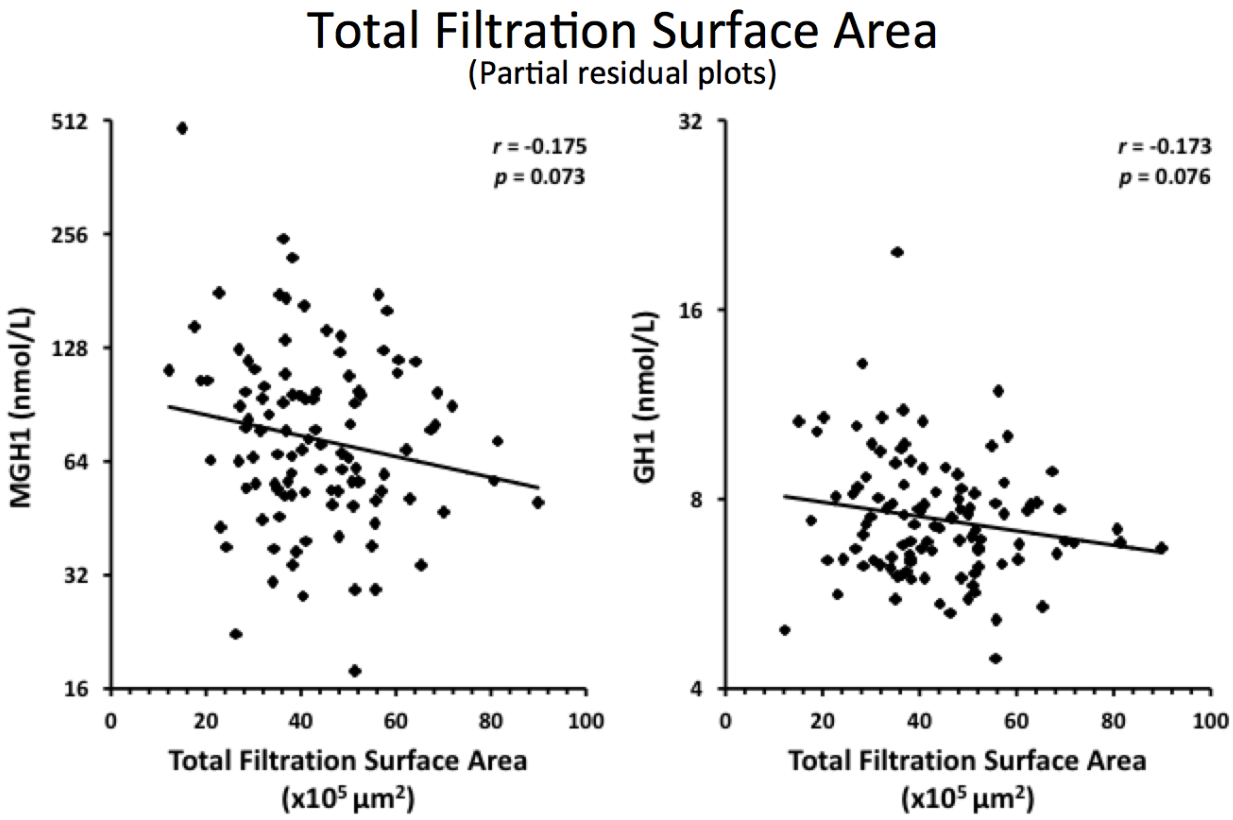

Fractional Mesangial Volume
(Partial residual plots)
MGH1 (nmol/L)
512
256
128
64
32
16
r = 0.201
p = 0.039
0
10
20
30
40
Fractional Mesangial Volume (%)
CEL (nmol/L)
128
64
32
16
r = 0.161
p = 0.099
0
10
20
30
40
Fractional Mesangial Volume (%)

Figure 2 B



Adjusted for age, sex, treatment assignment, diabetes duration, HbA1c, MAP, GFR, and ACR