A Hyaluronan Synthesis Inhibitor Delays the Progression of Diabetic Kidney Disease in A Mouse Experimental Model

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Key Points
- Nonfasting plasma glucose positively correlates with hyaluronan levels in kidneys.
- Hyaluronan content in kidneys positively correlates with urine albumin-creatinine ratio.
- Hyaluronan synthesis inhibitor, 4-methylumbelliferone, slows the progression of diabetic kidney disease.

Abstract
Background The role of hyaluronan (HA) in the development and progression of diabetic kidney disease (DKD), and the precise mechanisms and consequences of HA involvement in this pathology are still to be clarified.

Methods In this study, we assayed the effects of the HA synthesis inhibitor 4-methylumbelliferone (4-MU) on the development of DKD. Diabetic type 2 model mice (eNOS−/−C57BLKS/J) were fed artificial diets containing 5% 4-MU or not for 9 weeks. Plasma glucose, GFR, albumin-creatinine ratio (ACR), and biomarkers of kidney function and systemic inflammation were measured at baseline and after treatment. Diabetic nephropathy was further characterized in treated and control mice by histopathology.

Results Treated animals consumed a daily dose of approximately 6.2 g of 4-MU per kg of body weight. At the end of the experimental period, the 4-MU supplemented diet resulted in a significant decrease in nonfasting plasma glucose (516; interquartile range, 378–1170; versus 1149; interquartile range, 875.8–1287 mg/dl, P=0.05) and a trend toward lower HA kidney content (5.6±1.5 versus 8.8±3.1 ng/mg of kidney weight, P=0.07) compared with the control diet, respectively. Diabetic animals treated with 4-MU showed significantly higher GFR and lower urine ACR and plasma cystatin C levels than diabetic controls. Independent histologic assessment of DKD also demonstrated a significant decrease in mesangial expansion score and glomerular injury index in 4-MU-treated mice compared with controls. Plasma glucose showed a strong correlation with kidney HA levels (r=0.66, P=0.01). Both total hyaluronan (r=0.76, P=0.007) and low molecular weight hyaluronan content (r=0.64, P=0.04) in the kidneys correlated with urine ACR in mice.

Conclusions These results show the hyaluronan synthesis inhibitor 4-MU effectively slowed the progression of DKD, and constitutes a potential new therapeutic approach to treat DKD.

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Introduction
The morphologic features of diabetic renal lesions are similar in type 1 and type 2 diabetes mellitus (1). Diabetic nephropathy is characterized by diffuse or nodular glomerulosclerosis, afferent and efferent hyaline arteriolar sclerosis, tubulointerstitial fibrosis, and atrophy (2). Hyalinosis is an important morphologic feature distinguishing diabetic nephropathy from hypertensive nephropathy (3).

Hyaluronan (HA) is a nonsulfated linear glycosaminoglycan composed of repeating units of D-glucuronic acid and N-acetylglucosamine (4). It is recognized as a relevant structural component of the extracellular matrix, but it also interacts with cells during embryonic development, wound healing, inflammation, and cancer, which are important aspects of normal and pathologic conditions (5–7). HA accumulates in kidneys during diabetes and could play an important role in the pathogenesis of diabetic nephropathies (8–10). However, the involvement of HA in the development and progression of diabetic kidney disease (DKD) remains obscure, because most research efforts have been focused on the mechanisms, proinflammatory cytokines, and transcription factors implicated in the induction of

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HA synthases during development of the disease (3–7,10–12).

Lewis et al. (9) highlighted the unclear role of HA in nephropathies when revealing that increased HA levels in the kidney are not predictive of DKD progression. Moreover, they concluded that “interstitial HA is not involved in inflammatory cell recruitment and is unlikely that it plays a direct role promoting the fibrotic response” in diabetic kidneys. However, other researchers suggest HA may participate in the pathogenesis of diabetic nephropathy on the basis of the facts that HA amass in kidneys during DKD, and that several kidney cells produce HA at an increased rate in vitro under hyperglycemia (13–16). Other scientists postulate that HA may have protective disease-limiting and anti-inflammatory effects in the diabetic kidney (17–21). It is generally believed that native (high mol wt; HMW) HA is anti-inflammatory in the setting of various pathologic conditions (22). However, increased HA turnover and fragmentation has been observed in inflammatory and fibrotic diseases, giving rise to lower mol wt byproducts that activate innate immune cell receptors (23). Then, it seems there is a connection between HA synthesis and catabolism and diabetic nephropathy, but the precise mechanisms and role of HA in this pathology are still to be clarified.

HA biosynthesis can be inhibited by 4-methylumbelliferone (hymecromone, 4-MU) through lowering the supply of UDP glucuronic acid (24–26) and downregulating the expression of HA synthases (27). Hymecromone has been proposed as a promising therapeutic agent for preventing metastasis of different types of malignant tumor cells in vitro and in animal models, and to treat immunologic disorders (22). In this study, we hypothesized that inhibiting HA by 4-MU treatment can slow the progression of DKD.

Materials and Methods
Mouse Model
We use the moderately hypertensive and diabetic endothelial nitric oxide synthase/leptin receptor (LEPR)–deficient (eNOS−/−/db/db) mice (28), which is one of the best murine models of type 2 diabetes (29,30). Mice were obtained by crossbreeding of eNOS−/− C57BLKS/J db (mouse strain 008340; the Jackson Laboratory). Mice were produced, cared for, and studied within the Animal Resources Facility at Albany Medical College. Our animal facilities are accredited and inspected annually by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Albany Medical College.

Treatment with 4-MU
At 9 weeks old, double homozygous mice were separated into two similar groups regarding sex, body weight, nonfasting plasma glucose concentrations, and consanguinity. Each experimental group had at least four animals housed in pairs. Mice cohorts were fed ad libitum identical artificial diets formulated by Envigo-Teklad (Indianapolis), containing 5% of 4-MU sodium salt (4-MU diet) or not (control diet) for 9 weeks. Experiments were repeated at least twice, and heterozygous siblings fed with commercial mouse chow were used as controls.

Statistical Analyses
GraphPad Prism 8.0 was used for statistical analyses. After evaluating the normality of data groups, a two-tailed unpaired t test with or without Welch’s correction for unequal variances (or Mann–Whitney two-tailed test when normality tests were not satisfied) was performed for comparison between two groups. One-way ANOVA followed by Tukey test and Sidak’s post-test for pair comparisons was used to compare more than two groups (or Kruskal-Wallis followed by Dunn’s test for multiple comparisons, when normality tests were not satisfied). When the statistical significance values were >0.05 and <0.1, the effect size indexes according to Cohen’s d or Hedges’ g were calculated to assess the magnitude of the difference between groups and the meaningfulness of the observed difference. Values are presented as mean±SD or median and interquartile range (IQR).

Other Analyses
Detailed descriptions about sample collection and processing, HA quantification in kidneys, histology analyses, and kits used to measure nonfasting plasma glucose, cystatin C, and HA in plasma, serum C-reactive protein (CRP), GFR, and spot urine albumin and creatinine are disclosed in Supplemental Methods.

Results
Treatment with 4-MU, Weight Variations, Plasma Glucose, and Serum CRP Levels
Preliminary measures of 4-MU food consumption for 2 weeks indicated that our diabetic double homozygous mice (12 weeks old) ate about 5.4±1 g of food daily (0.123 g/g of body wt) (Supplemental Figure 1). This amount of food corresponds to a dose of approximately 270±50 mg per day of 4-MU (around 6.2 g/kg body wt per day for our obese animals). This dose is similar to previous animal studies of oral 4-MU administration (31,32). Mice required about a week to adapt to the 4-MU food taste. As a result, 4-MU–fed mice gained less weight during this time, and reached their maximum weight later compared with control animals (Figure 1A). However, it is noteworthy that, after reaching the maximum weight, 4-MU–fed mice kept their body weight longer than mice on the control diet (Figure 1, A and B). This difference can be appreciated in the steeper slope of the average weight curve for control animals after reaching their maximum weight (~0.97 versus ~0.71 in 4-MU–fed mice; Supplemental Figure 2A), and when comparing the relationship between animal weight at the end of the experiments and its maximum weight (Figure 1B). At 18 weeks, the median weight of 4-MU treated mice was 99% (IQR, 93–100) of the maximum weight they reached during the experimental period, compared with 88% (IQR, 77–97) in diabetic controls (P=0.003). This trend in weight change over time was independent of animal sex (Supplemental Figure 2B). Weights for female and male heterozygous non-diabetic (nonobese) mice ranged from 19 to 28 g and 24–35 g, respectively, similar to the wild type C57BLKS/J strain.

Most of our mice were clearly diabetic at the beginning of the experiment (9 weeks of age). At this moment, the average nonfasting plasma glucose levels for both groups of animals was above 250 mg/dl (366±190 and 338±179 mg/
when average plasma glucose for nondiabetic LEPR heterozygous siblings was 112 ±7 mg/dL (Figure 1C). Moreover, plasma glucose levels increased during the experimental period for both groups of diabetic animals reaching a median of 516 mg/dL (IQR, 378–1170) and 1149 mg/dL (IQR, 876–1287) for 4-MU–fed and control groups, respectively, at 18 weeks of age. At this later time point, nonfasting plasma glucose was significantly lower in 4-MU–treated mice compared with controls (P<0.05; Figure 1C). This last observation agrees with the fact that, during the experiment, plasma glucose levels did not change, or decreased, in over 50% (eight of 15) of mice fed with the 4-MU diet, whereas 10% (one of 16) of control mice showed this behavior (Supplemental Figure 3). Despite the above differences in hyperglycemia between treatment groups, both experimental arms were hyperglycemic and had similar levels of serum CRP (Supplemental Figure 4).

Assessment of 4-MU Treatment on Renal Function

At 8 weeks of age, the renal function of double homozygous mice was already affected compared with nondiabetic LEPR heterozygous siblings (Figure 2A, Supplemental Figure 5A). However, although spot urine albumin-creatinine ratios (ACR) were similar in double mutant eNOS<sup>−/−</sup>/db/db mice at week 8 (5669±3214 versus 7055±4875 µg/mg, P=0.48, in 4-MU and control mice, respectively), 4-MU–fed mice had significantly lower ACR (13,461±8751 versus 21,597±10,267 µg/mg, P<0.05) than diabetic controls at 17 weeks (Figure 2A, Supplemental Figure 5B).

GFR analysis showed a clear glomerular hyperfiltration in the double-mutant diabetic mice at the beginning of the experiment (9 weeks of age) compared with nondiabetic LEPR heterozygous siblings (P=0.006). At baseline, average GFR values between the diabetic experimental groups were not significantly different (378±91 versus 313±68 µl/min, P=0.12, in 4-MU–treated and control mice, respectively), whereas the mean for nondiabetic siblings was 227±42 µl/min (Figure 2B). However, 9 weeks after being fed with the special diets, the mean GFR in the control group (231±104 µl/min) was significantly lower than in 4-MU–treated mice (407±201 µl/min, P=0.04), a decline suggestive of kidney function deterioration (Figure 2B). Paired variations for 4-MU–treated and control groups showed that GFR did not change or increased for 56% (five of nine) of mice fed a diet containing 4-MU, whereas this only happened for 25% (two of eight) of diabetic control mice (Supplemental Figure 5C).
Plasma cystatin C values were similar for all three cohorts of studied animals at 9 weeks (469 ± 114, 519 ± 101, and 540 ± 147 ng/ml, P = 0.44, in 4-MU–treated, control, and nondiabetic mice, respectively; Figure 2C). At the end of the experimental period (week 17), plasma cystatin C values were significantly higher (863 ng/ml; IQR, 642–1225) in control diabetic mice than in animals treated with 4-MU (486 ng/ml; IQR 370–734, P = 0.05; Figure 2C). No statistical differences (P = 0.27) were detected between 4-MU–treated mice and their nondiabetic LEPR heterozygous siblings (440 ng/ml; IQR, 330–558) at 17 weeks.

To address the differences in food consumption between experimental groups during adaptation to a 4-MU diet and their potential effect on DKD development, a subset of control animals was subjected to caloric restriction from weeks 9 to 11 (75%–90% of daily food intake depending on the animal’s weight measured on alternate days), whereas the 4-MU–fed group started treatment on week 9 (Supplemental Figure 6). This regimen resulted in similar weight variation curves and final weight/maximum weight ratios between the experimental groups (Supplemental Figure 6, A and B). Nonetheless, there was a significant increase in plasma cystatin C levels from week 9 to 17 in control mice (549 ± 71 versus 1300 ± 713 ng/ml, P = 0.05) but not in the 4-MU–treated group (493 ± 117 versus 674 ± 309, P = 0.21), and a trend toward increased urine ACR in controls compared with 4-MU fed animals (P = 0.06, effect size = 1.31; Supplemental Figure 6, C and D).

Kidney Morphology and Histopathology Analysis

We found a significant increase in the average kidney weight (42%) of control diabetic animals (231 ± 63 mg) compared with diabetic 4-MU–treated mice (163 ± 26 mg, P = 0.03; Figure 2D) at the end of the experiment, and a 64% increase after normalizing by body weight of the animals (P = 0.05; Supplemental Figure 5D). The kidney weights of 4-MU–fed mice were similar to those of nondiabetic animals (171 ± 51 mg, P = 0.76). Examples of kidney morphology in the three experimental groups are presented in Supplemental Figure 7.
Figure 3 portrays representative images of histopathology analyses in 4-MU–treated and control mice. Glomerular injury was significantly higher in control eNOS<sup>−/−</sup>/db/db mice than in the 4-MU–fed group (38%, index=1.53 versus 30%, index=1.19 of glomerular tuft area affected, respectively, <i>P</i>=0.047; Table 1). Mice treated with 4-MU also showed less mesangial expansion (24%, score=0.94 versus 31%, score=1.24 of glomeruli affected, respectively, <i>P</i>=0.017) and a trend toward lower segmental glomerulosclerosis than diabetic controls (9.0%±1.8% versus 14.9%±7.5%, respectively, <i>P</i>=0.09), and lacked signs of severe arteriolar hyalinosis or nephritis. Both groups of animals showed similar degrees of mesangiolysis, interstitial fibrosis, and tubular atrophy, although their incidences were very low in both cohorts. Consistent with this diabetic mouse model (33), endothelial injury in the glomerular basement membrane was also present in all mice at variable degrees. In terms of macrophage-mediated inflammation and expression of the main HA receptor (CD44), immunohistochemistry analyses for CD68<sup>+</sup> and CD44<sup>+</sup> cells demonstrated a trend toward fewer CD68<sup>+</sup> macrophages in the glomeruli of 4-MU–treated animals compared with controls (<i>P</i>=0.08, effect size=0.85; Table 1). In contrast, there were similar counts of CD68<sup>+</sup> cells in the interstitium, and of CD44<sup>+</sup> cells in the glomeruli and interstitium from both groups of animals.

**HA Content Analysis**

The assessment of HA content in kidneys showed that total HA concentration was significantly higher in diabetic mice than in nondiabetic siblings (5.6±1.5, 8.8±3.1, and 2.2±0.4 ng/mg of wet tissue for 4-MU–fed, control, and nondiabetic mice, respectively, <i>P</i>=0.002; Figure 4A). This analysis also revealed a trend toward a 36% reduction in total organ HA content in mice treated with 4-MU compared with diabetic controls (<i>P</i>=0.07, effect size=1.31; Figure 4A). In contrast, the kidney concentration of low molecular mass HAs (<100 kDa) was similar among the three assessed cohorts (0.55; IQR, 0.40–1.40, 1.10; IQR, 0.58–2.72, and 0.43, IQR, 0.35–0.51 ng/mg wet tissue for 4-MU treated, control, and nondiabetic mice, respectively, <i>P</i>=0.21). We also found no statistical differences in the fraction of low mol wt (LMW) HA (as % of total kidney HA) in the three groups of animals (Supplemental Figure 8A).

HA content in plasma was similar between 4-MU–treated and diabetic control mice (1132; IQR, 691–1261 and 1089; IQR, 800–1382 ng/ml, <i>P</i>=0.74, at week 9 and 971; IQR, 357–1507 and 903; IQR, 389–1676, <i>P</i>=0.89, at week 17 for 4-MU–fed and control mice, respectively; Supplemental Figure 8B). Interestingly, there was a trend toward increased plasma HA in nondiabetic hypertensive LEPR heterozygous mice at 9 weeks (<i>P</i>=0.07, effect size=1.43), and a significant increase with respect to the diabetic groups at the end of the experimental period (<i>P</i>=0.02 versus diabetic controls; Supplemental Figure 8B).

Given that both lower levels of plasma glucose (Figure 1C) and the trend toward lower total HA kidney content (Figure 4A) in 4-MU–treated mice could contribute to the observed improvement in diabetic nephropathy in these mice compared with diabetic controls, we evaluated
Table 1. Histopathology and immunohistochemistry findings in kidneys from 4-methylumbelliferone–treated and control mice (n=5 and 7 respectively)

| Findings                                      | 4-Methylumbelliferone   | Control              | P value |
|-----------------------------------------------|-------------------------|----------------------|---------|
| Glomeruli                                     |                         |                      |         |
| Mesangial expansion score (%)                 | 0.94±0.21 (approximately 24% of glomeruli affected) | 1.24±0.16 (approximately 31% of glomeruli affected) | 0.017   |
| Glomerular injury index (%)                   | 1.19±0.29 (approximately 30% of glomerular tuft area affected) | 1.53±0.23 (approximately 38% of glomerular tuft area affected) | 0.047   |
| % of segmental glomerulosclerosis (%)         | 9.0±1.8 (0.0–13.0)      | 14.9±7.5 (0.0–8.0)   | 0.900   |
| % of nodular glomerulosclerosis (%) (Kimmelstiel-Wilson lesion) | 3.0 (0.0–13.0)          | 1.0 (0.0–8.0)        | 0.872   |
| % of global glomerulosclerosis (%)            | 6.2 (5.9–10.2)          | 7.6 (2.6–20.0)       | 0.76    |
| % of affected glomeruli (%)                   | 54.4±15.1               | 64.6±10.0            | 0.19    |
| % of mesangiolysis (%)                        | 5.6±2.4                 | 7.7±4.9              | 0.41    |
| Glomerular diameter (µm)                      | 92.8±5.5                | 96.2±5.7             | 0.33    |
| Vascular/Interstitial                         |                         |                      |         |
| Severe arteriolar hyalinosis                  | Not observed            | 42.9% (3/7)          | 0.20    |
| % of interstitial fibrosis and tubular atrophy| 6.7±3.9                 | 6.3±3.5              | 0.85    |
| Inflammation                                 |                         |                      |         |
| Nephritis and tubulitis                       | Not observed            | 28.6% (2/7)          | 0.47    |
| CD68+ cell count – glomeruli (%)              | 1.0 (0.5–4.1)           | 7.6 (2.4–21.0)       | 0.081   |
| CD68+ cell count – interstitium (%)          | 2.0 (1.1–6.3)           | 3.8 (1.1–74.2)       | 0.71    |
| CD44+ cell count – glomeruli (%)              | 27.0 (19.9–46.4)        | 34.1 (22.3–61.0)     | 0.57    |
| CD44+ cell count – interstitium (%)           | 100.0 (74.6–115.9)      | 138.5 (100.5–254.8)  | 0.18    |

4-MU, 4-methylumbelliferone.

the correlations between plasma glucose, HA levels, GFR, and urine ACR in mice (Figure 4, B–D). Plasma glucose strongly correlated with total kidney HA content at 17 weeks (r=0.66, P=0.01). In addition, both total HA and LMW HA levels in kidneys showed strong correlations with urine ACR (r=0.76, P=0.007 and r=0.64, P=0.04, respectively; Figure 4, C and D). In contrast, plasma glucose did not demonstrate a significant relationship with either GFR (r=0.26, P=0.19) or urine ACR (r=0.17, P=0.49) in animals.

Discussion

Patients with DKD have higher HA content and hyalination rate in their kidneys (8,9). In this study, we investigated the role of HA accumulation in the progression of diabetic nephropathy. Our model mice were diabetic and showed glomerular hyperfiltration, proteinuria, and elevated ACR at the beginning of the experiment as expected (34,35). Treatment with 4-MU significantly improved hyperglycemia and showed a trend toward lower kidney HA levels. These changes were associated with significantly reduced urine ACR in treated animals compared with the control group, which, along with lower mesangial expansion score and glomerular injury index, demonstrated a renoprotective effect of 4-MU administration on DKD development.

It has been reported that 4-MU can treat hyperglycemia in type 2 diabetic mice, significantly decreasing blood glucose and maintaining glycemic control (36). We observed a significant reduction in nonfasting plasma glucose in 4-MU-fed mice. However, hyperglycemia was not fully controlled during the experiment and rose, although at a slower rate than in nontreated animals. The observed differences could be due to the hypertensive characteristics of our eNOS−/−/db/db mice and/or differences in genetic backgrounds between our C57BLKS and the C57BL/6 mice used by others. In fact, hyperglycemia and renal changes are more significant in the C57BLKS background (30). Of note, HA accumulation in the pancreas is associated with islet destruction and reduced insulin production by β cells (37,38). In addition, increased HA levels are linked to insulin resistance in sensitive tissues, such as skeletal muscle and the liver (39,40).

As expected in eNOS−/−/db/db mice, spot urine ACR was already significantly elevated at 8 weeks of age compared with nondiabetic siblings (35). Premature GFR elevation is also characteristic of early stages of kidney disease in dB mice (41), and was observed in our diabetic mice at 7 weeks of age. Importantly, both urine ACR and cystatin C were significantly lower, and GFR was significantly higher, in 4-MU–treated mice compared with controls after 9 weeks of 4-MU administration. Our caloric restriction experiment ruled out any significant influence of the mice adaptation period to the drug on these results. Given that hyperfiltration may occur in the early stages of DKD, confirmatory studies of GFR changes are necessary, ideally in animal models in which longer follow-up periods are feasible.

Kidney morphology and histopathology analyses confirmed that 4-MU treatment can delay the progression of DKD in mice. Treated animals not only maintained the average kidney weight as nondiabetic mice, but they portrayed a lower incidence of typical DKD morphologic lesions in the organ. In agreement with kidney function parameters, kidneys of 4-MU–fed mice had significantly lower mesangial expansion scores and glomerular injury index, and did not present severe arteriolar hyalinosis or nephritis.

HA concentration in the kidneys of diabetic animals was two to seven times higher than in nondiabetic siblings, and 4-MU treatment showed a trend toward reduction of HA in
diabetic kidneys by 36%. Diabetic animals had a lower concentration of LMW HA because the ratio of LMW to total HA in kidneys was rather constant for all mice independent of their diabetic status or 4-MU treatment. This finding points to a tight balance between HA synthesis and degradation mechanisms in diabetic kidneys. However, it is not known whether there are differences in LMW to total HA ratio in different parts of the kidneys, or if 4-MU will equally affect HA content and size composition in them. Native (HMW) HA is typically associated with anti-inflammatory responses (22). However, HA synthesis can be upregulated by both inflammatory and anti-inflammatory cytokines (14,16,42,43), suggesting the effects of native HA are tissue and setting specific. Increased fragmentation of HA has been reported in inflammatory and fibrotic diseases (23). However, the inflammatory responses to LMW HA in tissues are implicated in both healing processes and chronic diseases, indicating the result of such stimulation also depends on the pathologic setting (23). Declèves et al. (44) showed an increase of HMW HA in kidneys immediately after ischemic injury and its subsequent degradation to LMW fragments during the regenerative period. Moreover, inhibition of HA degradation after renal ischemia in hyaluronidase knockout models worsened inflammatory responses, tubular damage, and fibrosis (45). Given the high turnover of HA in tissues (7), it is difficult to separate the potential anti-inflammatory and antifibrotic role of HMW HA from its role as a source of LMW molecules.

Both the reduction in plasma glucose and the trend in lower kidney HA levels in 4-MU–treated animals could contribute to the observed renoprotective effects. We demonstrated a strong correlation between plasma glucose and total kidney HA levels, in agreement with published data on the role of hyperglycemia on HA synthesis by kidney cells (13–16). Kidney HA content in turn strongly correlates with urine ACR, in contrast with the lack of direct correlation between plasma glucose and GFR or urine ACR. These results suggest plasma glucose affects diabetic nephropathy in mice by increasing HA levels. Previous studies have reported a lack of direct association between hyperglycemia and kidney HA levels, but our findings suggest that plasma glucose indirectly influences HA levels through its effects on renal function.
and renal function tests in animals (41). Despite the importance of hyperglycemia as a risk factor for diabetic nephropathy, it is possible direct correlations are obscured by the variability in disease development in mice, different timings for onset of hyperglycemia and kidney disease, and the distinct effects of hyperglycemia at different stages of DKD (e.g., hyperfiltration early on, impaired kidney function in advanced disease).

HAs are known to play a fundamental role in both normal renal physiology and disease etiology (10). Under normal conditions, HAs concentrate in the renal medulla, where they participate in the regulation of fluid balance (10). Lower HA levels also exist in the glomerular endothelial glyocalyx, where they are essential for vascular stability and barrier function (46). However, accumulation of HA in the kidney cortex has been reported in animal models of ischemic injury, autoimmune disease, graft rejection, and crescentic GN (47). For example, upregulation of HA in mesangial cells is associated with increased proliferation (15) and a disbalance in extracellular matrix components (48), leading to glomerulosclerosis. Hyperglycemia and excess HA are also thought to affect the proportion of sulfated glycosaminoglycans and charge selectivity of the glomerular glyocalyx (15,49). These factors, combined with arterial hyalinosis, predispose for impaired kidney function (49,50).

In line with these observations and with early morphometric abnormalities in kidneys from patients with DKD (51), most of the damage we observed in our animals at the time of tissue collection was located in the glomeruli. We did not observe significant differences in interstitial fibrosis or inflammation. It is possible that differences in interstitial injury will be evident later in DKD development (9), and that we missed the timepoint for most of the inflammatory infiltration. In contrast, histopathology studies in patients with diabetic nephropathy failed to detect a relationship between HA accumulation and macrophage infiltration (9). This suggests the protective mechanisms of 4-MU on glomerular function are not inflammation related, but likely the result of a combination of renal homeostatic mechanisms, including the preservation of endothelial barrier function and composition of the mesangial matrix.

The limitations of this study include the small number of animals and the use of FITC-inulin in obese mice to measure GFR (52). In conclusion, this study strongly suggests the accumulation of HA in kidneys is directly involved in the progression of DKD. Moreover, we have found that 4-MU can target the development of DKD in a mouse experimental model, revealing that inhibitors of HA synthesis could be effective drugs for preventing or delaying the progression of diabetic nephropathy. In-depth studies are necessary to address the pending fundamental questions about the localization and role of HA size in DKD.

Disclosures
L.H. Salman reports receiving research funding from Albany Medical Center, Roach funds, and Transonic Inc.; and has other interests/relationships through the American Society of Diagnostic and Interventional Nephrology, American Society of Nephrology, Renal Physician Association, and Data Safety Monitoring Board – Phraxis. L.H. Salman and G. Selman have filed a utility patent application regarding the findings disclosed in this work (application 16/653,665). All remaining authors have nothing to disclose.

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Author Contributions
L.H. Salman and G. Selman conceptualized the study; L. Martinez, L.H. Salman, G. Selman, and R.I. Vazquez-Padron were responsible for data curation; A. Lightle, L. Martinez, G. Selman, and R.I. Vazquez-Padron were responsible for formal analysis; L.H. Salman was responsible for funding acquisition and project administration; A. Aguilar, A. Lightle, G. Selman, and D. Woltmann were responsible for investigation; L.H. Salman, G. Selman, and Y. Xiao were responsible for methodology; L.H. Salman and G. Selman were responsible for resources, software, and validation; L.H. Salman and G. Selman provided supervision; A. Lightle and G. Selman were responsible for visualization; L.H. Salman and G. Selman wrote the original draft; A. Lightle, L. Martinez, L.H. Salman, G. Selman, and R.I. Vazquez-Padron reviewed and edited the manuscript; and all authors provided critical feedback and helped shape the research, analysis, and manuscript.

Supplemental Material
This article contains the following supplemental material online at http://kiden360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0004642020/-/DCSupplemental.
Supplemental Methods.
Supplemental Figure 1. Food consumption and 4-MU dose calculations.
Supplemental Figure 2. Weight changes in animal groups.
Supplemental Figure 3. Pairwise comparisons of non-fasting plasma glucose.
Supplemental Figure 4. Serum C-reactive protein in animals.
Supplemental Figure 5. Kidney function tests and weights.
Supplemental Figure 6. Caloric restriction experiment.
Supplemental Figure 7. Representative kidney morphologies.
Supplemental Figure 8. Kidney low mol wt HA and plasma HA.

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