A hyaluronan synthesis inhibitor delays the progression of diabetic kidney disease in a mouse experimental model

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KEY POINTS

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

ABSTRACT

Background:

The role of hyaluronan (HA) in the development and progression of diabetic kidney disease (DKD), as well as 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^{db}) were fed artificial diets containing 5% 4-MU or not for 9 weeks. Plasma glucose, glomerular filtration rate (GFR), albumin to 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 non-fasting plasma glucose (516 [interquartile range 378-1170] vs. 1149...
mg/dL, P=0.050) and a trend toward lower HA kidney content (5.6 ± 1.5 vs. 8.8 ± 3.1 ng/mg of kidney weight, P=0.070) compared to 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 histological assessment of DKD also demonstrated a significant decrease in mesangial expansion score and glomerular injury index in 4-MU-treated mice compared to controls. Plasma glucose showed a strong correlation with kidney HA levels (r=0.66, P=0.0098). Both total hyaluronan (r=0.76, P=0.0071) and low-molecular-weight hyaluronan content (r=0.64, P=0.036) in the kidneys correlated with urine ACR in mice.

Conclusion

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

The morphological 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 morphological feature distinguishing diabetic nephropathy from hypertensive nephropathy (3).

Hyaluronan (HA) is a nonsulfated linear glycosaminoglycan that is 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, i.e., important features in normal and pathological conditions (5-7). Hyaluronan accumulates in kidneys during diabetes and it 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, as most research efforts have been focused on the mechanisms, proinflammatory cytokines, and transcription factors implicated in the induction of HA synthases during development of the disease (3-7, 10-12).

Lewis and coauthors highlighted the unclear role of HA in nephropathies when revealing that increased HA levels in the kidney are not predictive of DKD progression (9). Moreover, they concluded that “interstitial HA is not involved in inflammatory cell recruitment and is unlikely that it plays a direct role in promoting the fibrotic response” in diabetic kidneys. However, other researchers suggest that HA may be participating in the pathogenesis of diabetic nephropathy based on the facts that HA amass in kidneys during DKD, and that several kidney cells produce HA at an increased rate \textit{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 molecular weight [HMW]) HA is anti-inflammatory in the setting of various pathological conditions (22). However, increased HA turnover and fragmentation has been observed in inflammatory and fibrotic diseases, giving rise to lower molecular weight byproducts that activate innate immune cell receptors (23). Then, it seems that 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.

Hyaluronan biosynthesis can be inhibited by 4-methylumbelliferone (hymecromone, 4-MU) through lowering the supply of UDP glucuronic acid (24-26). Moreover, 4-MU also downregulates 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, as well as to treat immunological disorders (22). In this study, we hypothesized that inhibiting HA by 4-MU treatment can slow the progression of DKD.

MATERIALS AND METHODS

Mice 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 (Jackson Laboratories, mouse strain no. 008340). Mice were produced, cared for, and studied within the Animal Resources Facility at Albany Medical College (AMC). Our animal facilities are AAALAC accredited and inspected annually. All animal protocols were previously reviewed and approved by the IACUC at AMC.
**4-MU treatment.** At 9 weeks old, double homozygous mice were separated into two similar groups regarding sex, body weight, non-fasting plasma glucose concentrations, and consanguinity. Each experimental group had at least 4 animals housed in pairs. Mice cohorts were fed *ad libitum* identical artificial diets formulated by Envigo-Teklad (Indianapolis, USA), 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 above 0.05 and below 0.1, the effect size indexes according to Cohen’s or Hedges’ were calculated to assess the magnitude of the difference between groups and the meaningfulness of the observed difference. Values are presented as mean ± standard deviation (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 non-fasting plasma glucose, cystatin C and HA in plasma, serum C-reactive protein (CRP), glomerular filtration rate
RESULTS

4-MU treatment, weight variations, plasma glucose, and serum CRP levels

Preliminary measures of 4-MU food consumption for two weeks indicated that our diabetic double homozygous mice (12-week-old) ate about 5.4 ± 1 g of food daily (0.123 g/g of body weight) (Supplementary Fig. S1). This amount of food corresponds to a dose of approximately 270 ± 50 mg per day of 4-MU (around 6.2 g/kg body weight 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 to control animals (Fig. 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 (Fig. 1A-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 vs. -0.71 in 4-MU-fed mice; Supplementary Fig. S2A), as well as when comparing the relationship between animal weight at the end of the experiments and its maximum weight (Fig. 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 to 88% [77-97] in diabetic controls (P=0.0033). This trend in weight change over time was independent of animal sex (Supplementary Fig. S2B). Weights for female and male heterozygous non-diabetic (non-obese) mice ranged from 19 to 28 g and 24 to 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 non-fasting plasma glucose levels for both groups of animals...
was above 250 mg/dL (366 ± 190 and 338 ± 179 mg/dL, P=0.68, for 4-MU and control groups, respectively), when average plasma glucose for non-diabetic leptin receptor (LEPR) heterozygous siblings was 112 ± 7 mg/dL (Fig. 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 [876-1287] for 4-MU-fed and control groups, respectively, at 18 weeks of age. At this later time point, non-fasting plasma glucose was significantly lower in 4-MU treated mice compared to controls (P=0.050; Fig. 1C). This last observation agrees with the fact that, during the experiment, plasma glucose levels did not change or decreased in over 50% (8/15) of mice fed with 4-MU diet, while less than 10% (1/16) of control mice showed this behavior (Supplementary Fig. S3). Despite the above differences in hyperglycemia between treatment groups, both experimental arms were hyperglycemic and had similar levels of serum CRP (Supplementary Fig. S4).

**Assessment of 4-MU treatment on renal function**

At 8 weeks of age, the renal function of double homozygous mice was already affected compared to non-diabetic LEPR heterozygous siblings (Fig. 2A and Supplementary Fig. S5A). However, while spot urine albumin to creatinine ratios (ACR) were similar in double mutant eNOS−/−/db/db mice at week 8 (5,669 ± 3,214 vs. 7,055 ± 4,875 ug/mg, P=0.48, in 4-MU and control mice, respectively), 4-MU fed mice had significantly lower ACR (13,461 ± 8,751 vs. 21,597 ± 10,267 ug/mg, P=0.049) than diabetic controls at 17 weeks (Fig. 2A and Supplementary Fig. S5B).

Glomerular filtration rate analysis showed a clear glomerular hyperfiltration in the double mutant diabetic mice at the beginning of the experiment (9 weeks of age) compared to non-
diabetic LEPR heterozygous siblings (P=0.0062). At baseline, average GFR values between the diabetic experimental groups were not significantly different (378 ± 91 vs. 313 ± 68 uL/min, P=0.12, in 4-MU-treated and control mice, respectively), while the mean for non-diabetic siblings was 227 ± 42 uL/min (Fig. 2B). However, nine weeks after being fed with the special diets, the mean GFR in the control group (231 ± 104 uL/min) was significantly lower than in 4-MU-treated mice (407 ± 201 uL/min, P=0.042), a decline suggestive of kidney function deterioration (Fig. 2B). Paired variations for 4-MU treated and control groups showed that GFR did not change or increased for 56% (5/9) of mice fed with 4-MU-containing diet, while this only happened for 25% (2/8) of diabetic control mice (Supplementary Fig. S5C).

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 non-diabetic mice, respectively; Fig. 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 [370-734], P=0.045; Fig. 2C). No statistical differences (P=0.27) were detected between 4-MU-treated mice and their non-diabetic LEPR heterozygous siblings (440 ng/mL [330-558] at 17 weeks.

To address the differences in food consumption between experimental groups during adaptation to 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), while the 4-MU fed group started treatment on week 9 (Supplementary Fig. S6). This regimen resulted in similar weight variation curves and non-statistically significant final weight/maximum weight ratios between the experimental groups (Supplementary Fig. S6A-B). Nonetheless, there was a significant increase
in plasma cystatin C levels from week 9 to 17 in control mice (549 ±71 vs. 1300 ± 713 ng/mL, P=0.050) but not in the 4-MU-treated group (493 ± 117 vs. 674 ± 309, P=0.21), and a trend toward increased urine ACR in controls compared to 4-MU fed animals (P=0.058, effect size=1.31; Supplementary Fig. S6C-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 to diabetic 4-MU-treated mice (163 ± 26 mg, P=0.032; Fig. 2D) at the end of the experiment, and a 64% increase after normalizing by body weight of the animals (P=0.048; Supplementary Fig. S5D). The kidney weights of 4-MU-fed mice were similar to those of non-diabetic animals (171 ± 51 mg, P=0.76). Examples of kidney morphology in the three experimental groups are presented in Supplementary Figure S7.

Figure 3 portrays representative images of histopathology analyses in 4-MU-treated and control mice. Glomerular injury was significantly higher in control eNOS−/−/db/db mice than in the 4-MU-fed group (38% [index=1.53] vs. 30% [index=1.19] of glomerular tuft area affected, respectively, P=0.035; Table 1). Mice treated with 4-MU also showed less mesangial expansion (24% [score=0.94] vs. 31% [score=1.24] of glomeruli affected, respectively, P=0.039) and segmental glomerulosclerosis than diabetic controls (9 ± 2% vs. 15 ± 7%, respectively, P=0.048), 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+ and CD44+ cells demonstrated a trend toward fewer CD68+ macrophages in the glomeruli of 4-MU-treated animals compared to controls (P=0.081, effect size=0.85; Table 1). On the other hand, there are similar counts of CD68+ cells in the interstitium, and of CD44+ cells in the glomeruli and interstitium from both groups of animals.

**Hyaluronan content analysis**

The assessment of HA content in kidneys showed that total HA concentration was significantly higher in diabetic mice than in non-diabetic 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 non-diabetic mice, respectively, P=0.0021; Fig. 4A). This analysis also revealed a trend toward a 36% reduction in total organ HA content in mice treated with 4-MU compared to diabetic controls (P=0.070, effect size=1.31; Fig. 4A). In contrast, the kidney concentration of low molecular weight (LMW) hyaluronans (<100 kDa) was similar among the three assessed cohorts (0.55 [IQR 0.40-1.40], 1.10 [0.58-2.72], and 0.43 [0.35-0.51] ng/mg wet tissue for 4-MU treated, control, and non-diabetic mice, respectively, P=0.21). We also found no statistical differences in the fraction of LMW HA (as % of total kidney HA) in the three groups of animals (Supplementary Fig. S8A).

Hyaluronan content in plasma was similar between 4-MU-treated and diabetic control mice (1132 [IQR 691-1261] and 1089 [800-1382] ng/mL, P=0.74, at week 9 and 971 [357-1507] and 903 [389-1676], P=0.89, at week 17 for 4-MU-fed and control mice, respectively; Supplementary Fig. S8B). Interestingly, there was a trend toward increased plasma HA in non-diabetic hypertensive LEPR heterozygous mice at 9 weeks (P=0.066, effect size=1.43), and a significant increase with respect to the diabetic groups at the end of the experimental period.
Given than both lower levels of plasma glucose (Fig. 1C) and the trend toward lower total HA kidney content (Fig. 4A) in 4-MU treated mice could contribute to the observed improvement in diabetic nephropathy in these mice compared to diabetic controls, we evaluated the correlations between plasma glucose, HA levels, GFR, and urine ACR in mice (Fig. 4B-D). Plasma glucose strongly correlated with total kidney HA content at 17 weeks \((r=0.66, \ P=0.0098)\). In addition, both total HA and LMW HA levels in kidneys showed strong correlations with urine ACR \((r=0.76, \ P=0.0071\) and \(r=0.64, \ P=0.036, \) respectively; Fig. 4C-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 hyalinosis 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 to 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 non-fasting plasma glucose in 4-MU-fed mice. However, hyperglycemia
was not fully controlled during the course of the experiment and rose, although at a slower rate than in non-treated 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 to non-diabetic siblings (35). Premature GFR elevation is also characteristic of early stages of renal disease in db mice (41), and it 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 to 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 non-diabetic mice, but they portrayed a lower incidence of typical DKD morphological lesions in the organ. In agreement with kidney function parameters, kidneys of 4-MU-fed mice had significantly lower mesangial expansion score and glomerular injury index, and did not present severe arteriolar hyalinosis or nephritis.
Hyaluronan concentration in the kidneys of diabetic animals was two to seven times higher than in non-diabetic siblings, and 4-MU treatment showed a trend toward reduction of HA in diabetic kidneys by 36%. Diabetic animals had a higher 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 impact HA content and size composition in them. Native (HMW) HA are 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 that 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 that the end result of such stimulation also depends upon the pathological setting (23). Declèves et al. showed an increase of HMW HA in kidneys immediately after ischemic injury and its subsequent degradation to LMW fragments during the regenerative period (44). 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 anti-fibrotic 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 that plasma glucose affects diabetic nephropathy in mice by increasing HA levels. Previous studies have reported a lack of direct association between hyperglycemia and renal function tests in animals (41). Despite the importance of hyperglycemia as a risk factor for diabetic nephropathy, it is possible that 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).

Hyaluronans are known to play a fundamental role in both normal renal physiology and disease etiology (10). Under normal conditions, HA concentrate in the renal medulla where they participate in the regulation of fluid balance (10). Lower HA levels also exist in the glomerular endothelial glycocalyx 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 glomerulonephritis (47). For example, upregulation of HA in mesangial cells is associated with increased proliferation (15) and a disbalance in ECM 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 glycocalyx (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 DKD patients (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. On the other hand, histopathology studies in patients with diabetic nephropathy failed to detect a relationship between HA accumulation and macrophage infiltration (9). This suggests that 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 that 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

G. Selman and L.H. Salman have filed a utility patent application regarding the findings disclosed in the present work (application number 16/653,665). L. Salman reports Research Funding: Transonics Inc., Roach funds, Albany Medical Center; Patents and Inventions: patent application (pending review) for the use of 4-Methylumbelliferone in Diabetic Kidney Disease;
Other Interests/Relationships: American Society of Diagnostic and Interventional Nephrology, American Society of Nephrology, Renal Physician Association, Data Safety Monitoring Board – Phraxis. All remaining authors have nothing to disclose.

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Author Contributions

G Selman: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing - original draft; Writing - review and editing

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A Lightle: Formal analysis; Investigation; Visualization; Writing - review and editing

A Aguilar: Investigation

D Woltmann: Investigation

Y Xiao: Methodology

R Vazquez-Padron: Data curation; Formal analysis; Writing - review and editing

L Salman: Conceptualization; Data curation; Funding acquisition; Methodology; Project administration; Supervision; Writing - original draft; Writing - review and editing
All authors provided critical feedback and helped shape the research, analysis, and manuscript.

**Supplemental Materials**

Supplementary Methods

Supplementary Figure S1

Supplementary Figure S2.

Supplementary Figure S3.

Supplementary Figure S4.

Supplementary Figure S5.

Supplementary Figure S6. Caloric restriction experiment.

Supplementary Figure S7. Representative kidney morphologies.

Supplementary Figure S8.
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Table 1. Histopathology and immunohistochemistry findings in kidneys from 4-MU-treated and control mice (n=5 and 7 respectively).

<table>
<thead>
<tr>
<th></th>
<th>4-MU</th>
<th>Control</th>
<th>P-value</th>
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<tr>
<td><strong>Glomeruli</strong></td>
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<tr>
<td>Mesangial expansion score</td>
<td>0.94 ± 0.21 (24% of glomeruli affected)</td>
<td>1.24 ± 0.16 (31% of glomeruli affected)</td>
<td>0.017</td>
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<td>Glomerular injury index</td>
<td>1.19 ± 0.29 (~30% of glomerular tuft area affected)</td>
<td>1.53 ± 0.23 (~38% of glomerular tuft area affected)</td>
<td>0.047</td>
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<td>% of segmental glomerulosclerosis</td>
<td>9.0 ± 1.8%</td>
<td>14.9 ± 7.5%</td>
<td>0.090</td>
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<tr>
<td>% of nodular glomerulosclerosis (Kimmelstiel-Wilson lesion)</td>
<td>3.0% [0.0-13.0]</td>
<td>1.0% [0.0-8.0]</td>
<td>0.87</td>
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<td>% of global glomerulosclerosis</td>
<td>6.2% [5.9-10.2]</td>
<td>7.6% [2.6-20.0]</td>
<td>0.76</td>
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<tr>
<td>% of affected glomeruli</td>
<td>54.4 ± 15.1%</td>
<td>64.6 ± 10.0%</td>
<td>0.19</td>
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<tr>
<td>% of mesangiolysis</td>
<td>5.6 ± 2.4%</td>
<td>7.7 ± 4.9%</td>
<td>0.41</td>
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<tr>
<td>Glomerular diameter (um)</td>
<td>92.8 ± 5.5</td>
<td>96.2 ± 5.7</td>
<td>0.33</td>
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<tr>
<td><strong>Vascular/Interstitium</strong></td>
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<td></td>
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<tr>
<td>Severe arteriolar hyalnosis</td>
<td>Not observed</td>
<td>42.9% (3/7)</td>
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<td>% of interstitial fibrosis and tubular atrophy</td>
<td>6.7 ± 3.9%</td>
<td>6.3 ± 3.5%</td>
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<td><strong>Inflammation</strong></td>
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<tr>
<td>Nephritis and tubulitis</td>
<td>Not observed</td>
<td>28.6% (2/7)</td>
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<td>CD68+ cell count - glomeruli</td>
<td>1.0 [0.5-4.1]</td>
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<td>CD68+ cell count - interstitium</td>
<td>2.0 [1.1-6.3]</td>
<td>3.8 [1.1-74.2]</td>
<td>0.71</td>
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<td>CD44+ cell count - glomeruli</td>
<td>27.0 [19.9-46.4]</td>
<td>34.1 [22.3-61.0]</td>
<td>0.57</td>
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<tr>
<td>CD44+ cell count - interstitium</td>
<td>100.0 [74.6-115.9]</td>
<td>138.5 [100.5-254.8]</td>
<td>0.18</td>
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FIGURE LEGENDS

Figure 1. Body weight changes and plasma glucose in 4-MU-treated and control diabetic mice. A. Average weight in 4-MU-treated (n=10) and control diabetic animals (n=8) by week. Error bars indicate the mean and standard deviation (SD). B. Ratio of final weight (week 18) over maximum weight in 4-MU-treated (n=16) and control mice (n=15). Error bars indicate the median and interquartile range (IQR). Groups were compared using a Mann-Whitney test. C. Non-fasting plasma glucose in 4-MU-treated (n=15) and control mice (n=16) at baseline (week 9) and after nine weeks of drug-supplemented or control diets. Heterozygous non-diabetic littermate mice (n=16) are included for comparison. Error bars indicate the median and IQR. Groups were compared using Student’s t-tests or Mann-Whitney tests.

Figure 2. Renal function tests and kidney morphology in 4-MU-treated and control diabetic mice. A. Urine albumin to creatinine ratio (ACR) in 4-MU-treated (n=10-12) and control mice (n=8-12) at baseline (week 8) and after nine weeks of drug-supplemented or control diets. Heterozygous non-diabetic littermate mice (n=10-12) are included for comparison. Error bars indicate the mean and standard deviation (SD). Groups were compared using Student’s t-tests. B. Glomerular filtration rate (GFR) in 4-MU-treated (n=9) and control mice (n=8) at baseline (week 9) and after nine weeks of drug-supplemented or control diets. Heterozygous non-diabetic littermate mice (n=9) are included for comparison. Error bars indicate the mean and SD. Groups were compared using Student’s t-tests. C. Plasma cystatin C levels in 4-MU-treated (n=10-11), control (n=10), and heterozygous non-diabetic littermate mice (n=8) at weeks 9 and 17. Error bars indicate the median and interquartile range (IQR). Groups were compared using Student’s t-tests or Mann-Whitney tests. D. Weight of the right kidney in 4-MU-treated (n=6), control (n=7),
and heterozygous non-diabetic littermate mice (n=4) at week 18. Error bars indicate the mean and SD. Groups were compared using Student’s t-tests.

**Figure 3. Kidney histopathology in diabetic mice.** Examples of A) a normal glomerulus, B) glomerulus with mesangial expansion, segmental glomerulosclerosis, and a Kimmelstiel-Wilson nodule (arrow), C) glomerular mesangiolysis, and D-E) arteriolar hyalinosis. A and E correspond to periodic acid-Schiff (PAS) stainings, while B-D are stained with hematoxylin and eosin (H&E). Image A was obtained from a 4-MU-treated mice, while B-E originate from diabetic animals treated with control diet.

**Figure 4. Kidney hyaluronans and correlation with plasma glucose and renal function tests.**
A. Total and low molecular weight (LMW) hyaluronans (HA) in kidneys from 4-MU-treated (n=5), control (n=5), and heterozygous non-diabetic littermate mice (n=4) at week 18. Values are expressed as HA content per milligram of kidney wet weight. Error bars indicate the median and interquartile range (IQR). Groups were compared using a Student’s t-test or Mann-Whitney test. 
B. Correlation between non-fasting plasma glucose and total kidney HA content at week 18. C-D. Correlations between total HA (C) or LMW HA content (D) in kidneys and urine ACR at week 17. Y-axis values were not available for one control mice and two heterozygous animals in these analyses.
Figure 1

A. Graph showing the average weight (g) over time (weeks) for 4-MU and control groups.

B. Graph showing the final/maximum weight ratio with significance level P=0.0033.

C. Graph showing plasma glucose levels with significance levels P<0.0001, P=0.68, and P=0.038.