SUPPLEMENTARY METHODS

Mice model. The eNOS^{-}/db double homozygous mutant mice are not fertile and the strain used to produce them (eNOS$^{-/-}$ C57BLKS/J ^{db}) has significant breeding difficulties. In our hands, over 30% of the females failed to have survivor pups, and overall, only one double homozygous mutant was obtained per female with survivor pups, close to the 25% expected for this breeding (Supplementary Table 1). Double mutant eNOS/db mice were selected by PCR genotyping according to Mohan and co-authors (1).

Supplementary Table 1. Summary of breeding efforts to obtain the double homozygous eNOS^{-}/db mice.

Breeding attempt	Number of mothers	Mothers with survivor pups	Total number of pups	Number of double homozygous mutants	Pups per mom with survivor pups	Double homozygous mutants per pup	Double homozygous mutants per mom with survivor pups
1	8	5	26	8	5.2	0.31	1.6
2	15	10	62	10	6.2	0.16	1
3	15	8	34	6	4.3	0.18	0.8
4	10	7	33	7	4.7	0.21	1
5	19	14	52	14	3.7	0.27	1
Totals	**67**	**44**	**207**	**45**	**4.8**	**0.23**	**1.1**

4-MU treatment. Both Envigo-Teklad diets provided 3.5 kcal/g and contained by weight: 17.7 % of protein, 53.9 % of carbohydrate, and 7.2 % of fat. It is known that 4-MU gives bad taste to the food and mice will not eat it without additives to make the food pellets palatable (2), then, we incorporated peanut butter (5%) and sucralose (0.1%) into the diets to mask the bitter taste of 4-MU. Diets were air-dried, sterilized by radiation, and kept sealed under vacuum at -20ºC protected from light. Every week, the old food was removed, and animals were provided fresh food pellets. Food was protected from light when in the animal cages. Heterozygous siblings were fed with commercial mouse chow (LabDiet 5P76 providing 3.45 Kcal/g, and containing 22.5% protein, 51.9% carbohydrate, and 6.9% fat). The weight and appearance of the animals were monitored at least weekly. 4-MU was administered orally in the food, and treated mice had a calculated daily dose of approximately 270 mg, which is close to values reported by others (1, 3).

Caloric restriction test. 4-MU gives a bad taste to food and mice place themselves in voluntary fasting for one to two weeks until they adapt to the drug's flavor (2). During this period, some mice lose weight. To evaluate the impact of a caloric restriction period on the outcome of our experiments, diabetic control mice on the control diet were kept in a temporal fasting regime for two weeks while the treated animals were put on the 4-MU diet (week 9). According to the CR-night protocol described by Acosta-Rodriguez et al. (4) mice received during the temporal fasting period between 75% to 90% (depending on the animal's weight variations measured on alternate days) of ad libitum daily food intake every day at evening to keep alike the weight variations between the 4-MU-treated and control animals.

Non-fasting plasma glucose, cystatin C, and hyaluronan determinations in plasma. Blood was collected from a tail snip using a 20 uL heparinized microcapillary approximately at the same hour of the day every time and kept on ice until centrifugation at 10,000 g for 5 minutes at 16ºC; the produced plasma was aliquoted and stored at -86 ºC until use. Glucose levels were assayed diluting plasma (1/80) and using a commercial kit (EIAGLUC, Invitrogen). For cystatin C determination, a 1/200 dilution was used in a commercial immunoassay kit (MSCTC0, R&D Systems). For hyaluronan determination, diluted plasma (1/80) was used with a Hyaluronan Immunoassay kit (R&D, cat. no. DHYAL0). Samples were analyzed in duplicates.

Urine albumin and creatinine. Spot urine was collected from individually caged mice using a 96-well plate covered with a stainless-steel mesh (1680 microns) at the bottom of the cage to avoid contamination of urine with feces at weeks 8 and 17. Collected urine was kept on ice until centrifuged at 6,000 g for 5 minutes at 4ºC. After centrifugation, the upper portion of the sample (3/4 of the volume) was collected and stored at -86 ºC until assayed using the Albuwell M and Creatinine companion kits (Ethos Biosciences cat. no. 1011 and 1012 respectively). Urine was diluted 1/80 for albumin determination, and 1/10 (and spiked with 1 mg/dL solution) for the creatinine assay. Samples were analyzed in duplicates.

Glomerular filtration rate measurements. GFR was determined before (week 9) and after 9 weeks of treatment (week 18) by a single bolus injection method according to a published protocol (5), using fluorescein isothiocyanate-(FITC) labeled inulin and collecting approximately 15 uL of blood from a tail snip at 3, 5, 7, 10, 15, 25, 50, and 75 minutes after the injection in heparinized microcapillaries. Collected blood was kept on ice until centrifuged (10,000g, 5 min) to obtain the

plasma. At all times, the FITC-inulin solution and blood containing the fluorophore were kept protected from light. Plasma from heterozygous sibling animals, fed with the same diet as the assayed animals, was used to prepare the standard curves. Measurements were done using a Nanodrop 3300 (Thermo scientific). GFR was calculated from circulating FITC-inulin concentrations versus time using the two-phase exponential decay function option in the GraphPad Prism application software.

C-reactive protein determination. At weeks 8 and 17, approximately 15 uL of blood was let to clot for 2 hours at room temperature after collecting it from a tail snip and then, centrifuged at 2,000 g for 20 minutes at 16 ºC. The collected serum was stored at -86 ºC until CRP levels were assayed by using a commercial immunoassay (MCRP00, R&D Systems). The serum was diluted 1/2000 for the assay. Samples were analyzed in duplicates.

Hyaluronan measurements in kidneys. Hyaluronan in kidneys was assayed by a modified protocol based on Declèves et al. (6) Briefly, left kidney was washed with 1X PBS (pH-7.2), dried, cut in small pieces, weighed, homogenized in two volumes of 1X PBS using a polytron (2250 rpm, for 30 sec. 3 times), weighted again, and the same vol. of TST buffer (100mM Tris pH 8, NaCl 100mM, Triton X-100 0.5%) was added as well as proteinase K (20 mg/mL in water) at a final concentration of 250 ug/mL. After overnight incubation at 55 ºC, proteinase K was inactivated by heating at 100 ºC x 20 min. The supernatant was collected after centrifugation (1000g x 20 min, RT), and stored at -80 ºC. Before using it, the collected supernatant was thawed and mixed well. To obtain the low molecular weight hyaluronans, a Sartorius Centrisart I (MWCO 100 kDa) device was used and the filtrate collected. The HA content in the extracts was determined

(diluting 1/100 and 1/1000, or 1/40 and 1/400 for the LMW fraction) using the Hyaluronan Immunoassay (R&D, cat. no. DHYAL0). Samples were analyzed in duplicates.

Histopathology analysis. Kidneys were collected from 4-MU-treated and control mice at the end of the experiment (18-week-old mice). Organs were fixed in 10% neutral buffered formalin for 44 hours, embedded in paraffin, and 5 um sections were stained with Hematoxylin and Eosin, Periodic acid–Schiff, or Picrosirius Red, before being examined following a semi-quantitative index similar to one proposed by Zhao and co-authors (7) to evaluate the glomerular injury (degree of glomerular mesangial expansion and sclerosis). Briefly, 50 glomeruli on a single section (25 from each kidney) were scored from 0 to 4, where 0 represents no lesions and 1, 2, 3, and 4, represent mesangial matrix expansion or glomerular sclerosis (glomerulosclerosis) involving less than or equal to 25, 26-50, 51-75, or more than 75% of the glomerular tuft area, respectively. The mesangial expansion alone was also scored following a similar semi-quantitative index. End-stage segmental and nodular (Kimmelstiel-Wilson lesion) glomerulosclerosis were counted, as well as interstitial fibrosis, tubular atrophy, arteriolar hyalinosis, mesangiolysis, and nephritis were assessed. Interstitial fibrosis was calculated in Picrosirius Red-stained sections as percent area of the total cortical parenchyma. The percentage of affected glomeruli was also calculated. Glomerular diameter was measured in 50 glomeruli per animal. Histopathology staining were done by an independent service (HistoWiz, Inc. New York) and the analyses were performed by pathologists that were blind regarding the source of the samples.

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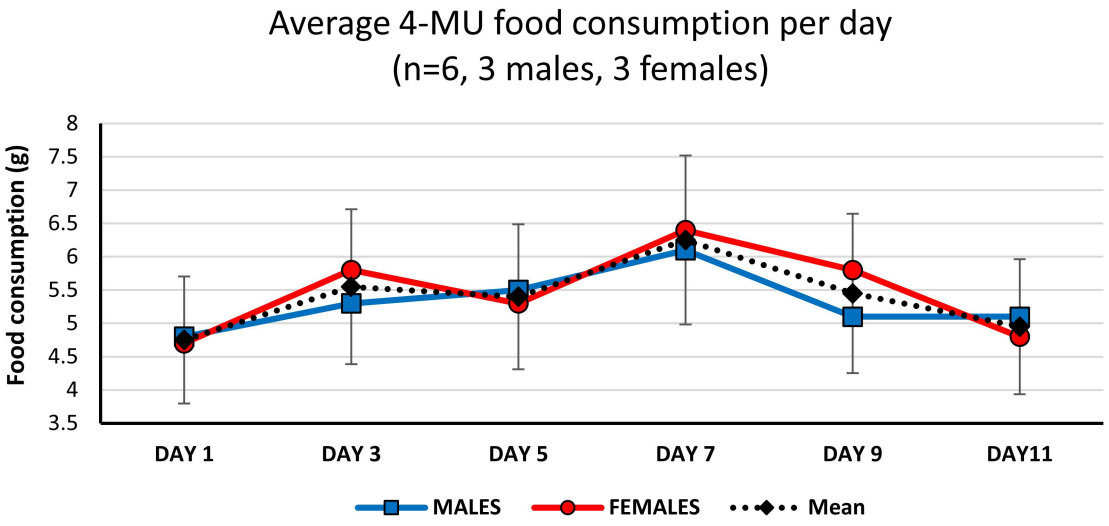
Supplementary Figure S1. Average daily 4-MU food consumption (12-week-old mice; 3 males, 3 females) and data used to calculate the amount of food eaten and 4-MU dose taken by treated mice. Means with standard deviations (SD) are plotted.



Food consumption per day (g)

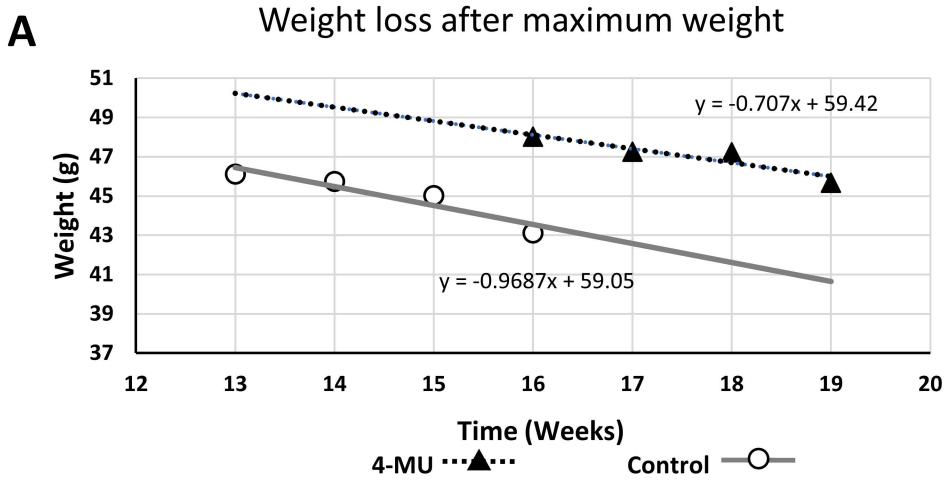
	DAY 1	DAY 3	DAY 5	DAY 7	DAY 9	DAY 11	Average
MALES	4.8	5.3	5.5	6.1	5.1	5.1	5.3
FEMALES	4.7	5.8	5.3	6.4	5.8	4.8	5.5
Average	4.8	5.6	5.4	6.3	5.5	5.0	5.4
SD*	0.95	1.16	1.09	1.27	1.20	1.01	1.1

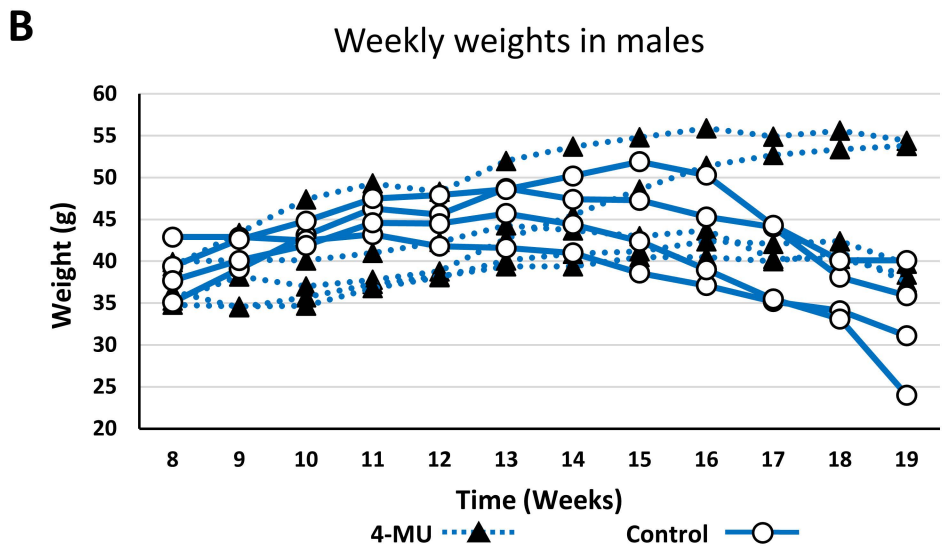
** Deviation considering 20% food nibbled and not eaten*

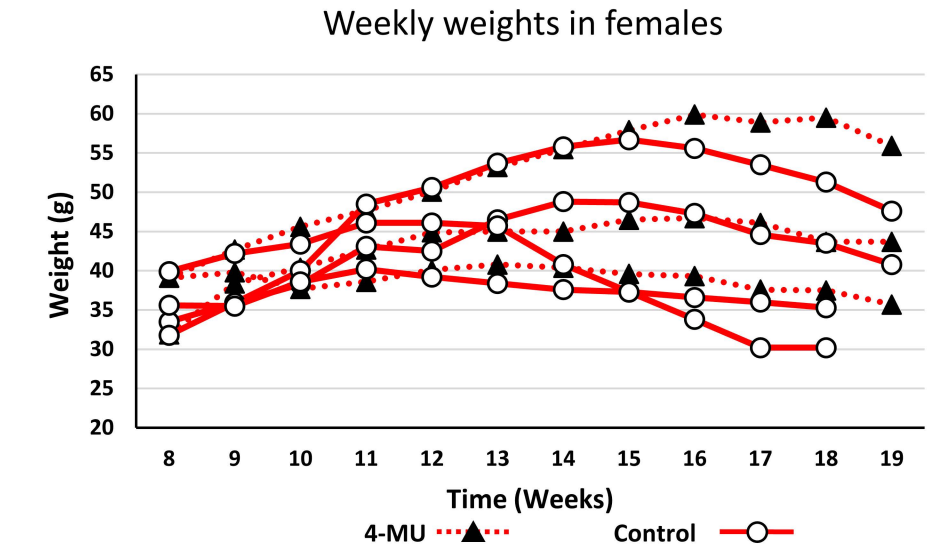
Mice weight (g)

MALES	42.8	41.8	41.5	42.4	42.7	42.6	42.3
SD	0.6	0.9	0.8	1.4	1.6	1.8	1.2
FEMALES	45.8	44.6	44.0	46.1	46.0	45.7	45.4
SD	2.6	2.5	1.8	2.2	1.7	1.5	1.9
Weight average (males and females)							43.8
SD							2.2

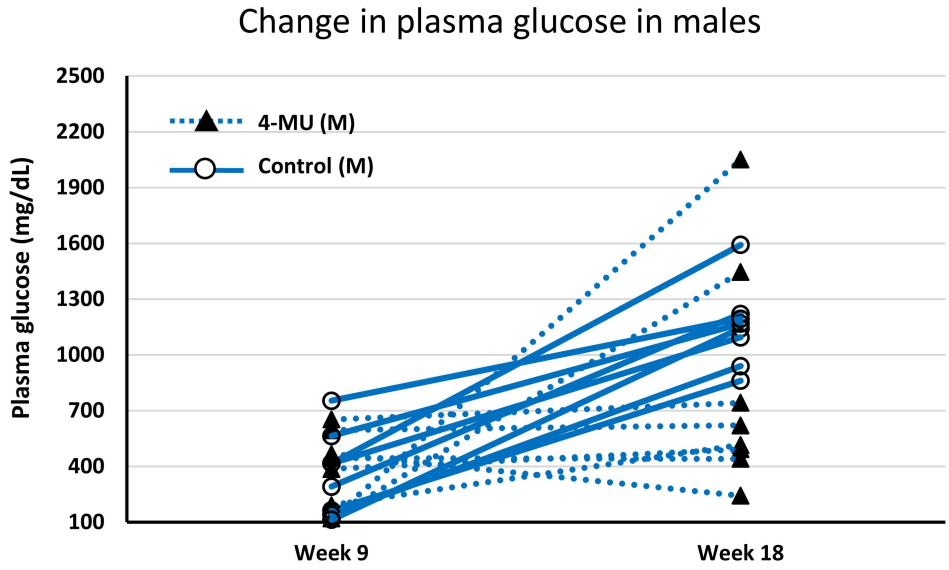
Supplementary Figure S2. A) Weight decline after reaching the maximum weight in mice fed with 4-MU or control diets. **B)** Weekly weights in representative male and female mice fed with 4-MU or control diets.

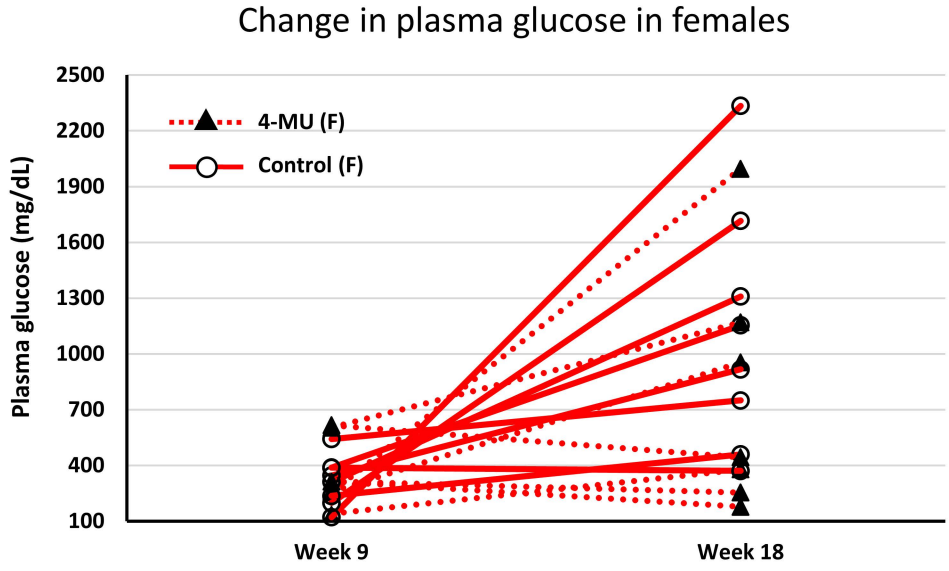




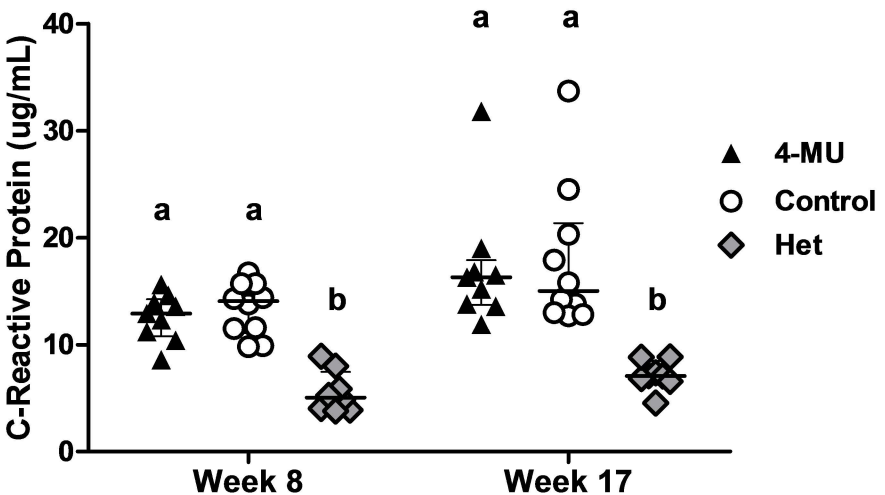


Supplementary Figure S3. Pairwise comparisons of non-fasting plasma glucose in male and female mice at baseline (week 9) and after 9 weeks of 4-MU or control diets (week 18).

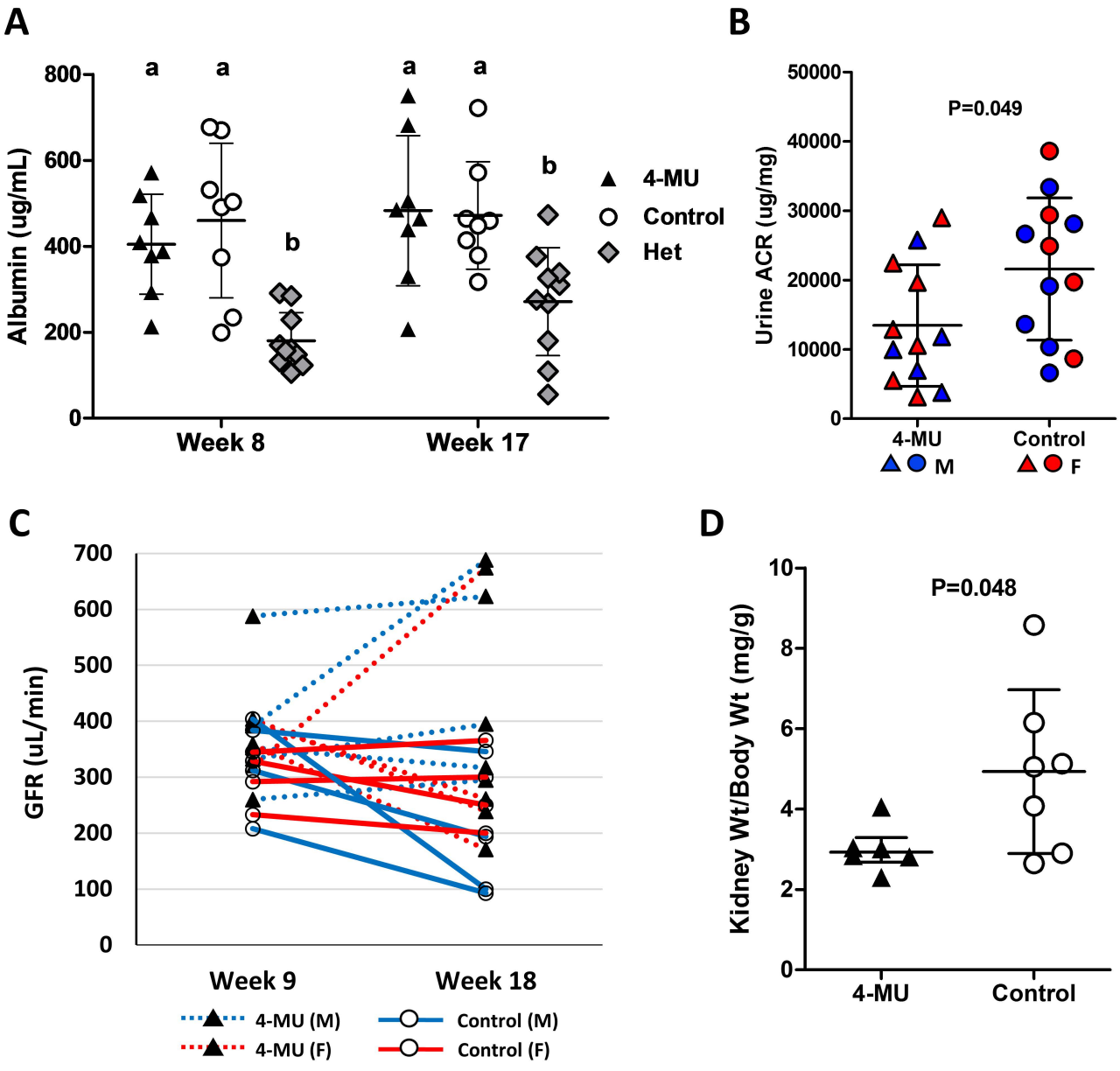




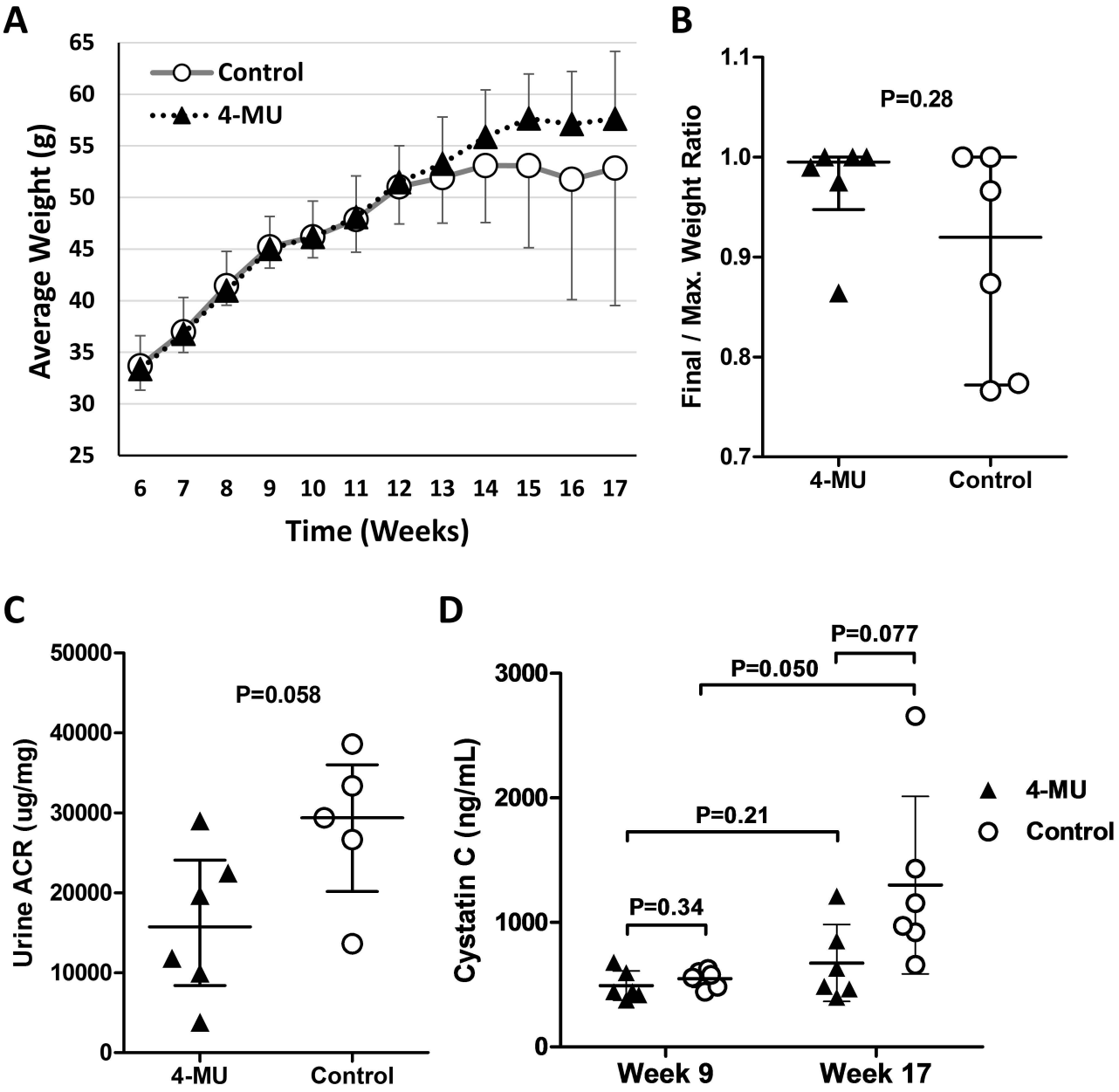
Supplementary Figure S4. Serum C-reactive protein levels at baseline (week 8) and after 9 weeks of 4-MU or control diets (week 17). Heterozygous non-diabetic littermate controls are included for comparison. Error bars indicate the median and interquartile range. Groups were compared using the Kruskal-Wallis test followed by the Dunn's post-test for multiple comparisons. Different letters above the groups indicate statistically significant differences ($p<0.05$).



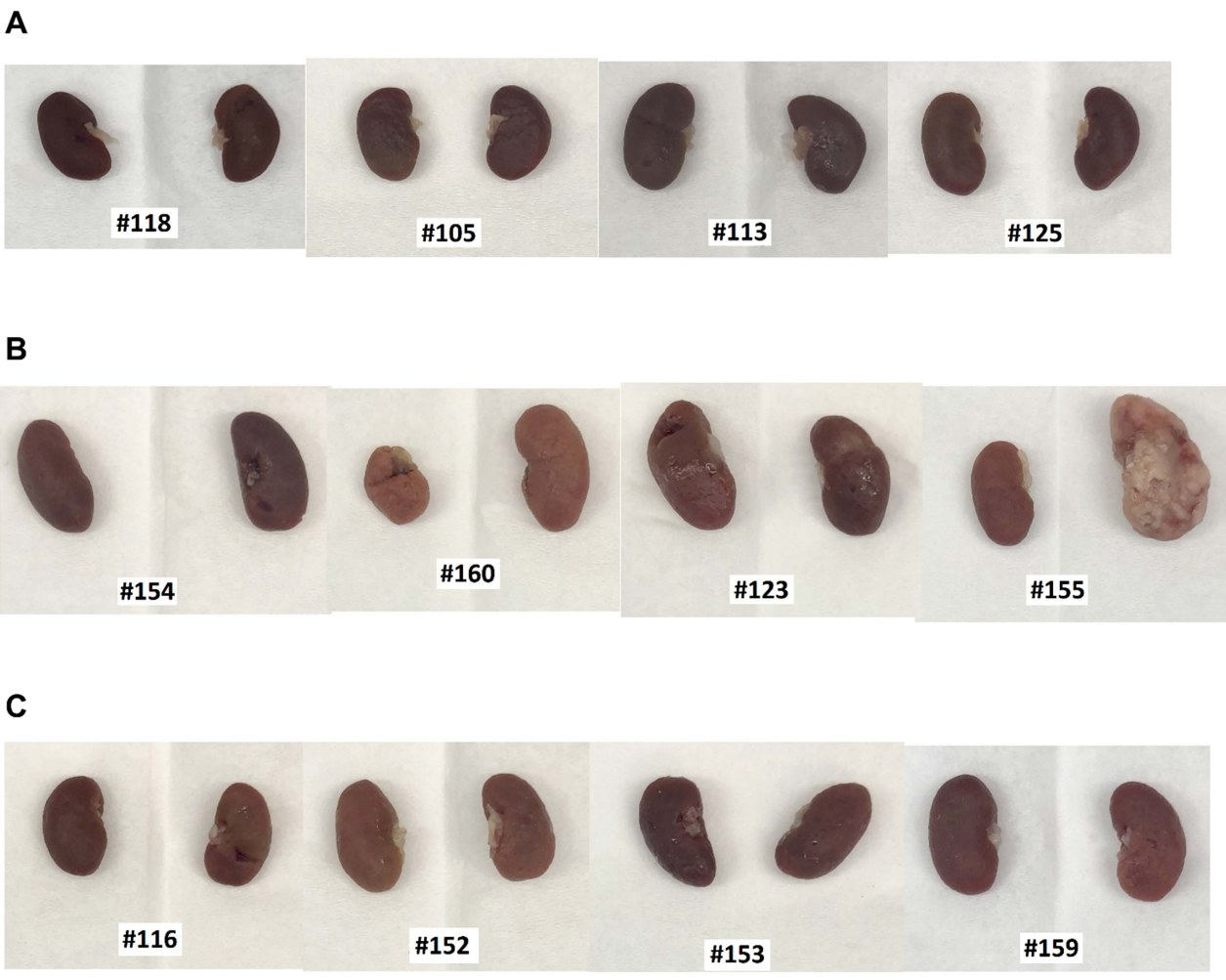
Supplementary Figure S5. A) Urine albumin levels at baseline (week 8) and after 9 weeks of 4-MU or control diets. Heterozygous non-diabetic littermate controls are included for comparison. Error bars indicate the mean and SD. Groups were compared using a one-way ANOVA followed by the Tukey's post-test for multiple comparisons. Different letters above the groups indicate statistically significant differences ($p<0.05$). **B)** Urine ACR at week 17 in male (blue) and female (red) mice. Error bars indicate the mean and SD. Groups were compared using a Student's t-test. **C)** Pairwise GFR comparisons in male and female mice at baseline and after 9 weeks of 4-MU or control diets. **D)** Kidney weights in mice after 9 weeks of 4-MU or control diets. Values were normalized to body weight. Error bars indicate the mean and SD. Groups were compared using a Student's t-test.



Supplementary Figure S6. Caloric restriction experiment. A) Weekly average weights in mice fed with 4-MU diet starting on week 9 or control diet following a caloric restriction regimen from weeks 9 to 11. Error bars indicate the mean and SD. **B)** Final weight (week 17) to maximum weight ratio in mice fed with 4-MU diet or control diet following caloric restriction. Error bars indicate the median and IQR. Groups were compared using the Mann Whitney test. **C)** Urine ACR at week 17 in experimental groups. Error bars indicate the mean and SD. Groups were compared using a Student's t-test. **D)** Plasma cystatin C levels at baseline (week 9) and at the end of the experiment (week 17). Error bars indicate the mean and SD. Groups were compared using Student's t-tests.



Supplementary Figure S7. Representative kidney morphologies. Examples of kidneys from **A)** non-diabetic LEPR heterozygous mice, **B)** diabetic control mice, and **C)** 4-MU-treated mice. Photos were taken from the same distance, with similar light conditions, and using the same magnification settings.



Supplementary Figure S8. A) Fraction of low molecular weight hyaluronans (HA) in kidneys at week 17 expressed as percentage of total HA kidney content. Heterozygous non-diabetic littermate controls are included for comparison. Error bars indicate the median and IQR. Groups were compared using the Mann Whitney test. **B)** Total HA in plasma at baseline (week 9) and after treatment with 4-MU or control diets (week 17). Error bars indicate the median and IQR. Groups were compared using the Mann Whitney test or Student's t-test.