

Supplementary Materials: Plasma Metabolomics Reveals a Shared Metabolomic Profile in Experimental and Human Chronic Kidney Disease

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Supplemental Figure S1. H&E and AQP1 staining.

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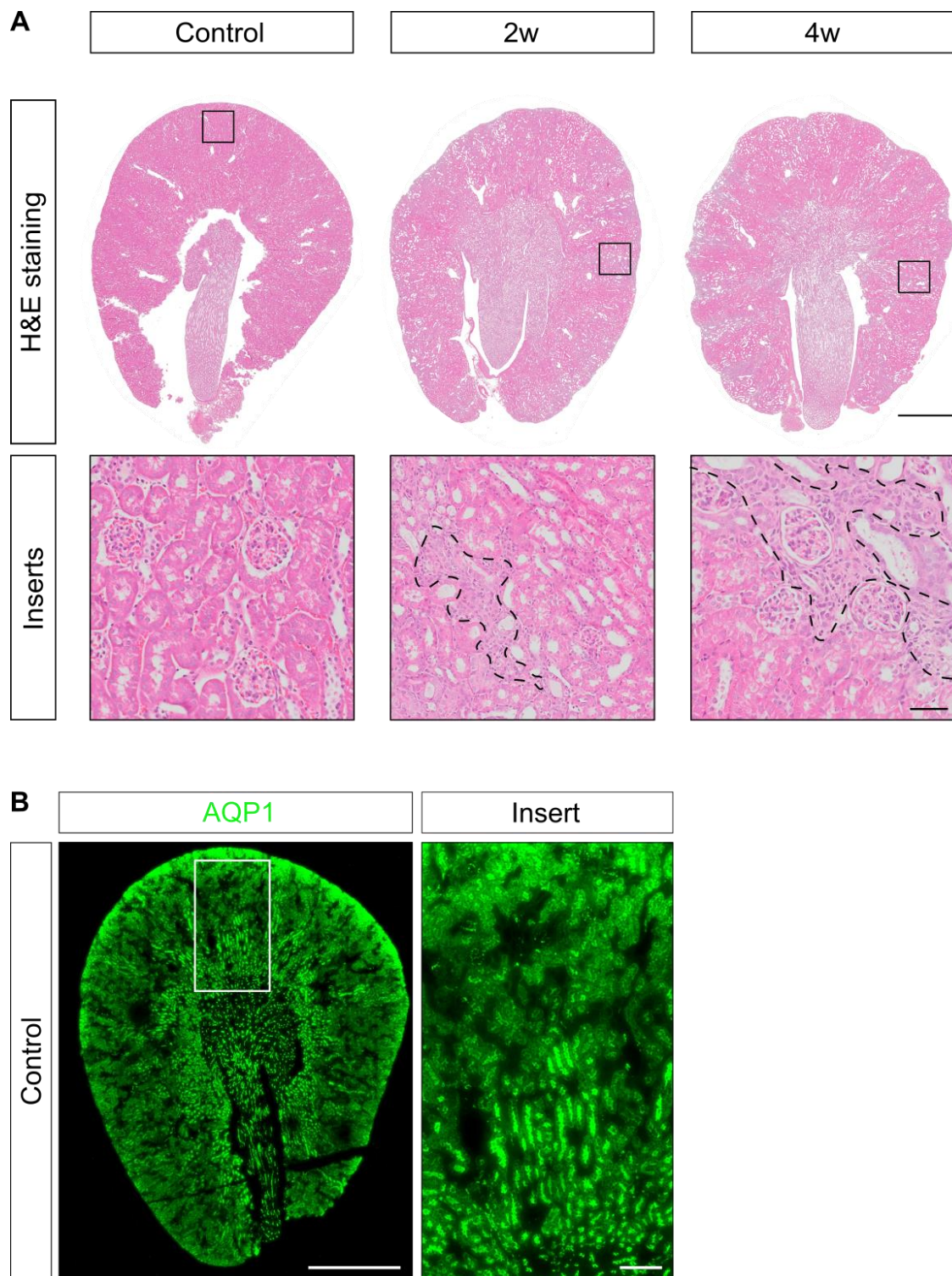
Supplemental Table S2. Primer sequences used for qPCR.

Supplemental Table S3. Antibodies used for immunoblotting, immunohistochemistry, and immunofluorescence.

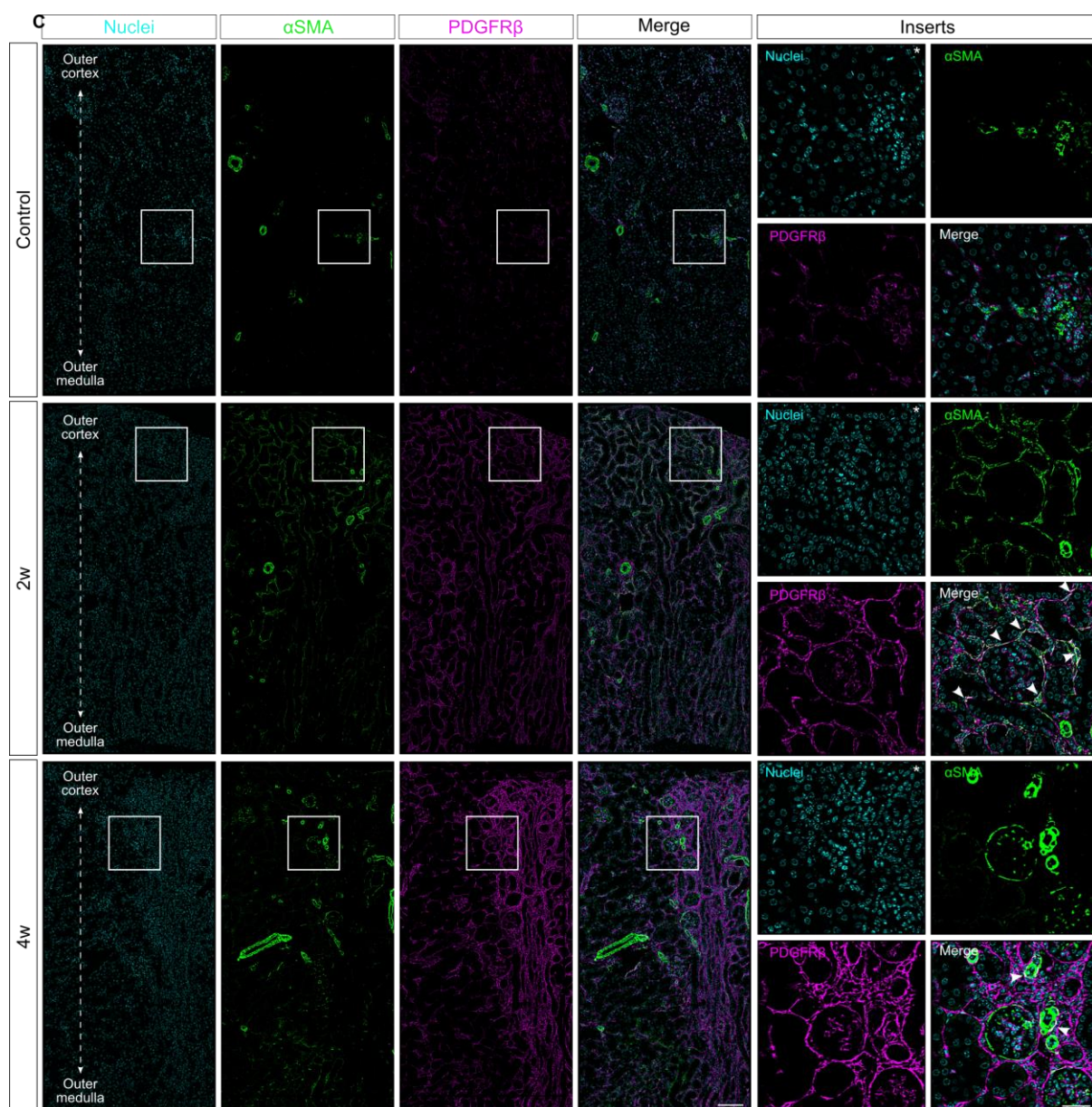
Supplemental Table S4. Pure chemicals used as standards for the targeted metabolomics.

Supplemental Table S5. Pipeline of the statistical methodology for identification of features in Compound Discover.

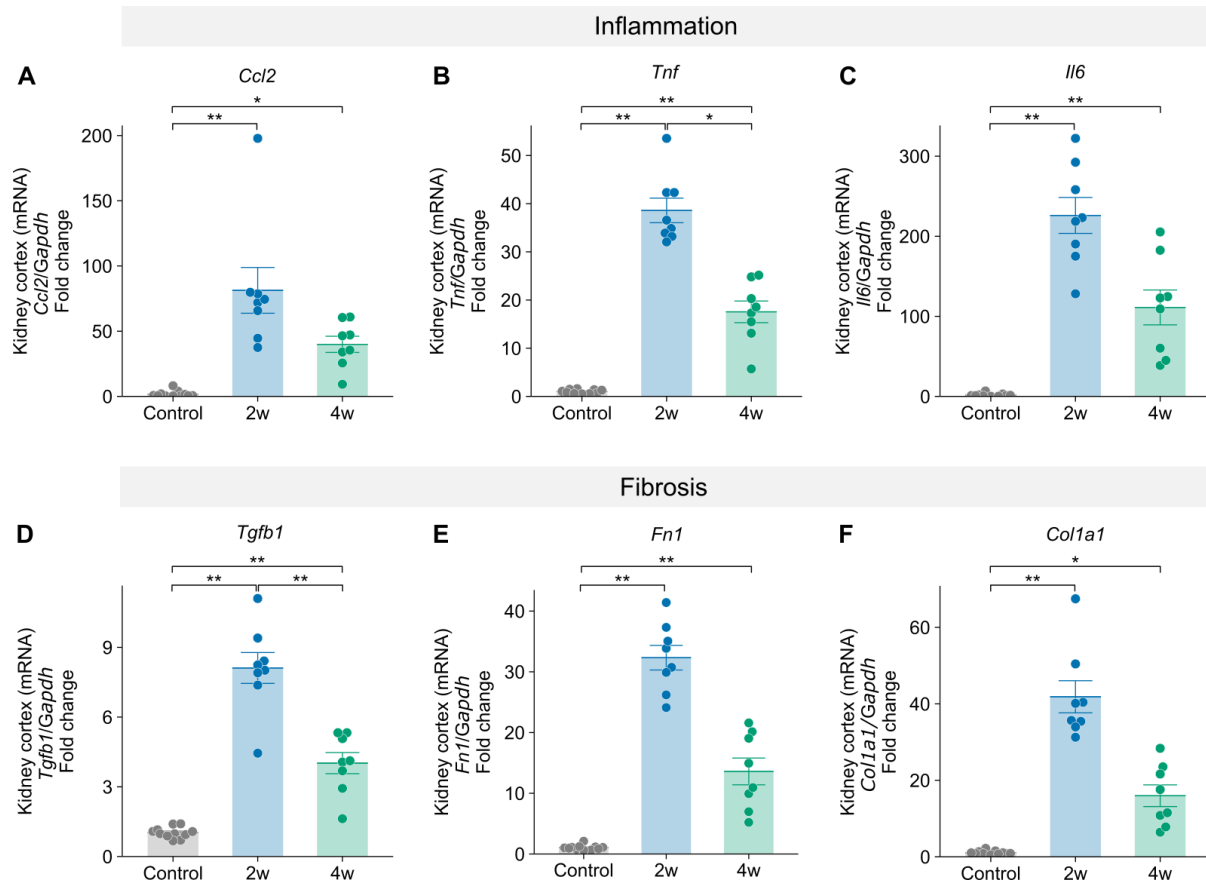
Supplementary Figures



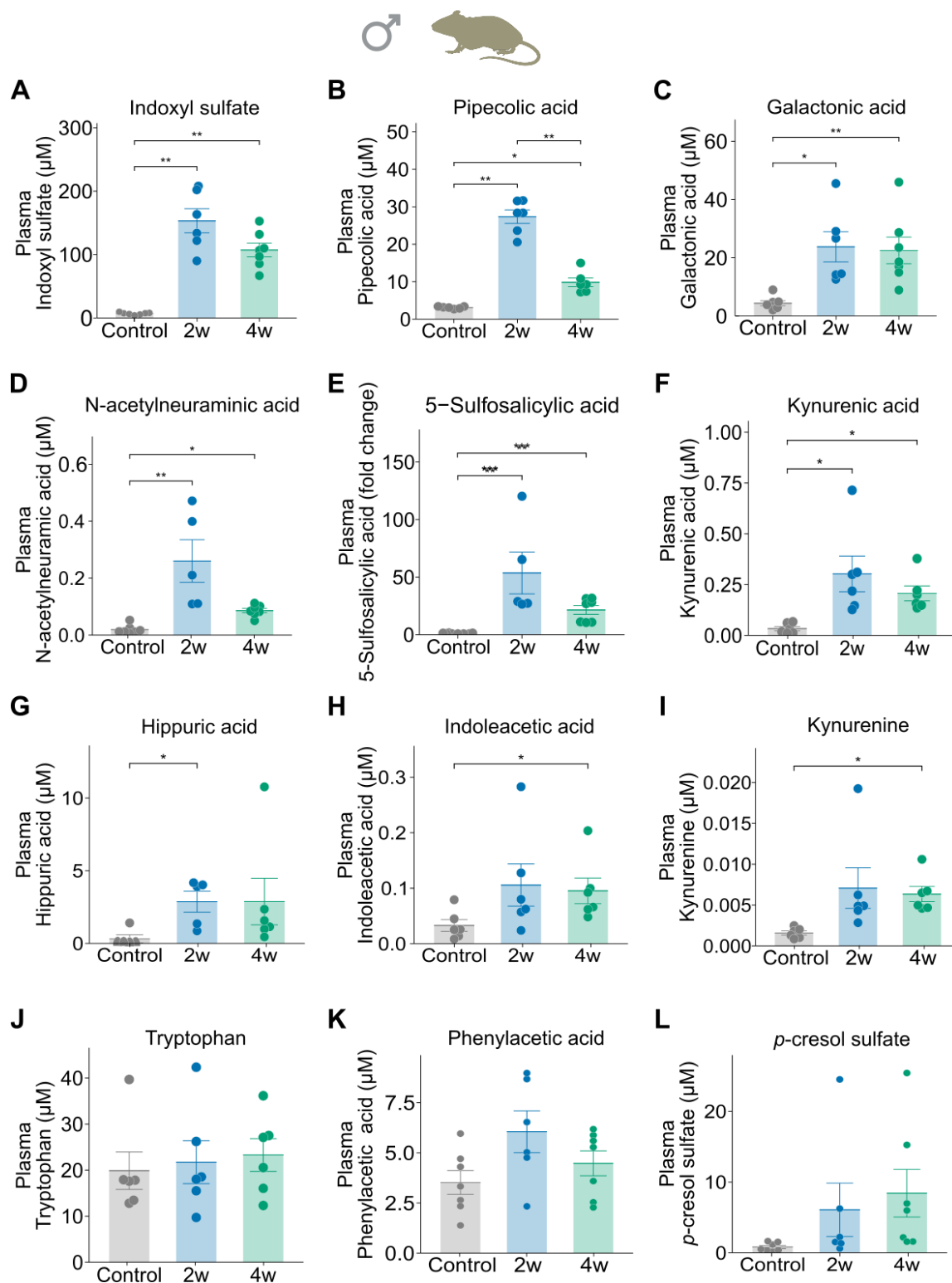
Supplemental Figure S1. Histology and proximal tubule health. **(A)** H&E staining of kidney sections in the control, 2-week, and 4-week groups (top panels). Inserts show magnified regions (bottom panels). Areas of fibrosis and tubulointerstitial injury are encircled with a dashed line; representative images are shown ($n=5$); top scale bar = 1 mm; bottom scale bar = 50 μm . Images were acquired on an Olympus VS120 Virtual Slide Scanner (40 \times air objective). **(B)** Immunofluorescence staining for Aquaporin 1 (AQP1) in kidney sections mounted on coverslips from controls, highlighting healthy proximal tubules. Insert shows the magnified region (white rectangle); representative images are shown ($n=5$); left scale bar = 1 mm; right scale bar = 100 μm . Images were acquired on a Nikon Eclipse Ti2 microscope (60 \times oil objective). **(A-B)** Images from the control and 4-week group have previously been published [23].



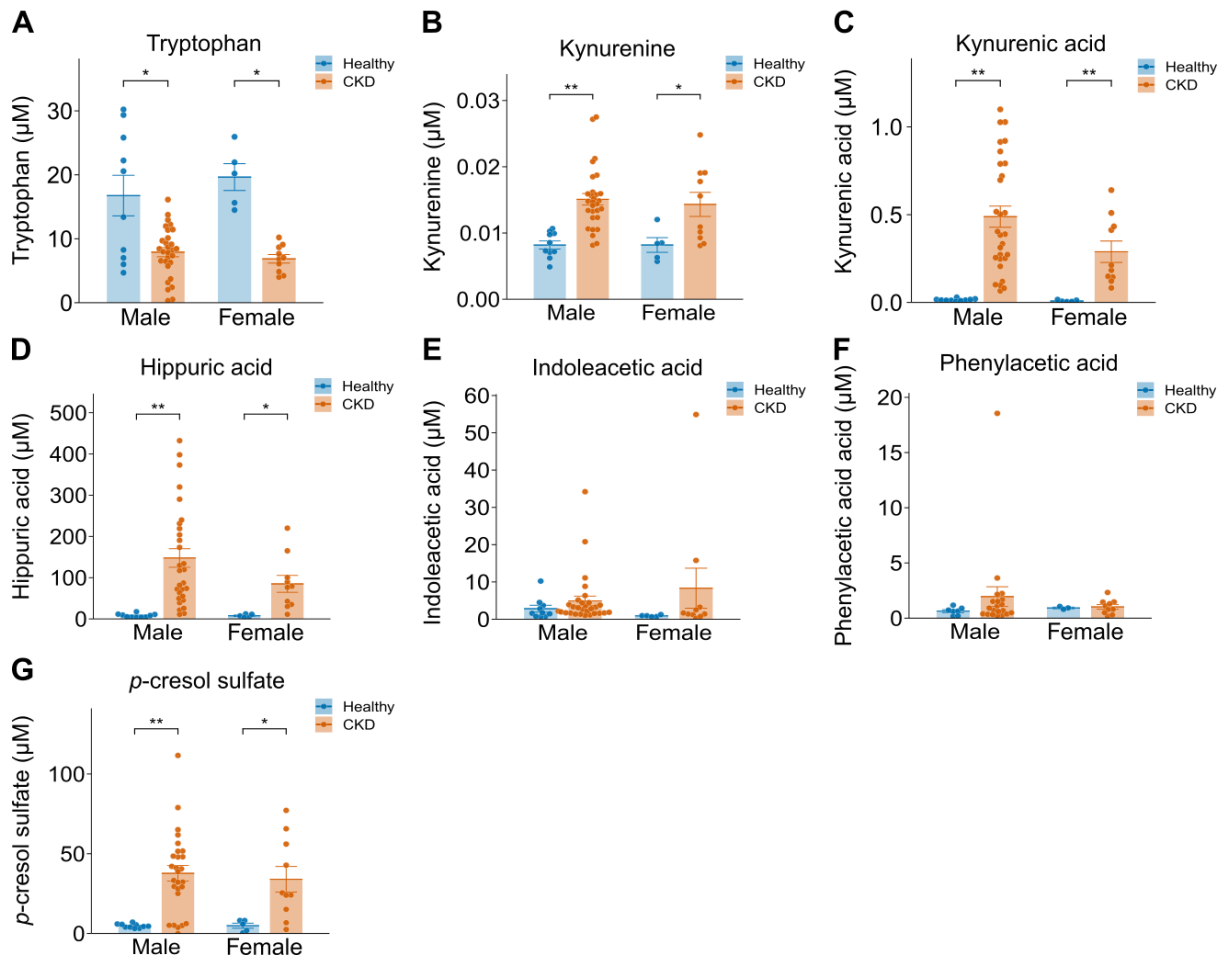
Supplemental Figure S2. α Smooth muscle actin and PDGFR β co-staining. Immunofluorescence staining of kidney tissue sections for Hoechst (cyan, nuclei), α Smooth muscle actin (α SMA; green, marker of myofibroblasts) and PDGFR β (magenta, marker of fibroblasts) in the control, 2-week, and 4-week groups. The images cover the outer cortex (top) and outer medulla (bottom); the stainings are shown individually and merged; inserts show magnified regions (white rectangles); white arrow heads point at overlapping α SMA and PDGFR β staining, indicating activated myofibroblasts. Representative images are shown ($n=5$); left scale bar = 100 μ m; right scale bar = 40 μ m. Images were acquired on an Olympus VS120 Virtual Slide Scanner (40 \times air objective). Images from the control and 4-week group have previously been published [23].



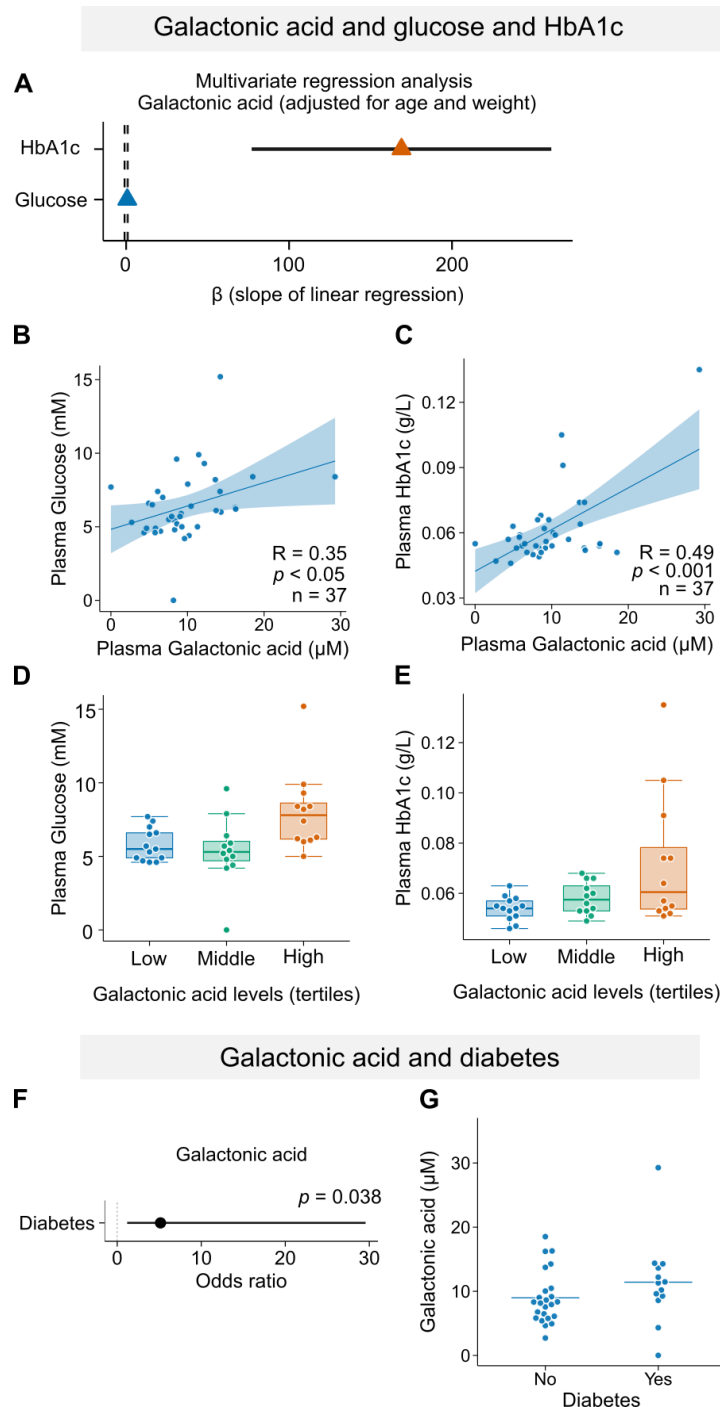
Supplemental Figure S3. Kidney cortex inflammation and fibrosis. (A-F) Kidney cortex mRNA expression levels of (A) *Ccl2*, (B) *Tnf*, (C) *Il6*, (D) *Tgfb1*, (E) *Fn1*, and (F) *Col1a1* were assessed by qPCR, normalized to *Gapdh* expression, and expressed as fold change compared with control levels (2-week and 4-week groups $n=8$; controls $n=10$); data are presented as mean \pm SEM and statistical significance was evaluated by either one-way ANOVA followed by Tukey-Kramer post hoc test or Kruskal-Wallis test; *: $p < 0.05$; **: $p < 0.001$. (A-F) Data from the control and 4-week group have previously been published [23].



Supplemental Figure S4. Targeted plasma metabolomics from adenine-fed mice. (A–L) Targeted quantification of plasma metabolites in control ($n=7$), 2-week ($n=6$), and 4-week ($n=7$) groups of (A) Indoxyl sulfate, (B) Pipecolic acid, (C) Galactonic acid, (D) N-acetylneuraminic acid, (E) 5-Sulfosalicylic acid, (F) Kynurenic acid, (G) Hippuric acid, (H) Indoleacetic acid, (I) Kynurenine, (J) Tryptophan, (K) Phenylacetic acid, and (L) *p*-cresol sulfate. Data are presented as mean \pm SEM and statistical significance was assessed using one-way ANOVA followed by Tukey–Kramer post hoc test or Kruskal–Wallis test; *: $p < 0.05$; **: $p < 0.001$.



Supplemental Figure S5. Targeted plasma metabolomics from CKD patients. (A-G) Targeted quantification of plasma metabolites in human CKD patients (CKD men, $n=29$; CKD women, $n=10$,) and healthy controls (healthy men, $n=10$; healthy women, $n=5$). (A) Tryptophan, (B) Kynurenine, (C) Kynurenic acid, (D) Hippuric acid, (E) Indoleacetic acid, (F) Phenylacetic acid, and (G) *p*-cresol sulfate. Data are presented as mean \pm SEM and statistical significance was assessed using one-way ANOVA followed by Tukey–Kramer post hoc test; *: $p < 0.05$; **: $p < 0.001$.



Supplemental Figure S6. Galactonic acid and diabetes. (A) Multivariate linear regression analyses of galactonic acid against HbA1c and glucose adjusted for age and body weight. Triangles indicate direction and significance of associations: blue triangles represent non-significant associations ($p \geq 0.05$), with upward- and downward-pointing symbols for positive and negative slopes, respectively; orange triangles represent significant associations ($p < 0.05$), again indicating directionality. β values and 95% confidence intervals are shown for each regression. (B-C) Linear regression analyses showing the relationship between plasma galactonic acid (B) plasma glucose and (C) plasma HbA1c. (D-E) plasma galactonic acid levels divided in tertiles from indicating low ($n=15$), middle ($n=13$) and high ($n=13$) levels of galactonic acid and the corresponding plasma concentrations of (D) glucose and (E) HbA1c. (F) The odds ratio for having diabetes between patients with galactonic acid levels below median compared with those above. The odds ratio is shown with 95% confidence intervals. (G) Galactonic acid concentrations stratified by diabetes status, either not having (No, $n=24$) or having diabetes (Yes, $n=13$).

Supplemental Table S1. Patient characteristics.

| | Value (±SD) |
|--|--------------------|
| Basic characteristics | |
| Patients (n) | 39 |
| Sex, male (%) | 74.4 |
| Age (years) | 61 (14.3) |
| Height (cm) | 173 (9.6) |
| Smoking, n (%) | 28.21 |
| Plasma potassium (mmol/L) | 4.2 (0.7) |
| Plasma sodium (mmol/L) | 138.4 (3.2) |
| Dialysis parameters | |
| Haemodialysis frequency (treatments/week) | 2.82 (0.45) |
| Dialysis time (hours/week) | 10.7 (3.0) |
| Dialysis vintage (days) | 175 (102) |
| Duration of dialysis (hours) | 3.77 (0.56) |
| Primary kidney disease | |
| Diabetic nephropathy, n (%) | 28 |
| Glomerulonephritis, n (%) | 7.7 |
| Hypertensive renal disease, n (%) | 13 |
| Kidney graft failure, n (%) | 2.6 |
| Other, n (%) | 13 |
| Polycystic kidney disease, n (%) | 13 |
| Unknown, n (%) | 23 |
| Kidney disease parameters | |
| Plasma creatinine (μmol/L) | 620 (127) |
| Measured glomerular filtration rate (ml/min/m ²) | 4.63 (3.16) |
| Plasma urea (mmol/L) | 18.9 (5.73) |
| Hormones | |
| Plasma adrenaline (nmol/L) | 0.11 (0.08) |
| Noradrenaline (nmol/L) | 2.21 (1.3) |
| Plasma parathyroid hormone (pmol/L) | 25.4 (24.2) |
| Cardiometabolic/diabetes parameters | |
| Plasma total cholesterol (mmol/L) | 4.08 (0.92) |
| Plasma phosphate (mmol/L) | 1.63 (0.44) |
| Plasma glucose (mmol/L) | 6.33 (2.32) |
| Plasma HDL (mmol/L) | 1.13 (0.36) |
| Plasma LDL (mmol/L) | 2.28 (0.81) |
| HbA1c (g/L) | 0.06 (0.02) |
| Plasma triglycerides (mmol/L) | 1.53 (0.95) |
| Diabetes, n (%) | 35.9 |
| Cardiovascular parameters | |
| Diastolic blood pressure (mmHg) | 74.4 (12.4) |
| Heart rate (dialysis end) (beats/min) | 70.8 (21.8) |
| Heart rate (dialysis start) (beats/min) | 73.1 (14.1) |
| Heart rate variability (HRV) mean N-N interval (ms) | 689 (377) |
| Left ventricular ejection fraction (%) | 61.7 (10.0) |
| Left ventricular end diastolic diameter (cm) | 4.9 (0.56) |

| | |
|---|--------------|
| Left ventricular end systolic diameter (cm) | 3.3 (0.59) |
| Left ventricular mass index (g/m ²) | 120.9 (34.9) |
| Systolic blood pressure (mmHg) | 144 (19.9) |
| Cardiovascular disease biomarkers | |
| NT-proBNP (mmol/L) | 3.62 (4.6) |
| Troponin (nmol/L) | 66.2 (63.8) |
| Inflammatory parameters | |
| CRP (mg/L) | 4.19 (6.3) |
| IL-1 β (pg/mL) | 1.36 (1.27) |
| IL-6 (pg/mL) | 16.9 (16.6) |
| IL-8 (pg/mL) | 38.9 (34.2) |
| Cardiovascular disease outcomes | |
| Angina, n (%) | 5 |
| Arrhythmia, n (%) | 13 |
| Heart disease, n (%) | 39 |
| Heart valve disease, n (%) | 5 |
| Hypertension, n (%) | 95 |
| Ischemic heart disease, n (%) | 26 |
| Peripheral atherosclerosis, n (%) | 23 |

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, c-reactive protein; NT-proBNP, N-terminal pro b-type natriuretic peptide; HbA1c, hemoglobin A1c; IL, interleukin.

Supplemental Table S2. Primer sequences used for qPCR.

| Target gene | Direction | Sequence (5' to 3') |
|------------------------------|-----------|-------------------------|
| <i>Abcc4</i> (MRP4) | Sense | GAGATGGTGCAGAAGGGGAC |
| | Antisense | ACCAAATTGAGGCCTCGGAG |
| <i>Abcg2</i> (BRCP) | Sense | TGGACTCAAGCACAGCGAAT |
| | Antisense | GGGTTGTTGTAGGGCTCACA |
| <i>Acta2</i> (α SMA) | Sense | CTGACAGAGGCACCACTGAA |
| | Antisense | CATCTCCAGAGTCCAGCACA |
| <i>Aqp1</i> (Aqp1) | Sense | GTCCAGGACAACGTGAAGGT |
| | Antisense | ACACACTGGGCGATGATGTA |
| <i>Gapdh</i> (GAPDH) | Sense | TAAAGGGCATCCTGGGCTACACT |
| | Antisense | TTACTCCTTGAGAGCCATGTAGG |
| <i>Havcr1</i> (KIM1) | Sense | CGGTACAACCTTAAAGGGGCA |
| | Antisense | GACGTGTGGGAATCTCTGGT |
| <i>Slc22a2</i> (OCT2) | Sense | AAATGGTCTGCCTGGTCAAC |
| | Antisense | AGGCCAACCACAGCAAATAC |
| <i>Slc22a6</i> (OAT1) | Sense | CTTCCTGTACACCGGAGAGC |
| | Antisense | AGCGCCGAAGATGAAGAGAG |
| <i>Slc22a6</i> (OAT3) | Sense | TCTGGCCTGGTTTGCTACTG |
| | Antisense | CGCCGGCCCAGATAACTTAT |
| <i>Slc47a1</i> (MATE1) | Sense | TCTTCAGACAGGACCCGGAT |
| | Antisense | GCGTTGACAAGGTTAGCTGC |
| <i>Slco4c1</i> (OATP4c1) | Sense | ATCCCCGCCTTCTTCGAATC |
| | Antisense | TGCTAATGTTTACCAGGCCGT |

Supplemental Table S3. Antibodies used for immunoblotting, immunohistochemistry, and immunofluorescence.

| Target | Source | Dilution | Method | Manufacturer |
|-----------------------------|--------|----------|----------------------|-----------------------------|
| <i>Primary antibodies</i> | | | | |
| OCT2 | Rabbit | 1:250 | Immunohistochemistry | ab230629; Abcam |
| | | 1:500 | Western blotting | |
| OAT1 | Rabbit | 1:4,000 | Immunohistochemistry | 26574-1-AP; Proteintech |
| | Rabbit | 1:500 | Western blotting | ab135924; Abcam |
| PDGFR β | Rabbit | 1:400 | Immunofluorescence | ab32570; Abcam |
| α SMA | Mouse | 1:500 | Immunofluorescence | ab215368; Abcam |
| | | | Western blotting | M0851; DAKO |
| AQP1 | Rabbit | 1:200 | Immunofluorescence | AB2219; Sigma-Aldrich |
| KIM1 | Goat | 1:400 | Immunofluorescence | AF1817; R&D Systems |
| <i>Secondary antibodies</i> | | | | |
| Rabbit | Goat | 1:2,000 | Western blotting | P0488; DAKO |
| Rabbit | Goat | 1:400 | Immunohistochemistry | P0488; DAKO |
| Mouse | Goat | 1:500 | Immunofluorescence | Alexa Fluor 488; Invitrogen |
| Rabbit | Donkey | 1:500 | Immunofluorescence | Alexa Fluor 647; Invitrogen |
| Goat | Donkey | 1:500 | Immunofluorescence | Alexa Fluor 680; Invitrogen |

Supplemental Table S4. Pure chemicals used as standards for targeted metabolomics.

| Chemical | Manufacturer | Concentration range (μM) | ESI mode |
|-------------------------|--|--------------------------|----------|
| L-Pipecolic acid | P2519, Sigma Aldrich, St. Louis, MI, USA | 500 μM – 0.9 μM | Positive |
| D-Galactonic acid | S960098, Sigma Aldrich, St. Louis, MI, USA | 500 μM – 2.1 μM | Negative |
| L-Kynurenic acid | K3375, Sigma Aldrich, St. Louis, MI, USA | 100 μM – 0.14 μM | Positive |
| L-Kynurenine | K8625, Sigma Aldrich, St. Louis, MI, USA | 400 nM – 0.5 nM | Positive |
| Hippuric acid | 112003, Sigma Aldrich, St. Louis, MI, USA | 500 μM – 0.7 μM | Positive |
| L-Tryptophan | T0254, Sigma Aldrich, St. Louis, MI, USA | 300 μM – 0.4 μM | Positive |
| 3-Indoleacetic acid | I2886, Sigma Aldrich, St. Louis, MI, USA | 500 μM – 0.7 μM | Positive |
| Phenylacetic acid | P16621, Sigma Aldrich, St. Louis, MI, USA | 450 μM – 0.6 μM | Negative |
| Indoxyl sulfate | I3875, Sigma Aldrich, St. Louis, MI, USA | 100 μM – 15.2 nM | Negative |
| p-cresol sulfate | 29504, Cayman Chemical, Ann Arbor, Michigan 48108, USA | 450 μM – 0.6 μM | Negative |
| 5-Sulfosalicylic acid | S2130, Sigma Aldrich, St. Louis, MI, USA | 1000 μM – 20 nM | Negative |
| N-Acetylneuraminic acid | A2388, Sigma Aldrich, St. Louis, MI, USA | 1000 μM – 20 nM | Negative |

ESI: Electric spray ionization

Supplemental Table S5. Pipeline of the statistical methodology for identification of features in Compound Discover.

| Node name | Processing step | Method description |
|------------------------------------|--------------------------------------|--|
| Align Retention Times (ChromAlign) | Retention Time Alignment | Retention time alignment performed by the ChromAlign algorithm: Sadygov et al. (https://doi.org/10.1021/ac060923y). |
| Group Compounds | [Compounds] Apply Peak Rating Filter | Only compounds with an Original Peak Rating greater or equal to 3 in at least 5 samples are kept for further processing. |
| Fill Gaps | Similar Features Search | Features search within a tolerance of 5 ppm. |
| Fill Gaps | Centroids Filtering | Filtered centroids with S/N threshold = 1.5. |
| Fill Gaps | Detection | Real detection (for more accurate areas) was performed. |
| Apply SERRF QC Correction | QC Correction Method | QC correction performed by using SERRF method: Fan et al. (https://doi.org/10.1021/acs.analchem.8b05592). |
| Apply SERRF QC Correction | Random Forest Settings | Random Forest was run with 200 trees. |
| Apply SERRF QC Correction | Gap Filled Values | Gap filled areas from QC samples were used for QC. |
| Apply SERRF QC Correction | Interpolation | Values were interpolated with a non-linear regression. |
| Apply SERRF QC Correction | Correction Acceptance | The maximum QC RSD allowed before correction was set to 30%, and the maximum allowed after correction was set to 25%. |
| Apply SERRF QC Correction | Correction Acceptance | The minimum percentage of usable hits (actual signals found) per compound is 50%. |
| Differential Analysis | [Compounds] Input Data | Normalized Peak Area. |
| Differential Analysis | [Compounds] Group Area | Median. |
| Differential Analysis | [Compounds] Data Transformation | Log-10 areas for p-value estimation. |
| Differential Analysis | [Compounds] Statistical Test | The p-value of per group ratio calculated by a one-way ANOVA model with Tukey as post hoc test. |
| Differential Analysis | [Compounds] p-value Correction | p-value adjusted using Benjamini–Hochberg correction for the false-discovery rate. |

Abbreviations: Ppm, parts per million; QC, quality control; RSD, relative standard deviation; S/N, Signal-to-noise.