ONLINE SUPPLEMENT

IDENTIFICATION OF CATHEPSIN L AS A POTENTIAL SEX-SPECIFIC BIOMARKER FOR RENAL DAMAGE

Yasmina Bauer*1, Patrick Hess*2, Changbin Qiu2, Axel Klenk1, Bérengère Renault1, Daniel Wanner2, Rolf Studer1, Nina Killer1, Anna Katharina Stalder1, Manuel Stritt1, Daniel Stefan Strasser1, Hervé Farine1, Katalin Kauser2, Martine Clozel2, Walter Fischli1 and Oliver Nayler1

1Drug Discovery Biology and 2Drug Discovery Pharmacology and Preclinical Development, Actelion Pharmaceuticals Ltd, Gewerbestrasse 16, CH-4123 Allschwil, Switzerland
* Both authors contributed equally to this work

Running Title: Biomarkers for renal damage in dTGR

Corresponding author: Yasmina Bauer
Actelion Pharmaceuticals Ltd
Gewerbestrasse 16
CH-4123 Allschwil, Switzerland
Phone: +41 61 565 6365
Fax: +41 61 565 65 00
Email: yasmina.bauer@actelion.com
EXPANDED MATERIALS AND METHODS

Renal Clearance Experiments
At week 8, after 24-hour urine collection, renal clearance measurements were performed as previously described \(^1\text{-}^5\). Briefly, the rats were anesthetized by intraperitoneal injection of 100 mg/kg thiobutabarbital-Na (Inactin, Byk-Gulden, Konstanz, Germany) and placed on a thermostatically controlled heating table to maintain body temperature at 36–38°C. A catheter was placed into the left femoral vein for infusion of synthetic plasma, inulin, and p-aminohippurate (PAH) and the left femoral artery was prepared for arterial blood pressure monitoring and periodic blood sampling. Through a small, suprapubic incision, a flanged catheter was placed in the urinary bladder for collection of urine. After a 45-minute equilibration period, two consecutive 20-minute urine collections were performed, with midpoint arterial blood sampling. Urine volume was measured gravimetrically, and inulin concentrations in urine and plasma were determined using the anthrone method \(^6\text{-}^7\). p-Aminohippurate concentrations in urine and plasma were measured colorimetrically \(^8\). These measurements allowed calculation of inulin clearance (equal to glomerular filtration rate, GFR), PAH clearance (equal to renal plasma flow, RPF, when adjusted for renal extraction of PAH), renal vascular resistance (RVR) and filtration fraction (FF) \(^1\text{-}^5\). The results of the two clearance periods were averaged.

Renal Histopathologic and Morphometric Analyses
The left kidney was embedded in paraffin. Sections of 5 \(\mu\)m thickness were stained with hematoxillin eosin and examined under light microscopy. Investigators, blinded as to experimental groups, assessed the severity of the morphological changes, i.e. the presence of glomerulosclerosis, tubulointerstitial lesions, and vascular lesions in the renal cortex. Each type of lesion was graded semiquantitatively as previously described \(^9\).

To assess glomerulosclerosis, 50 glomeruli in each kidney were observed at \(\times 400\) magnification and graded from 0 to 4+ by a semiquantitative score, according to the following criteria: 0, normal; 1+, slight glomerular damage such as a mild increase in the mesangial matrix and/or hyalinosis with focal adhesion, involving < 25% of the glomerulus; 2+, sclerosis of 25-50%; 3+, sclerosis of 50-75%; 4+, sclerosis of > 75% of the glomerulus. A glomerular damage index was calculated by averaging the grades assigned to all glomeruli.

Tubulointerstitial lesions (interstitial inflammation and fibrosis, tubular atrophy, and dilation with casts) were assessed at \(\times 100\) magnification in every third field of each kidney (total of 10 fields/kidney) and assigned an injury grade from 0 to 3: grade 0, normal; 1, lesions involving < 25%; 2, lesions involving 25-50%; 3, lesions involving > 50% of the field. A score for tubulointerstitial lesions for each kidney was obtained by averaging the grades given to all fields.
Vascular lesions in each kidney were attributed grades of severity from 0 to 4 in 20 fields at × 200 magnification. This grade was based on both the severity of vascular wall thickening and the extent of fibrinoid necrosis in afferent arterioles, interlobular arterioles, and small arteries: grade 0, normal vessel; 1, mild vascular wall thickening; 2, moderate thickening; 3, severe thickening (onion skin pattern); and 4, fibrinoid necrosis. The vascular lesion score was obtained using the same procedure as described above.

Quantitative morphometry was performed in order to assess total glomerular surface area, delimited by the internal edge of the Bowman’s capsule. In each kidney section, 30 consecutive glomeruli, randomly distributed over the depth of the cortex, were measured. Glomerular polar sections were not measured. The glomerular surface area was used as an indicator of glomerular hypertrophy.

REFERENCES
### Table S1. Renal handling of sodium and creatinine from weeks 4 to 7 after birth in male (n=13) and female (n=15) double transgenic rats (dTGRs).

Data are expressed as mean ± SEM. S$_{Na}$, serum sodium concentration; S$_{Cr}$, serum creatinine concentration; Ccr, creatinine clearance rate; V, urine flow rate; FE$_{Na}$, fractional excretion of sodium; T$_{Na}$, net tubular reabsorption of sodium. *, *P* < 0.05; ** *P* < 0.01, *** *P* <0.001, vs. female dTGRs.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time (weeks)</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>S$_{Na}$ (mmol/L)</td>
<td>144 ± 0.3</td>
<td>146 ± 0.3*</td>
<td>143 ± 0.3</td>
<td>144 ± 0.3**</td>
<td>139 ± 0.3</td>
</tr>
<tr>
<td>S$_{Cr}$ (mg/dl)</td>
<td>0.56 ± 0.03</td>
<td>0.60 ± 0.04</td>
<td>0.55 ± 0.03</td>
<td>0.54 ± 0.01</td>
<td>0.53 ± 0.02</td>
</tr>
<tr>
<td>Ccr (ml/min/100g)</td>
<td>0.31 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.35 ± 0.01</td>
<td>0.37 ± 0.01</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>V (ml/24h)</td>
<td>8.3 ± 0.9</td>
<td>10.9 ± 1.2</td>
<td>10.8 ± 1.3</td>
<td>16.0 ± 1.9*</td>
<td>11.4 ± 1.7</td>
</tr>
<tr>
<td>U$_{Na}$V (mmol/24h)</td>
<td>0.44 ± 0.02</td>
<td>0.58 ± 0.03***</td>
<td>0.52 ± 0.04</td>
<td>0.72 ± 0.04***</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>FE$_{Na}$ (%)</td>
<td>0.80 ± 0.06</td>
<td>0.97 ± 0.09</td>
<td>0.59 ± 0.03</td>
<td>0.68 ± 0.03</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>T$_{Na}$ (µmol/min)</td>
<td>39.6 ± 2.5</td>
<td>44.0 ± 3.3</td>
<td>63.0 ± 2.6</td>
<td>73.3 ± 2.6**</td>
<td>84.6 ± 3.6</td>
</tr>
</tbody>
</table>
Table S2. Body weight, relative kidney weight, and renal structural damage in male (n=13) and female (n=15) double transgenic rats (dTGRs) at week 8 after birth. Data are expressed as mean ± SEM. BW, body weight; KW, kidney weight. *, P < 0.05; **, P < 0.01; ***, P < 0.001, vs. female dTGRs.
Supplementary Figures
Figure S1. Body weight (top panel) and daily food intake (bottom panel) from weeks 4 to 7 after birth in male and female double transgenic rats (dTGRs). *, $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$, vs. female dTGRs.
Figure S2. Glomerular filtration rate (GFR), renal plasma flow (RPF), filtration fraction (FF) and renal vascular resistance (RVR) in male (n = 8) and female (n = 15) double transgenic rats (dTGRs) at week 8 after birth. *, P < 0.05; ** P < 0.01, vs. female dTGRs.
**Figure S3. Summary of the main biological responses involved in the expression dimorphism in dTGR.** The genes are grouped into 3 categories (i) genes that show sex-related expression changes in both dTGR and Sprague-Dawley (SD) (yellow), (ii) genes that show sex-related expression changes in dTGR but not in SD (red) and (iii) genes that show sex-related expression changes in SD but not in dTGR (blue).
Figure S4. Relative expression levels of genes that show gender-related expression changes in dTGR (VEGFA), or in both dTGR and SD (CTSL2 and SOD3) and that were verified by qPCR. The x-axis shows the log10 of gene expression; i.e., a value of 5 corresponds to an expression value of 100,000. In all cases the differences between dTGR and SD are significant at a threshold of 0.01 using a t-test.
Figure S5. Boxplot presentation of morphometric analysis of immunohistochemical staining for CTSL in the rat kidney. Relative values of CTSL staining normalized to the tubular area in a longitudinal section of the kidney. Left, comparison of female dTGR versus SD controls; right, males dTGR versus SD controls. Significance was calculated with a Welch one sided t-test and reached significance for male rats, $P = 0.004$