NIR fluorescent probe for the detection of renal damage based on overrepresentation of Alanine Aminopeptidase enzyme

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Materials. Nile Blue perchlorate (NB), Fmoc-L-alanine (Fmoc-Ala-OH), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), piperidine, triethylamine, dimethyl sulfoxide (DMSO), dichloromethane, $MgCl_2$, K_2CO_3, $NaHCO_3$, NH_4Cl, H_2O_2, vitamin C, glutathione, cysteine, β-galactosidase from *Escherichia coli* (β-Gal), phosphatase from bovine intestinal mucosa, nitroreductase from *Escherichia coli* and leucine Aminopeptidase from porcine kidney (LAP) were obtained from Sigma-Aldrich. Recombinant Human Aminopeptidase N/CD_{13} Protein (APN) from mouse myeloma cell line, were purchased from R&D Systems. Invitrogen™ Human Aminopeptidase N/ANPEP ELISA Kit was obtained from Sigma-Aldrich. Urine samples employed for this work were from voluntary individuals and informed consents were obtained for experimentation. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker FT-NMR Avance 400 (Ettlingen, Germany) spectrometer at 300 K, using TMS as an internal standard. Fluorescence spectroscopy was carried out in a JASCO spectrofluorometer FP-8500 and using a PerkinElmer multimode plate reader (EnSpire). Absorption spectra were collected in a JASCO V-650 spectrophotometer. High-pressure liquid chromatography coupled to mass spectrometry (HPLC-MS) was recorded with an Agilent 1620 Infinity II HPLC coupled to a mass spectrometer Agilent Ultivo equipped with a triple QTOF detector.

Table S1. Comparison of the **NB-ALA** probe with other fluorescent probes for the detection of APN in urine as a biomarker of renal damage.

Probe	Excitation (nm)	Emission (nm)	Time (min)	LOD (water) (ng/ml)	LOD (urine) (ng/ml)	Lineal range (ng/ml)	*In vivo* model	
NB-ALA	530	630	30	0.5	2	0-200	Mice Folic Acid -Induced Acute Kidney Injury Model	This work
MUR2	480	590	60	Not reported	Not reported	0-100	Mice Cisplatin/ Doxorubicin-Induced Acute Kidney Injury Model	Pu et al, Anal. Chem. 2020, 92, 8, 6166–6172
CVN	525	575/626	30	0.033	Not reported	0–6	Not reported	He et al, Anal. Chem. 2017, 89, 5, 3217–3221

Synthesis and characterization of NB-ALA

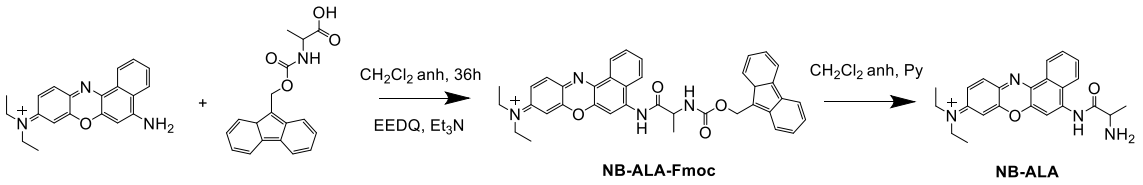

Figure S1. Synthetic route for the **NB-ALA** probe.

*Synthesis of **NB-ALA-Fmoc***: Fmoc-Ala-OH (500 mg, 1.6 mmol), EEDQ (643 mg, 2.6 mmol), and triethylamine (360 µL, 2.6 mmol) were dissolved in dry CH_2Cl_2 (5 mL) with stirring at room temperature for 1 h. Then, NB (960 mg, 2.3 mmol) dissolved in CH_2Cl_2 (5 mL) was added and the reaction mixture was further stirred at room temperature for 36 h. The solvent was evaporated under reduced pressure and the residue was purified by silica gel chromatography with hexane as eluent, yielding compound **NB-ALA-Fmoc** (500 mg, yield 52%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69 (d, J = 4.7 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 7.88 (d, J = 8.0 Hz, 1H), 7.56 (d, J = 8.3 Hz, 2H), 7.48 (q, J = 8.0 Hz, 3H), 7.30 (t, J = 7.2 Hz, 2H), 7.20 – 7.04 (m, 4H), 6.52 (dd, J = 8.5, 6.1 Hz, 1H), 5.98 (dt, J = 14.3, 5.3 Hz, 1H), 4.14 (m, 1H), 3.93 (qd, J = 7.1, 2.0 Hz, 2H), 3.59 – 3.40 (m, 1H), 3.10 (d, J = 19.1 Hz, 1H), 1.83 (d, J = 7.6 Hz, 2H), 1.24 – 1.02 (m, 6H), 1.00 – 0.84 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.79, 141.33, 129.53, 127.70, 127.06, 125.11, 124.08, 123.52, 119.99, 66.95, 61.37, 52.54, 47.23, 41.91, 31.93, 31.62, 29.70, 22.70, 21.89, 14.18. ESI-MS: Calculated for $C_{38}H_{35}N_4O_4^+$ ($M+H^+$): 611.72m/z; ($M+H^+$) measured 612.26 m/z.

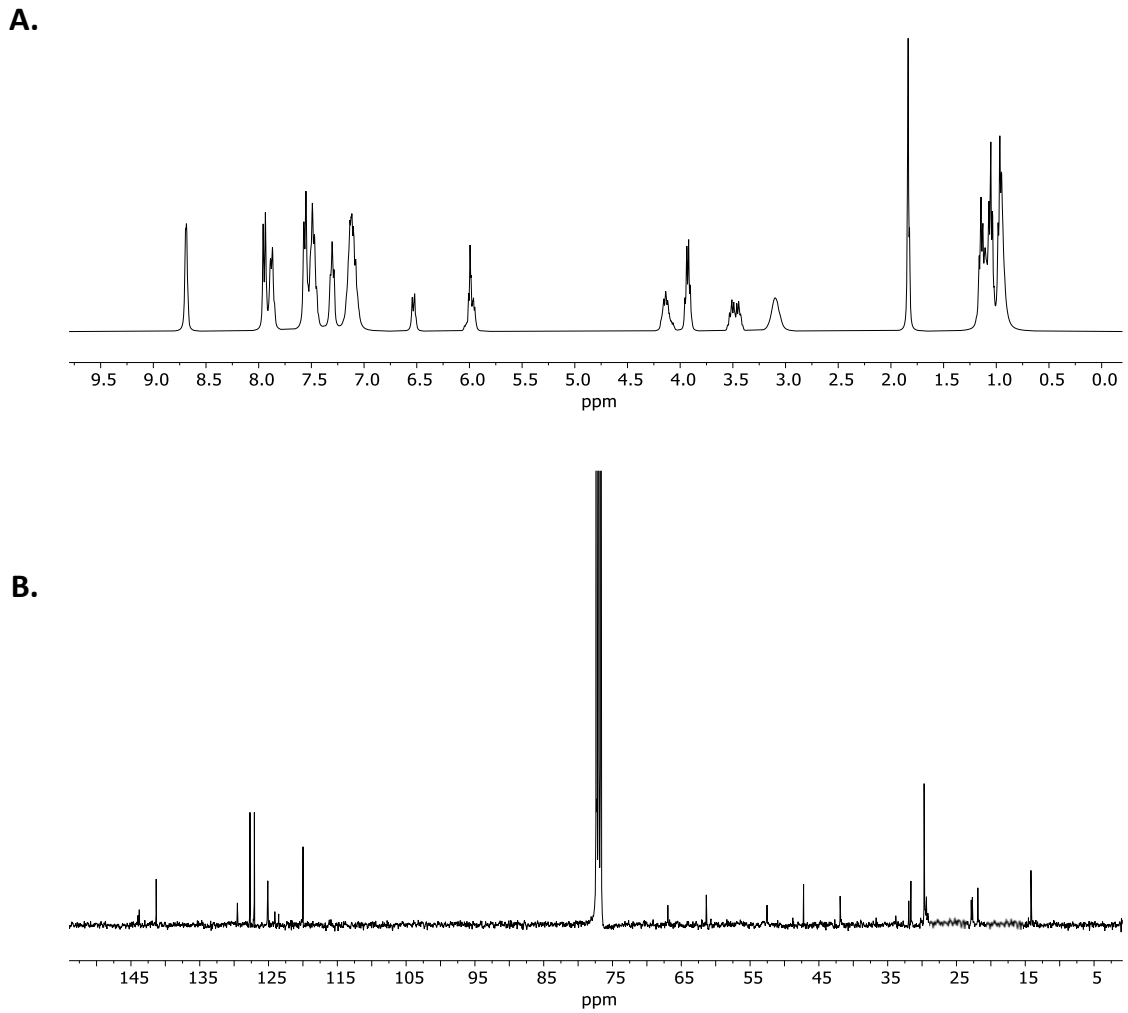

Figure S2. ^{1}H-NMR and ^{13}C-NMR of **NB-ALA-Fmoc.**

*Synthesis of **NB-ALA***: Compound **NB-ALA-Fmoc** (500 mg, 0.8 mmol) was dissolved in CH_2Cl_2 (10 mL) containing piperidine (160 µL, 1.63 mmol) and stirred at room temperature overnight. The solvent was removed by evaporation under reduced pressure, and the crude product was purified by silica gel chromatography eluted with hexane-ethyl acetate (v/v, 1:1), affording probe **NB-ALA** (300 mg, yield 60%).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8,93 (d, J =2,9 Hz, 1H); 8,17 (d, J =8,1 Hz, 1H); 8,12 (d, J =8,6 Hz, 1H); 7,83 (d, J = 8,2 Hz, 1H); 7,73 (ddd, J = 8.5, 6.7, 1.5 Hz, 1H); 7,55 (t, J = 13,5; 6,0 Hz, 1H); 7,41 (dd, J= 8,3; 4,2 Hz, 1H); 7,26 (s, 2H); 3,48 (dd, J=14,1; 7,0 Hz, 1H) ; 3,38 (dd, J=14,1; 7,1, 1H);2,88 (s,1H); 2.43 (s, 4H); 1,55 (dd, J= 10,7; 5,4 Hz, 6H) ; 1,43 (dd, J=13,9; 8,8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 150.91, 148.81, 136.52 , 129.95 , 128.78 , 128.26 , 127.01 , 121.55 , 53.51 , 30.16, 26.33 , 25.29 . ESI-MS: Calculated for $C_{23}H_{25}N_4O_2^+$ ($M+H^+$): 389.47 m/z; ($M+H^+$) measured 390.18 m/z.

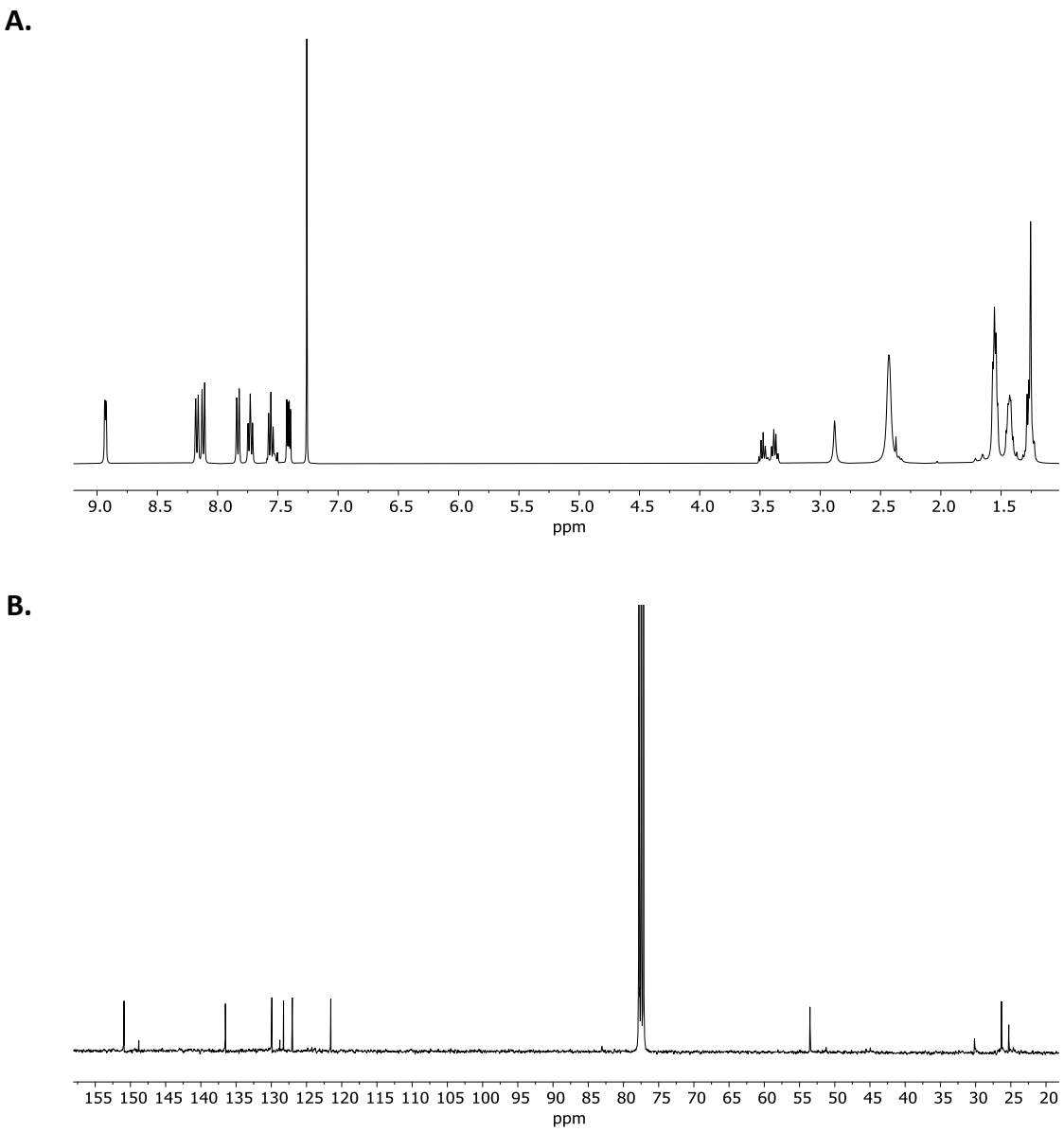

Figure S3. ^{1}H-NMR and ^{13}C-NMR of **NB-ALA**.

Photophysical characterization of NB and NB-ALA. Molar extinction coefficients, stokes shift and quantum yields were determined for **NB** and **NB-ALA** (Table S1). Quantum yields values were measured using **NB** dissolved in water as standard (Φ = 0.01) while molar extinction coefficients were obtained in the adsorption maximum at 530 nm.

Table S2. Photophysical parameters for **NB**, **NB-ALA**

	NB	NB-ALA
Molar extinction coefficient [L/(mol/cm)]	4.30×10^4	1005
Quantum yield	0.01	0.00028
Stokes shift	37	45

Hydrolysis of NB-ALA by APN. 4.5 µl of APN water solution were added to a water solution of **NB-ALA** (20 µM). Hydrolysis was monitored, after 15 min of incubation, through HPLC-MS measurements using a Kromasil C18 column, 0.7 ml/min, with acetonitrile-MeOH gradient elution from 70:30 at 0 min to 50:50 at 15 min. Besides, chromatograms of **NB** and **NB-ALA** alone were also obtained using the same experimental conditions.

HPLC-MS characterization of NB and NB-ALA

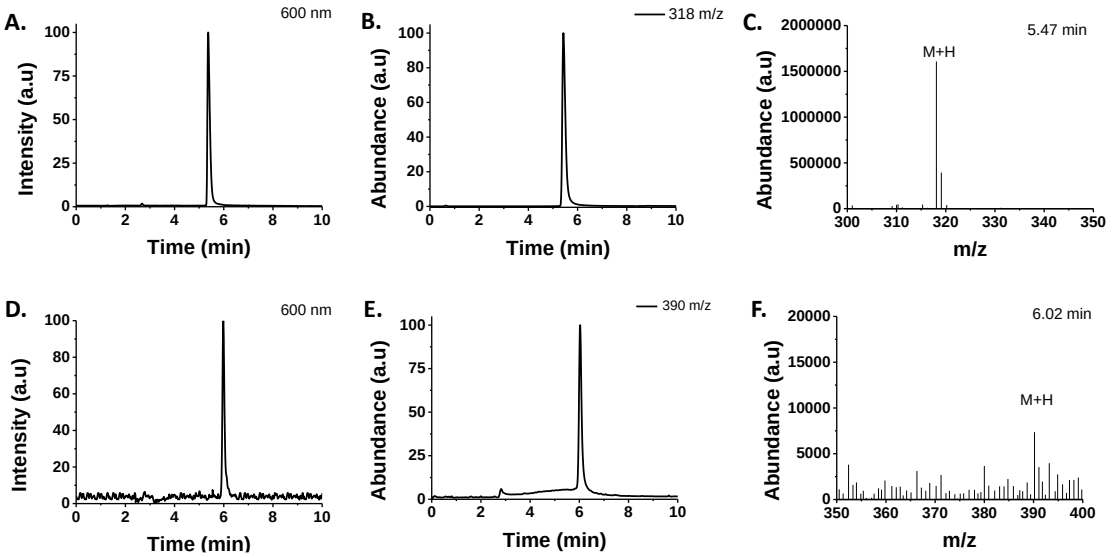

Figure S4. (A) UV chromatogram of **NB** at 600 nm; (B) Mass spectrum for **NB** obtained from the peak at 5.47 min; (C) Mass spectrum of B showing a signal at 318 m/z corresponding to M+H of **NB**; (D) UV chromatogram of **NB-ALA** at 600 nm; (E) Mass spectrum for **NB-ALA** obtained from the peak at 6.02 min; (F) Mass spectrum of E showing a signal at 390 m/z corresponding to M+H of **NB-ALA**.

APN activity measurement. The APN enzyme used in the different experiments have an activity of 22.1 mU/ml. The activity of the enzyme was determined by its ability to cleave the fluorogenic peptide substrate, Ala-7-amido-4-methylcoumarin (Ala-AMC). For this purpose, solutions of 0.2 µg/mL APN enzyme and 200 µM Ala-AMC in 50 mM Tris, pH 7.0 were prepared. Next, 50 µL of 0.2 µg/mL APN enzyme was loaded onto a plate and the reaction was initiated by adding 50 µL of 200 µM Ala-AMC. An Ala-AMC blank containing 50 µL of assay buffer and 50 µL of substrate was included. Fluorescence on the multiplate reader (380 nm and 460 nm excitation and emission) in kinetic mode for 5 min was measured. Specific enzyme activity was determined using equation S1.

$$\textbf{Specific Activity}\ ((\text{pmol/min})/\ (\mu\text{g})) = \frac{\textbf{Adjusted Vmax}^{*}\textbf{x Conversion Factor}^{**}}{\textbf{Amount of enzyme}} \quad \text{(S1)}$$

*Adjusted for substrate blank

**Derived using calibration standard Ala-7-amido-4-methyilcoumarin

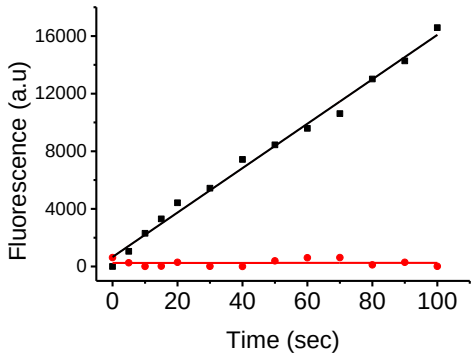

Figure S5. Monitoring of fluorescence at 460 nm due to alanine aminopeptidase activity, which induced the hydrolysis of fluorogenic peptide substrate Ala-7-amido-4-methyilcoumarin (Ala-AMC). Commercial enzyme solution (black) and blank (red).

General procedure for APN detection. Fluorescence emission measurements of **NB-ALA** were carried out with 4.0 µL of the probe from a stock solution (1.0 x 10^{-3} M in DMSO), followed by addition of APN solution in water. Final volume was adjusted to 200 µl with distilled H_2O at pH 7.4. After incubation at 37°C for 30 min in a thermostat, solution was transferred to a quartz cell of 1 cm optical length to measure the fluorescence (λ_{ex} = 530 nm). A blank solution without APN was prepared and measured under the same conditions.

Calibration curve in water/DMSO. The limit of detection (LOD) was obtained from the plot of fluorescence intensities at 630 nm upon excitation at 530 nm versus APN concentration in ng/ml. LOD was calculated by using the equation (S2), where K=3; Sb is the standard deviation of the blank and m is the slope of the calibration curve. The resulting LOD was 1 ng/ml.

$$LOD = K * \frac{Sb}{m} \qquad \text{(S2)}$$

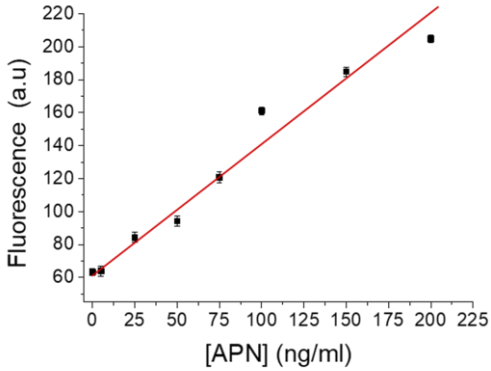

Figure S6. Calibration curve of **NB-ALA** (20 µM) at different APN concentrations in water-DMSO 99:1 v/v at pH 7.4. Fluorescence measures were taken 30 min after APN addition. Error bars are expressed as 3σ for three independent experiments.

Detection of NB-ALA in human doped urine. In this study a sample of urine was employed. Urine sample was taken from a healthy volunteer of 28 years old, from whom informed consent was obtained. In a common experiment of urine (10 µL) were diluted with distilled H_2O (1:20 v/v) and doped whit different amounts of APN followed by the addition of 4.0 µL of **NB-ALA** stock solution (1.0 x 10^{-3} M in DMSO). Distilled H_2O was added upon a final volume of 200 µl per vial. After incubation at 37°C for 30 min in a thermostat, the reaction solution was transferred to a quartz cell of 1 cm optical length to measure the fluorescence (λ_{ex}= 530 nm). Blank solutions without APN enzyme were prepared and measured under same conditions.

APN-induced hydrolysis of NB-ALA probe. The emission intensity at 630 nm (excitation at 530 nm) after APN-induced hydrolysis of **NB-ALA** at different times and in the presence of different enzyme amounts was recorded. The obtained results are shown in Table S3.

Table S3. Hydrolysis of **NB-ALA** probe in the presence of several amounts of APN enzyme.

	Normalized Fluorescence at 630 nm (a.u.)			
Time (min)	0.05 (μg/mL)	0.2 (μg/mL)	1 (μg/ml)	2 (μg/ml)
0	0.18	6.40	11.79	11.50
5	0.67	9.28	21.33	39.78
10	1.29	12.63	30.81	54.01
15	1.82	21.58	38.09	72.52
20	15.89	27.30	49.19	80.55
30	24.19	35.93	53.40	90.16
40	36.56	47.14	66.70	99.99
50	46.89	58.50	83.10	-
60	59.83	63.36	-	-

Determination of APN in Urine by Human Aminopeptidase N/ANPEP ELISA Kit. The concentration of APN in health human urine was determined by measuring the absorbance at 450 nm using a commercial Human Aminopeptidase N/ANPEP ELISA kit. A series of 100 μL of standard solutions of APN and 100 μL of diluted urine were added to the ELISA kit wells. After incubation with the antigen at room temperature for 2.5 h, the solution in each well was removed and washed 4 times with washing buffer, followed by addition of 100 μL of biotin conjugate and incubation at room temperature for 1 h. Later, all of the wells were washed 4 times with 300 μL of the washing buffer. Then, 100 μL of Streptavidin-HRP solution was added to each well and incubated at room temperature for 45 min. After that, all of the wells were washed 4 times with 300 μL of the washing buffer, followed by addition of 100 μL of TMB color developing agent solution. After further incubation at room temperature for 30 min in the dark, 50 μL of stop solution was added to each well, and the absorbance of each well was measured immediately on a microplate reader at 450 nm (see Table S4).

Table S4. APN in human urine determined by a human aminopeptidase N/ANPEP ELISA kit.

APN spiked (ng/mL)	APN detected by ELISA (ng/mL)	% of APN found by ELISA
0	0.10	-
1.0	0.98	98.0
2.5	2.41	96.0
5.0	4.85	97.0

Mouse model. C57BL/6 J male mice were maintained at the Institut de Recerca Biomèdica (IRB). All animal procedures were carried out in compliance with the regulations of the Animal Care and Use Ethical Com-

mittee of the Barcelona Science Park (CEEA-PCB) and the Catalan Government under the recommendations of the FELASA (11054-P2). To generate renal fibrosis, 8-10-week-old male BL6/J mice were treated intraperitoneally once with a high dose (250 mg/kg body weight) folic acid (F7876, Sigma; dissolved in 300 mM sodium bicarbonate (S5761, Sigma)). After 2 days, blood was taken by puncturing the sub-mandibular vein and serum was generated (centrifugation of blood samples at 2500 g, RT for 15 min). Mice were monitored longitudinally by measuring the body weight and urine. Urine was taken in the early evening (= spot urine) and urine density was determined using a refractometer. At 7 and 15 days, mice were sacrificed, and kidneys were harvested, paraffin-embedded and sectioned. Sections were stored at RT until histochemical staining's were performed with a single dose of either 250 mg/kg of folic acid or vehicle. Sirius red/ fast green staining was performed in order to corroborate the presence of renal fibrosis. Briefly, paraffin-embedded 6um kidney slides were incubated with the mordant thiosemicarbazide 99% (TSC) (Sigma, T33405) for 10 min. Then they were washed in distilled water for 10 min prior incubation with 0.1% Fast green (Sigma, FCF F7552) for 20 min and rinsed with 1% acetic acid (Sigma, 320099) for 1 min. Finally, the stained samples were dehydrated and mounted with Mounting Medium, toluene-free (CS705, Dako, Agilent) using a Dako CoverStainer.

Acute injury mice model characterization

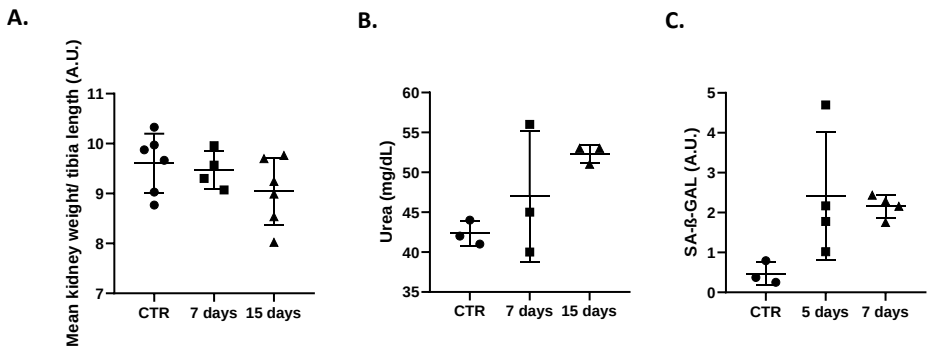

Figure S7. Macroscopic phenotyping (A), biochemical phenotyping (B) and senescence phenotyping for acute injury mice (C).