SUPPLEMENTAL MATERIALS:

Renal function

Average kidney weight was 5.8 ± 0.2 mg/g bw. in untreated wild-type mice and 5.7 ± 0.2 mg/g bw. in untreated *kl/kl* mice. NH_4Cl treatment increased the kidney mass significantly ($p < 0.001$) to 7.1 ± 0.2 mg/g bw. in wild-type mice and to 7.0 ± 0.1 mg/g bw. in *kl/kl* mice. Neither before nor after NH_4Cl treatment the ratio of kidney mass over body weight was significantly different between wild-type mice and *kl/kl* mice. Measurements in metabolic cages were performed in wild-type mice, NH_4Cl treated wild-type mice and NH_4Cl treated *kl/kl* mice. Because untreated *kl/kl* mice do not survive this procedure, in a fourth group of NH_4Cl treated *kl/kl* mice the treatment was stopped at the age of seven weeks. After a 3 week wash-out phase the animals were housed in the metabolic cages. According to the measurements (Supplemental Figures 1-3) body weight, food and fluid intake, urinary volume and pH, fecal mass, plasma creatinine concentration, creatinine excretion, creatinine clearance, plasma protein, plasma albumin, urinary albumin excretion, plasma cystatin C, urinary cystatin C excretion, urinary urea excretion and urinary ammonia excretion were similar in *kl/kl* mice and wild-type mice. Plasma urea concentration was, however, significantly higher and plasma ammonia concentration tended to be higher in the previously NH_4Cl treated *kl/kl* mice than in wild-type mice. In both, *kl/kl* mice and wild-type mice NH_4Cl treatment significantly decreased urinary pH, and significantly increased plasma ammonia concentration, urinary urea excretion, urinary ammonia excretion and, in wild-type mice, plasma urea concentration. All other measured parameters were not significantly modified by the NH_4Cl treatment.

Detailed description of Materials and Methods

Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities (Regierungspräsidium Tübingen). Male klotho-hypomorphic mice (*kl/kl*) were compared to male wild-type mice (*WT*). Origin, breeding and genotyping of the mice were described previously.[1] The mice had access to either tap water or a solution of NH_4Cl (15 g/l) in tap water ad libitum and were fed a standard chow diet. The lifelong NH_4Cl treatment started with the mating of the parental generation and was maintained from pregnancy until the offspring was sacrificed. Where not otherwise indicated, blood was taken and the animals sacrificed for histology and quantitative RT-PCR at the age of 7-8 weeks.

Blood chemistry

Blood specimens were obtained from male klotho-hypomorphic mice (*kl/kl*) and male wild-type mice (*WT*) by puncturing the retro-orbital plexus. Ammonia was determined enzymatically using glutamate dehydrogenase with NADPH as Cofactor. The reaction was photometrically followed and quantified using an ADVIA 1650 analyser (Siemens, Fernwald, Germany). The plasma phosphate and calcium concentrations were determined utilizing a photometric method (FUJI FDC 3500i, Sysmex, Norsted, Germany). The plasma aldosterone concentrations (Alpha Diagnostics International, Texas; USA), 1,25$(OH)_2$-vitamin D_3 concentrations (IDS, Boldon, UK), C-terminal FGF23 (ImmunDiagnostics, Boston, USA), intact FGF23 (Kainos Laboratories, Tokyo, Japan) and PTH (Immutopics, San Clemente, USA) were determined by ELISA. The plasma ADH concentration was determined utilizing a commercial EIA-Kit (AVP EIA Kit, Phoenix Europe, Karlsruhe, Germany). For the determination of blood pH, blood CO_2 and electrolytes a blood gas analyzer was used (EDAN care lab i15, EDAN Instruments, China)

Metabolic cage studies

To assess metabolic parameters male wild-type mice and klotho-hypomorphic mice were housed in metabolic cages at the age of 10 weeks. The wild-type mice were either untreated or

lifelong treated with NH_4Cl solution (0.28 M). Due to their strong phenotype untreated klotho-hypomorphic mice cannot be kept in metabolic cages. For this reason only klotho-hypomorphic mice with lifelong NH_4Cl treatment (0.28 M) and klotho-hypomorphic mice with NH_4Cl treatment until the the age of 7 weeks and subsequent 3 weeks without treatment were used for this experiment. After a three day training phase specimens were collected in a four day acquisition phase. Bodyweight, fluid intake, food intake as well as urine volume and fecal mass were measured daily at the same time. At the last day of the experiment blood was drawn by puncturing the retro-orbital plexus. Urinary pH was measured by a pH microelectrode (Mettler-Toledo, Greifensee, Schweiz). Plasma creatinine was measured by an enzymatic procedure (Mouse Creatinine Kit, Crystal Chem, Downers Grove, USA). Urinary creatinine concentrations were determined with a urinary creatinine detection kit (Arbor Assays, Ann Arbor, USA) utilizing the Jaffe method. Plasma urea and total protein were measured photometrically (FUJI FDC 3500i, Sysmex, Norsted, Germany). ELISA was employed for the measurement of plasma and urinary albumin (ICL, Portland, USA) as well as plasma and urinary cystatin C (R&D Systems, Minneapolis, USA). Urinary urea was determined by a colorimetric detection kit (DetectX Urea Nitrogen, Arbor Assays, Ann Arbor, USA). Urinary ammonia was measured with the EnzyChrom Ammonia/Ammonium Assay Kit (BioAssay Systems, Hayward, USA)

Histology

To elucidate trachea, lung, renal, gastric and vascular pathology, tissues from male klotho-hypomorphic mice with or without treatment with NH_4Cl (0.28 M in tap water) have been embedded in paraffin, cut in 2–3 µm sections and stained with von Kossa and H&E.[2]

Cell culture of HAoSMCs

Primary human aortic smooth muscle cells (Invitrogen) were routinely cultured in Waymouth's MB 752/1 medium and Ham's F-12 nutrient mixture (1:1, Gibco, Life Technologies)

supplemented with 10% FBS (Gibco, Life Technologies) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies). HAoSMCs were grown to confluency and used in all experiments from passages 4 to 11. HAoSMCs were transfected with 2 µg DNA encoding human *NFAT5* in pcDNA6V5-HisC vector or with 2 µg DNA of empty pcDNA6V5-HisC vector using X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's protocol. The cells were used 48 hours after transfection. Transfection efficiency was verified by quantitative RT-PCR. HAoSMCs were treated for 24 hours with 2 mM β-glycerophosphate (Sigma-Aldrich), with 500 µM NH_4Cl (Sigma-Aldrich) or with 10 ng/ml human TGFB1 (R&D Systems).

The pH of the culture media was measured before and after 24 hours treatment of HAoSMCs with 2 mM β-glycerophosphate and/or 500 µM NH_4Cl. At the beginning of the treatment, the pH was 7.83 ± 0.03 in culture media from untreated HAoSMCs, 7.82 ± 0.03 in culture media from HAoSMCs treated with 2 mM β-glycerophosphate and 7.83 ± 0.03 in culture media from HAoSMCs treated with 2mM β-glycerophosphate and 500 µM NH_4Cl. After 24 hours of treatment, the pH was 7.68 ± 0.07 in culture media from untreated HAoSMCs, 7.58 ± 0.04 in culture media from HAoSMCs treated with 2 mM β-glycerophosphate and 7.63 ± 0.04 in culture media from HAoSMCs treated with 2mM β-glycerophosphate and 500 µM NH_4Cl.

Quantitative RT-PCR

After sacrificing the animals, tissues were immediately snap frozen in liquid nitrogen. Total RNA was isolated from aortic tissues of male klotho-hypomorphic mice (*kl/kl*) and male wild-type mice (*WT*) by using Trifast Reagent (Peqlab) according to the manufacturer's instructions. HAoSMCs were washed with PBS and total RNA was isolated using Trifast Reagent (Peqlab) according to the manufacturer's instructions. Reverse transcription of 2 µg RNA was performed using oligo$(dT)_{12-18}$ primers (Invitrogen) and SuperScriptIII Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed with the iCycler iQ^{TM} Real-Time PCR Detection System

(Bio-Rad Laboratories) and iQ™ Sybr Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. The following mouse primers were used (5'→3' orientation):

TN alkaline phosphatase fw: TTGTGCCAGAGAAAGAGAGAGA;

TN alkaline phosphatase rev: GTTTCAGGGCATTTTTCAAGGT;

Cbfa1 fw: AGAGTCAGATTACAGATCCCAGG;

Cbfa1 rev: AGGAGGGGTAAGACTGGTCATA;

Gapdh fw: AGGTCGGTGTGAACGGATTTG;

Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;

Glb1 fw: TGGCTGGGCTGAATGCTATC;

Glb1 rev: TGGTTGGGGTTCATGGAAGTT;

Nfat5 fw: CTGTAGGCGATCTGTTGGGG;

Nfat5 rev: CTGGTGCTCATGTTACTGAAGTT;

Sox9 fw: AGTACCCGCATCTGCACAAC;

Sox9 rev: ACGAAGGGTCTCTTCTCGCT.

p21 fw: CCTGGTGATGTCCGACCTG;

p21 rev: CCATGAGCGCATCGCAATC;

Pai-1 fw: TTCAGCCCTTGCTTGCCTC;

Pai-1 rev: ACACTTTTACTCCGAAGTCGGT;

Tgfb1 fw: CTCCCGTGGCTTCTAGTGC;

Tgfb1 rev: GCCTTAGTTTGGACAGGATCTG.

The following human primers were used (5'→3' orientation):

TN alkaline phosphatase fw: GGGACTGGTACTCAGACAACG;

TN alkaline phosphatase rev: GTAGGCGATGTCCTTACAGCC;

CBFA1 fw: GGAAGGGCTTGATTGACGTG;

CBFA1 rev: CAGAACCAAACATAGCACTGACT;

GAPDH fw: GAGTCAACGGATTTGGTCGT;

GAPDH rev: GACAAGCTTCCCGTTCTCAG;

GLB1 fw: TATACTGGCTGGCTAGATCACTG;

GLB1 rev: GGCAAAATTGGTCCCACCTATAA;

NFAT5 fw: GGGTCAAACGACGAGATTGTG;

NFAT5 rev: GTCCGTGGTAAGCTGAGAAAG;

SOX9 fw: AGCGAACGCACATCAAGAC;

SOX9 rev: CTGTAGGCGATCTGTTGGGG.

p21 fw: TGTCCGTCAGAACCCATGC;

p21 rev: AAAGTCGAAGTTCCATCGCTC;

PAI-1 fw: ACCGCAACGTGGTTTTCTCA;

PAI-1 rev: TTGAATCCCATAGCTGCTTGAAT;

TGFB1 fw: CAATTCCTGGCGATACCTCAG;

TGFB1 rev: GCACAACTCCGGTGACATCAA.

The specificity of the PCR products was confirmed by analysis of the melting curves and in addition by agarose gel electrophoresis. All PCRs were performed in duplicate, and mRNA fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method using GAPDH as internal reference.

Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using ANOVA followed by posthoc analysis or unpaired t-test, where appropriate. For the life span experiments, SAS Jmp version 10.0.2 (SAS Institute Inc., Cary, NC, USA) was used. Only results with $p < 0.05$ were considered statistically significant

Supplemental Figures

Supplemental Figure 1:

Basic metabolic parameters of wild-type mice with or without NH_4Cl treatment as well as kl/kl mice with NH_4Cl treatment or discontinued NH_4Cl treatment.

Arithmetic means ± SEM (n=5) of (**A**) bodyweight, (**B**) food intake, (**C**) fluid intake, (**D**) urine excretion, (**E**) urinary pH and (**F**) fecal mass of wild-type mice (*WT*, white bars) with (NH_4Cl) and without (Control) NH_4Cl treatment and klotho-hypomorphic mice (*kl/kl*, black bars) with NH_4Cl treatment (NH_4Cl) and discontinued NH_4Cl treatment (Control). Animals were kept in metabolic cages to obtain the specimens. #($p < 0.05$) indicate statistically significant differences from untreated mice.

Supplemental Figure 2:

The effect of NH_4Cl administration on kidney function of wild-type mice as well as kl/kl mice with NH_4Cl treatment or discontinued NH_4Cl treatment.

Arithmetic means ± SEM (n=5) of (**A**) plasma creatinine concentrations, (**B**) urinary creatinine excretion, (**C**) GFR, (**D**) plasma protein concentration, (**E**) plasma albumin concentration, (**F**) urinary albumin excretion, (**G**) plasma cystatin C concentration and (**H**) urinary cystatin C excretion of wild-type mice (*WT*, white bars) with (NH_4Cl) and without (Control) NH_4Cl treatment and klotho-hypomorphic mice (*kl/kl*, black bars) with NH_4Cl treatment (NH_4Cl) and discontinued NH_4Cl treatment (Control). Animals were kept in metabolic cages to obtain the specimens.

Supplemental Figure 3:

The effect of NH_4Cl on nitrogen metabolism of wild-type mice as well as kl/kl mice with NH_4Cl treatment or discontinued NH_4Cl treatment.

Arithmetic means ± SEM (n=5) of (**A**) plasma urea concentrations, (**B**) urinary urea excretion, (**C**) plasma ammonia concentrations and (**D**) urinary ammonia excretion of wild-type mice (*WT*, white

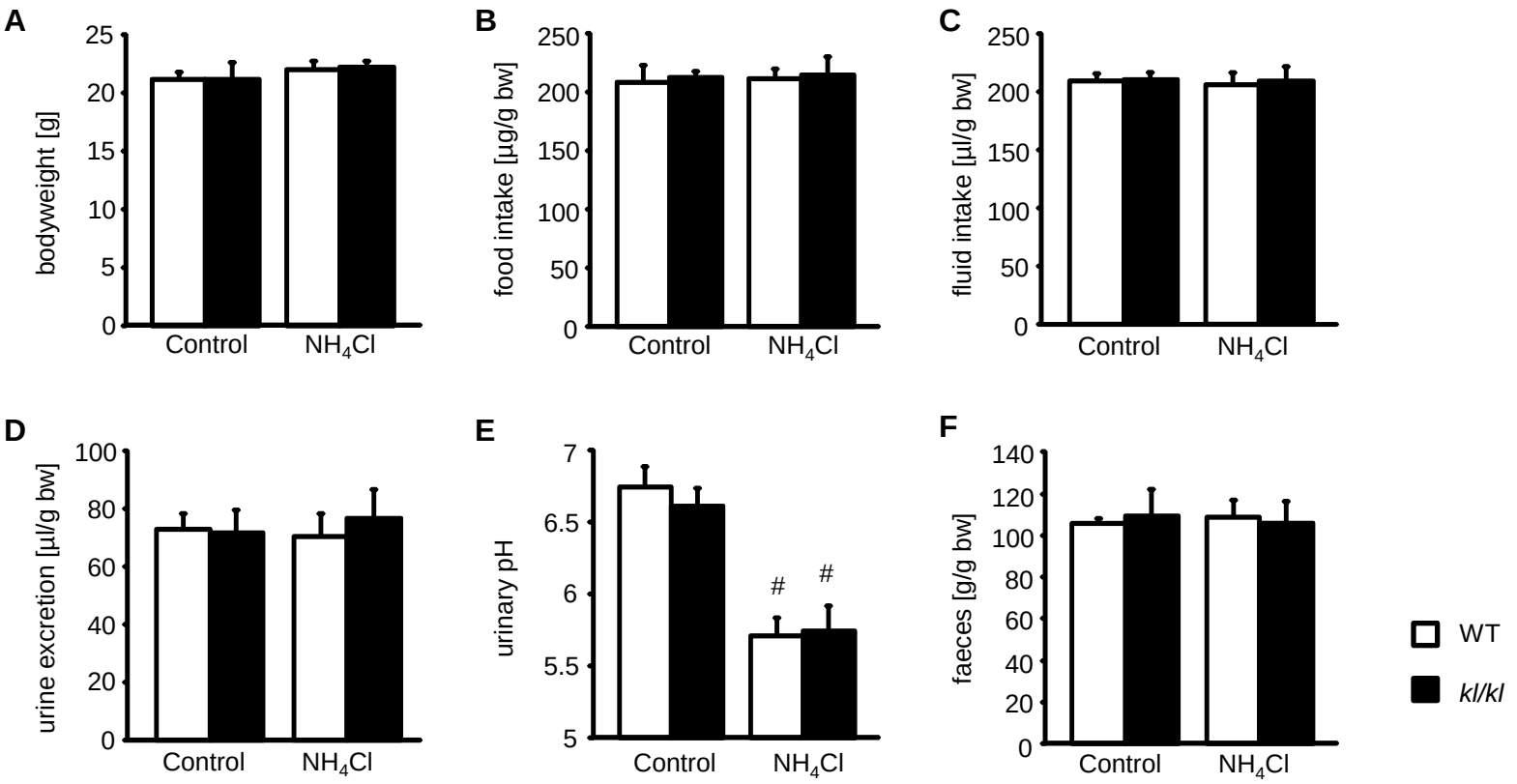
bars) with (NH_4Cl) and without (Control) NH_4Cl treatment and klotho-hypomorphic mice (*kl/kl*, black bars) with NH_4Cl treatment (NH_4Cl) and discontinued NH_4Cl treatment (Control). Animals were kept in metabolic cages to obtain the specimens. # ($p < 0.05$) indicate statistically significant differences from untreated mice.

Supplemental References

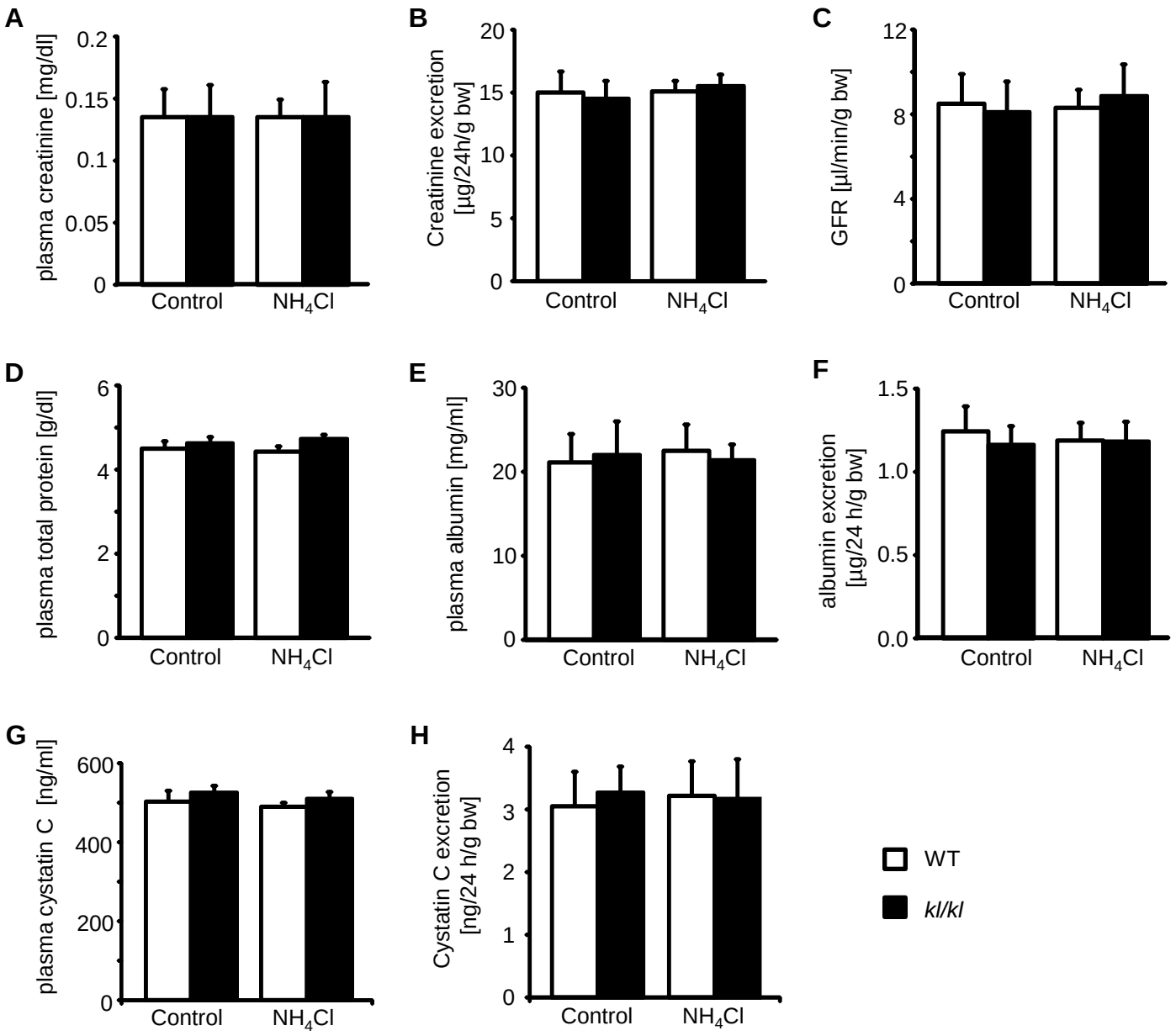
1. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, *et al.*: Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 390(6655):45-51, 1997.

2. Mossbrugger I, Hoelzlwimmer G, Calzada-Wack J, Quintanilla-Martinez L: Standardized morphological phenotyping of mouse models of human diseases within the German Mouse Clinic. *Verh Dtsch Ges Pathol* 91:98-103, 2007.

Supplemental Fig. 1



Supplemental Fig. 2



Supplemental Fig. 3