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Albumin downregulates Klotho in tubular cells

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DISCLOSURE

The authors report no conflict of interest

ABBREVIATIONS

- AKI: Acute Kidney Injury
- CKD: Chronic Kidney Disease
- FBS: Fetal Bovine Serum
- FGF-23: Fibroblast growth factor 23
- GFR: Glomerular Filtration Rate
- HDAC: Histone Deacetylases
- KDIGO: Kidney disease improving global outcomes
- MCP-1: Monocyte chemotactic protein 1
- mRNA: Messenger Ribonucleic acid
- NaPi2a: Sodium/phosphate cotransporter 2a
- NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
- PAN: Puromycin Aminonucleoside
- PTH: Parathyroid hormone
- RAS: Renin-angiotensin System
- TNF: Tumor necrosis factor
- TWEAK: Tumor necrosis factor-like weak inducer of apoptosis
- UACR: Urinary Albumin Creatinine Ratio

ABSTRACT

Kidney tubular cells are the main sources of Klotho, a protein with phosphaturic actions. Genetic Klotho deficiency causes premature cardiovascular aging in mice. Human chronic kidney disease (CKD) is characterized by acquired Klotho deficiency. Despite the lack of uremic toxin accumulation, category G1 CKD (normal glomerular filtration rate, GFR) is already associated with decreased Klotho and with premature cardiovascular aging. We have explored whether albuminuria, a criterion to diagnose CKD when GFR is normal, may directly decrease Klotho expression in human CKD, preclinical models and cultured tubular cells.

In a CKD cohort, albuminuria correlated with serum phosphate after adjustment for GFR, age and sex. In this regard, urinary Klotho was decreased in patients with pathological albuminuria but preserved glomerular filtration rate. Proteinuria induced in rats by puromycin aminonucleoside and in mice by albumin overload was associated with interstitial inflammation and reduced total kidney Klotho mRNA expression. Western blot disclosed reduced kidney Klotho protein in **proteinuric rats and** mice and immunohistochemistry localized the reduced kidney Klotho expression to tubular cells in proteinuric animals. In cultured murine and human tubular cells, albumin directly decreased Klotho mRNA and protein expression. This was inhibited by trichostatin A, an inhibitor of histone deacetylases (HDAC), but unlike cytokine-induced Klotho downregulation, not by NF- κ B inhibitors.

In conclusion, albumin directly decreases Klotho expression in cultured tubular cells. This may explain or at least contribute to decrease Klotho and to promote FGF-23 resistance in early CKD categories, as observed in preclinical and clinical proteinuric kidney disease.

Key words: aging, albuminuria, chronic kidney disease, inflammation, Klotho, phosphate

INTRODUCTION

The current definition and categorization of chronic kidney disease (CKD) reflects that below a certain threshold of glomerular filtration rate (GFR) and above a certain threshold of albuminuria there is an increased risk of CKD progression and of cardiovascular and all-cause death (1). Thus, CKD is associated with CKD progression and with accelerated cardiovascular aging. When GFR is decreased, accumulation of uremic toxins is thought to have a deleterious effect on cardiovascular aging. However, in early stage CKD, when GFR is still normal, uremic toxins do not accumulate and the pathogenic link between CKD and accelerated cardiovascular aging is unclear. In this regard, the loss of additional renal functions, beyond GFR, may account for the increased risk associated with category G1 CKD. One of the candidate functions is loss of Klotho expression. Klotho behaves as an anti-aging kidney-secreted hormone (2). Defective murine *klotho* gene expression results in a syndrome resembling human aging that includes hyperphosphatemia and accelerated cardiovascular disease and the phenotype is rescued by expression of a soluble Klotho transgene (3). Furthermore, kidney-specific Klotho deletion reproduced the accelerated aging phenotype (4). Indeed, kidney tubular cells are the main sites of Klotho expression. In addition, Klotho deficient mice develop renal failure, suggesting that Klotho deficiency is also deleterious for the kidney and could also contribute to CKD progression (5). In this regard, animal models of acute kidney injury (AKI) and CKD have uniformly shown decreased kidney Klotho as well as a nephroprotective role of Klotho (6–13).

Decreased Klotho has been reported in human category G1 CKD (9). However, such an early decrease in urinary Klotho is not expected to result from the loss of Klotho-producing cells, since at this stage the parenchymal kidney cell mass is essentially preserved. The observation of reduced Klotho already in category G1 human CKD suggests that there are factors that reduce Klotho in tubular epithelium beyond the loss of Klotho-producing cells. Some factors that suppress Klotho expression have been identified. Systemic or local inflammation may be one such factor. TNF superfamily inflammatory cytokines, TGF-β1 or angiotensin II decreased Klotho expression in cultured tubular cells (6–8,10,11). Systemic delivery of TWEAK or angiotensin II decreased kidney Klotho levels and targeting of TWEAK, TNF or the renin-angiotensin system (RAS) prevented kidney Klotho downregulation in animal models of kidney injury or systemic inflammation (7,10). These tubular cell stressors could decrease Klotho synthesis through modulation of common downstream transcription factors (NF-κB or Smad-3) or epigenetic modulation of Klotho expression (8,10). However, there is no information on the regulation of

Klotho expression by albuminuria, the main diagnostic criterion for CKD category G1. Albumin is a tubular cell stressor that promotes an inflammatory response and lethality in tubular cells (14) and, thus, is a candidate Klotho regulator.

A key anti-aging function of Klotho is to protect from excess dietary phosphate both by being a necessary co-receptor for the phosphaturic hormone FGF-23 and by directly inhibiting tubular phosphate reabsorption by the sodium-phosphate cotransporter NaPi2a (5–7). In this regard, higher serum phosphate levels were associated with a decreased nephroprotective response to RAS targeting in clinical trials (15), suggesting a potential involvement of Klotho deficiency in clinical CKD progression. More recently, albuminuria was found to predict higher serum phosphate levels independently from GFR, albuminuric patients displayed higher plasma FGF-23 and experimental glomerular proteinuria was associated with higher renal NaPi2a expression and decreased phosphorylation of FGF receptor substrate 2 α , a marker of FGF-23 signal transduction, suggesting renal FGF-23 resistance in proteinuric CKD (16). Interestingly, as in previous reports, experimental kidney injury was associated with lower renal Klotho protein expression (16). However, in vitro, albumin did not directly alter spontaneous or PTH-stimulated phosphate uptake in cultured proximal tubular cells (16) suggesting that albumin did not directly compete with NaPi-2a for endocytosis. Thus, additional molecular mechanism linking albuminuria to in vivo low Klotho levels and FGF-23 resistance should be explored. These include the proinflammatory effects of pathological albuminuria, promoting a local inflammatory response that leads to decreased Klotho expression (7) or a direct effect of albumin on tubular cells Klotho expression. This later hypothesis remains unexplored.

We have now explored the hypothesis that albuminuria directly decreases tubular cell Klotho, thus contributing to the observed FGF-23 resistance in proteinuric kidney disease. We report that albumin directly decreases Klotho in cultured tubular cells through epigenetic mechanisms, and this may have clinical impact, since urinary Klotho was low in patients with pathological albuminuria despite normal GFR. In this regard, albuminuria directly correlated with serum phosphate in human CKD and kidney Klotho was decreased in experimental proteinuric kidney disease.

METHODS

Cells and reagents

The murine proximal tubular epithelial (MCT) cell line was cultured in RPMI 1640 (GIBCO, Grand Island, NY, USA), 10% decompemented fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml

penicillin and 100 µg/ml streptomycin, in 5% CO₂ at 37 °C (17,18). Penicillin and streptomycin were from BioWhittaker (Waltham, Massachusetts) and FBS from Life Technologies (Carlsbad, California). Exposure of cultured tubular cells to bovine serum albumin (Sigma, St. Louis, MO) was used as a surrogate for the in vivo exposure of tubular cells to albumin in proteinuric nephropathies (14). Cells were cultured in serum-free media 24 hours prior to the addition of the stimuli and throughout the experiment. The NF-κB inhibitors parthenolide (10 µM, Sigma) and SN50 (0.1 µM, Merck, Millipore), and the HDAC inhibitor trichostatin A (TSA, 100 ng/ml) (Upstate Biotechnology, Millipore) were added 1 hour before albumin: Doses were derived from prior experience in the lab (10,19). Additional studies were performed in HK2 human proximal tubular epithelial cells, cultured as previously described (20).

Animal models

Studies were conducted in accord with the European Union normative and NIH Guide for the Care and Use of Laboratory Animals. For experimental murine protein-overload nephropathy, C57/BL6 (n=5/group) mice weighing 20 g were intraperitoneally injected daily with 0.2 g bovine serum albumin or saline for 7 days (21). Urinary albumin excretion was assessed by conventional Coomassie blue stains. This assay will detect both endogenous murine albumin and exogenous albumin.

In 10-week-old Wistar Kyoto rats (Criffa, Barcelona, Spain) nephrosis was induced by a single i.v. injection of 150 mg/Kg puromycin aminonucleoside (PAN, Sigma) or vehicle (saline) (n=5/group) and rats were euthanized 2 and 10 days later, following a 24-h urine collection to assess proteinuria (14,21,22). Under general anesthesia, kidneys were perfused in situ with cold saline before removal. One kidney was snap-frozen in liquid nitrogen for RNA and protein studies and the other fixed and paraffin embedded and used for immunohistochemistry (22).

Immunohistochemistry

Immunohistochemistry was carried out as previously described in paraffin-embedded tissue sections 5 µm thick (23). Primary antibodies were rabbit polyclonal anti-Klotho (1:100, Calbiochem, La Jolla, California, Merck Millipore #423500) (10,24,25) or anti-human Klotho monoclonal antibody (1:500, Clone KM2076, Hölzel Diagnostika), rat polyclonal anti-F4/80 antigen (1:50, Serotec, Oxford, UK) for murine macrophages and goat polyclonal anti-CD68 (1:100, Santa Cruz Biotechnology, Santa Cruz, California) for rat macrophages. Sections were counterstained with Carazzi's hematoxylin.

Negative controls included incubation with a non-specific immunoglobulin of the same isotype as the primary antibody. The total number of F4/80 positive macrophages and mouse monoclonal CD68 (1:100, Serotec) was quantitated in 20 randomly chosen fields (x40) using Image-Pro Plus software (Media Cybernetics, MD). Samples were examined in a blinded manner.

Quantitative reverse transcription-polymerase chain reaction

One µg RNA isolated with Trizol (Invitrogen, Carlsbad, CA) was reverse transcribed with High Capacity cDNA Archive Kit and real-time PCR was performed on a ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA) using the DeltaDelta Ct method (26). Expression levels are given as ratios to GAPDH. Pre-developed primer and probe assays were obtained for murine GAPDH and Klotho (Applied Biosystems).

Western blot in cells samples and tissues

Cell samples or tissue were homogenized in lysis buffer (50 mM TrisHCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40, 0.1 mM PMSF, and 1 µg/ml pepstatin A) and then separated by 10% SDS-PAGE under reducing conditions (26). After electrophoresis, samples were transferred to PVDF membranes (Millipore, Bedford, Massachusetts), blocked with 5% skimmed milk in PBS/0.5% vol/vol Tween 20 for 1 h, washed with PBS/Tween, and incubated with rabbit polyclonal anti-Klotho (1:500, Calbiochem, La Jolla, California, Merck Millipore #423500) (10,24,25) or anti-human Klotho monoclonal antibody (1:500, Clone KM2076, Hölzel Diagnostika) diluted in 5% milk PBS/Tween. Blots were washed with PBS/Tween and incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:2000, Amersham, Aylesbury, UK). After washing with PBS/Tween blots were developed with the chemiluminescence method (ECL, Amersham) and then probed with mouse monoclonal anti-α-tubulin antibody (1:2000, Sigma). Levels of expression were corrected for minor differences in loading. The 130 kDa Klotho band was assessed to be consistent with human data described below.

Urinary Klotho protein

The IIS-Foundation Jimenez Diaz Ethics Committee approved the protocol. Patients signed an informed consent according to the European Union directive and Spanish Law. Urinary samples were

obtained from four groups of patients donating to the IIS-Fundacion Jimenez Diaz biobank according to eGFR and urinary albumin creatinine ratio (UACR) categories following KDIGO categories as follows (1): Group 1 (n=6), G1-2 (eGFR >60 ml/min/1.73 m²) A1-2 (UACR <300 mg/g); Group 2 (n=6), G1-2 (eGFR>60 ml/min/1.73 m²) A3 (UACR >300 mg/g); Group 3 (n=5), G3-5 (eGFR <60 ml/min/1.73 m²) A1-2 (UACR <300 mg/g); Group 4 (n=6), G3-5 (eGFR <60 ml/min/1.73 m²) A3 (UACR >300 mg/g). Patients were selected to represent four different combinations of eGFR and UACR, and extreme eUACR values were favored in order to increase the chances of observing differences despite the low number of patients. In this regard, patient selection was not meant to represent the actual prevalence of the different combinations in a general CKD population, since two key combinations (high eGFR/high UACR and low eGFR/low UACR) would have been underrepresented. Key patient characteristics are shown in supplementary table 1. To assess urinary Klotho by Western blot, second morning, fresh human urine was immediately processed and aliquots containing the same amount of creatinine per sample were concentrated to 0.2 ml through Amicon Ultra-4 filters with a 100 kD cutoff (Millipore), and 50 µl concentrated urine was separated by 8% SDS-PAGE, transferred to nitrocellulose membrane (Biorad) and incubated with anti-human Klotho monoclonal antibody (1:500, Clone KM2076, Hölzel Diagnostika) and anti-rat antibody conjugated with horseradish peroxidase (1:2000) (6). Specific signal was visualized using the ECL chemiluminescence kit (Amersham). Since a 100 kD filter was used, we focused on the 130 kDa Klotho band as described by Hu et al (9).

Serum phosphate and albuminuria

In addition, a potential correlation between serum phosphate and albuminuria was assessed in a cohort of 351 patients from the Fundación Jimenez Diaz Nephrology Outpatient clinic database. These were all-comers and stable outpatient. In-patients were excluded. Main clinical characteristics of the cohort are shown in **Table 1**. Serum and spot urine samples were collected in the morning after a 12 h fast and used for routine biochemistry in an automatic workstation ADVIA Centaur XP-Siemens Heathineers Global. UACR was measured using routine immunoassay-based measurement and eGFR was calculated from serum creatinine by the CKD-EPI equation (27).

Statistics

Statistical analysis was performed using SPSS 11.0 statistical software. Results are expressed as mean \pm SD or as median [inter-quartile (IQR) range]. Significance at the $p < 0.05$ level was assessed by Student's *t* test for two groups of data and ANOVA for three or more groups. Associations between quantitative variables were assessed using Spearman correlation coefficient. In order to identify potential predictors of quantitative outcomes, multivariable linear regression models were fitted. UACR was log transformed to meet a normal distribution, as assessed by the Kolmogorov-Smirnov test. Models were built using forward stepwise procedures in order to maximize R-Squared with the smallest number of predictor variables. Age, sex and variables with statistically significant association in the univariate analysis were used. The statistical significance of variables in the models was assessed by ANOVA test.

RESULTS

Albuminuria correlated with serum phosphate in human CKD

A recent report has suggested that human proteinuric kidney disease is associated with FGF23 resistance, but the molecular underlying mechanisms are unclear (16). In a cohort of 351 CKD patients, a direct correlation was found between serum phosphate and UACR in univariate analysis (**Table 2, Figure 1**). Serum phosphate also correlated with serum magnesium and PTH and inversely with eGFR (**Table 2**). In multivariate analysis, eGFR and UACR were independent predictors of serum phosphate when adjusted for age and sex (**Table 3.A**). These data are consistent with previous reports of the presence of FGF23 resistance in human proteinuric kidney disease (16). In the multivariable model, a significant interaction between eGFR and UACR was found. Including the interaction term modified the results: when entering the eGFR \times UACR interaction term, the variable eGFR is no longer significant (**Table 3.B**): the effect that UACR has on serum phosphate depends on the value taken by eGFR, or, in other words eGFR modifies the relationship between UACR and serum phosphate. This is consistent with greater ability of kidneys with higher eGFR to excrete higher amounts of phosphate. In this regard, categorization by baseline eGFR disclosed that the impact of UACR on serum phosphate levels was more apparent at baseline eGFR < 30 ml/min/1.73 m² (**Suppl figure 1**).

Urinary Klotho is decreased in patients with severe albuminuria

Following the observation that albuminuria correlates with serum phosphate independently of eGFR, we set out to study the mechanisms of this link and, specifically, explored the hypothesis that albuminuria may decrease Klotho to promote FGF-23 resistance. As a first step, the impact of pathological albuminuria on urinary Klotho excretion was assessed in human CKD. Urinary Klotho was reported to be decreased in category G1 CKD in humans (9). While category G1 CKD usually implies the presence of pathological albuminuria, it is possible to have category G1 CKD with normoalbuminuria if abnormal kidney imaging or histology are present (1). Thus, we focused on the relationship between albuminuria and urinary Klotho in individuals with diverse degrees of GFR impairment and of severity of albuminuria, expanding G categories G1 through G5 and albuminuria categories A1 through A3. Urinary Klotho levels were highest in individuals with preserved eGFR and minimal albuminuria, but either the presence of severe albuminuria or decreased GFR resulted in a dramatic decrease in urinary Klotho (**Figure 2.A,B**). **Suppl figure 2** shows the full blot and Ponceau red staining. Despite the low number of patients a trend was observed for higher phosphate levels in patients with higher UACR (**Figure 2.B**).

Patients with UACR above a certain threshold had suppressed urinary Klotho and there was a trend toward a negative correlation between UACR and urinary Klotho (Correlation= -0.378, $p = 0.076$)(**Suppl fig. 3**). In addition, some patients with low UACR also had low urinary Klotho: those with low GFR. When urinary Klotho was plotted against eGFR and the magnitude of UACR incorporated into the graph as the size of the dots for individual patients, a pattern emerged that either high UACR or low eGFR was associated with low urinary Klotho values (**Figure 3**). **Suppl fig. 4** represents urinary Klotho vs the ratio eGFR/log UACR. These results suggest that an inverse relationship between albuminuria and Klotho is present in human CKD and becomes more apparent when renal function is still preserved, since when renal function and mass are lost, the decreased tubular cell mass may already account for lower Klotho levels.

Experimental proteinuric kidney disease is associated with Klotho downregulation

Since albuminuria was associated with decreased urinary Klotho in human CKD, the relationship between albuminuria and Klotho expression was explored in two animal models of pathological albuminuria in the presence of preserved global renal function.

In rats albuminuria was induced by a single injection of the podocyte toxin PAN (**Figure 4.A**) while in mice it was induced by albumin overload (**Figure 4.B**). PAN nephrosis is a classical model of

minimal change nephrotic syndrome while albumin overload results in massive albuminuria (28). In these animals, renal function was preserved as assessed by serum creatinine (control mice 0.26 ± 0.05 mg/dl, albumin overload mice 0.26 ± 0.05 mg/dl; control rats day 10, 0.48 ± 0.10 mg/dl, PAN rats day 10, 0.52 ± 0.11 mg/dl, ns).

In both animal models pathological albuminuria was associated with interstitial inflammation characterized by increased kidney MCP-1 mRNA levels (**Figure 4.C,D**) and interstitial infiltration by macrophages (**Figure 4.E,F**). Reduced total Klotho mRNA expression was observed both in rat PAN nephrosis (**Figure 5.A**) and in murine albumin overload proteinuria (**Figure 5.D**). A correlation between proteinuria and Klotho mRNA was observed in rats: above a certain threshold of proteinuria, Klotho mRNA decreased (**Suppl fig. 5**). This was similar to the observation in humans (**Suppl fig. 3**), although in rats renal function was homogeneous.

Further characterization of Klotho protein levels was carried out in the **mice and rats**. Western blot using the monoclonal KM2076 antibody confirmed the reduced total kidney Klotho protein expression in **rat PAN nephrosis (Figure 5.C) and in** albumin-overloaded mice (**Figure 5.F**). Immunohistochemistry using the monoclonal KM2076 antibody localized Klotho expression to tubular cells and confirmed reduced tubular cell Klotho expression in proteinuric animals (**Figure 5.B and 5.E**). **Supplementary figure 6** shows results obtained using the polyclonal antibody.

These results suggest that pathological albuminuria decreases Klotho expression in experimental proteinuric kidney disease with preserved renal function. Both local inflammation and albumin itself could drive this response.

Albumin directly decreases Klotho expression in cultured renal tubular cells

Since pathological albuminuria was associated with decreased kidney or urinary Klotho in both human and experimental proteinuric kidney disease, and both local inflammation or a direct albumin effect could explain the association, we explored the second alternative, whether albumin had direct effects on Klotho expression in cultured tubular cells since inflammation induced Klotho downregulation is already well characterized. Murine tubular cells were cultured in the presence of albumin to simulate exposure to albumin when the glomerular permeability to protein is increased. Albumin dose-dependently decreased Klotho mRNA expression (**Figure 6.A**). Other stressors had previously been reported to decrease Klotho expression in tubular cells. Thus, both inflammatory cytokines present in the injured

kidney environment, such as TWEAK, and activation of the transcription factor NF- κ B decreased Klotho expression by tubular cells (10). Indeed, TWEAK decreases Klotho mRNA through NF- κ B activation and promotion of histone deacetylation (10). However, the molecular mechanism of albumin-induced Klotho downregulation differed from that elicited by TWEAK. Thus, unlike cytokine-induced Klotho downregulation, two structurally different NF- κ B inhibitors did not prevent Klotho downregulation in response to albumin (**Figure 6.B**). By contrast the HDAC inhibitor trichostatin A prevented the decrease in Klotho mRNA and protein induced by albumin (**Figure 6.C,D**). Albumin also decreased Klotho expression in human proximal tubular cells, **although only a trend towards a dose-response was observed at 24h (Suppl figure 7)**. In these cells, trichostatin A also prevented the decrease in Klotho protein induced by albumin. These results suggest that albumin directly represses Klotho expression in tubular cells through epigenetic mechanisms.

DISCUSSION

The main finding is that exposure of proximal tubular cells to albumin in culture, a model that reproduces the exposure of proximal tubular cells to albumin in proteinuric nephropathies, resulted in decreased Klotho expression. This may be clinically significant since tubular Klotho was decreased in experimental proteinuric nephropathies, urinary Klotho was decreased in human proteinuric disease and proteinuria correlated with serum phosphate, a potential indication of FGF23 resistance, in human CKD.

Acquired Klotho deficiency has been described in human CKD and found to be present already in category G1 CKD (9). Category G1 CKD means that eGFR is normal, but there is evidence of kidney injury (1).The most frequent evidence of kidney injury in the clinic is pathological albuminuria or proteinuria. However, there are other criteria to define CKD when eGFR is normal. These include urine sediment abnormalities, electrolyte and other abnormalities due to tubular disorders, abnormalities detected by histology and structural abnormalities detected by imaging (1). Thus, while decreased urinary Klotho in category G1 CKD had already been described, this is, to our knowledge, the first study that addresses specifically the association between albuminuria and urinary Klotho and found an inverse relationship in humans and in preclinical models of kidney disease.

Albuminuria may theoretically decreases kidney Klotho by direct or indirect effects. In this regard, albuminuria is toxic to proximal tubular cells and may result in tubular cell death or in a sublethal stress response characterized by the secretion of inflammatory mediators and inflammation driven by

secretion of chemokines such as MCP-1 (14,29–31). Systemic or local inflammation reduces kidney Klotho and may account for low Klotho levels in patients with pathological albuminuria (7,10). In fact, we observed that in preclinical models of proteinuric kidney disease, increased MCP-1 mRNA expression and interstitial inflammation is a prominent feature, confirming prior observations. However, we have now shown that albuminuria also has a direct effect on kidney Klotho levels and that this effect is not mediated by the transcription factor NF κ B, thus, arguing against autocrine activation of inflammatory cytokines such as TWEAK. Indeed, TWEAK- and TNF-induced downregulation of Klotho expression in tubular epithelium is mediated by NF κ B (10). By contrast, cytokines and albumin share epigenetic mechanisms to downregulate Klotho expression. The HDAC inhibitor trichostatin A prevented Klotho downregulation induced either by TWEAK (10) or, as shown here, by albumin. Thus, HDAC inhibition may be an approach to preserve Klotho expression that protects against both the direct and the indirect (inflammation-mediated) effects of albuminuria on Klotho expression. The preclinical finding may be clinically relevant since in humans a decreased urinary Klotho excretion, generally accepted to represent kidney Klotho, was found in the presence of either albuminuria or decreased GFR. These findings confirm and extend the prior observation, using the same Western blot technique, of decreased urinary Klotho in category G1 CKD (9) and pinpoint pathological albuminuria as a factor associated with decreased urinary Klotho when GFR is normal. Unfortunately, Western blot is a time-consuming technique that is not well suited to study a large number of samples. In addition, in a cohort study we confirmed a recent observation of a direct correlation between albuminuria and serum phosphate (16). These authors identified FGF23 resistance as a potential driver of the relationship. We now pinpoint the problem to acquired kidney Klotho deficiency probably resulting from direct and indirect effects of albumin on tubular cells. Genetic Klotho deficiency has been previously shown to result in FGF-23 resistance and high circulating FGF-23 levels (4). In addition, urinary Klotho has direct phosphaturic effects on the proximal tubule, dependent on its enzymatic glycosidase activity that inactivates the NaPi2a phosphate transporters in proximal tubules (32). It is theoretically possible that albuminuria-induced FGF23 resistance may help preserve serum 1,25(OH) $_2$ D $_3$ levels. This in turn may contribute to higher serum phosphate levels that may not be excreted as required, given the loss of the direct effect of Klotho on phosphate transporters in proximal tubules and on phosphaturia.

Current therapy for proteinuric kidney disease is based on renin angiotensin system (RAS) blockade (33). However, residual albuminuria despite RAS blockade is a key prognostic factor. The

present result suggests that in addition to generating kidney inflammation, residual albuminuria may directly decrease kidney Klotho expression, thus potentially favoring CKD progression and accelerated cardiovascular aging despite normal GFR and no accumulation of uremic toxins. The sensitivity of this response to HDAC inhibitors, such as trichostatin A, suggests the involvement of epigenetic mechanisms, and, thus, the potential for chronification of Klotho downregulation driven by albuminuria. In this regard, epigenetic downmodulation of RASAL1 in fibroblasts is a key contributor to the AKI-to-CKD transition (34). Further characterization of intracellular signaling pathways that lead to a direct effect of albuminuria on tubular cell Klotho expression may identify novel therapeutic approaches aimed at preserving Klotho expression despite persistence of albuminuria. In this regard, two recent observations help illustrate the clinical relevance of our findings. First, RAS blockade does not efficiently prevent CKD progression in patients with higher serum phosphate levels (15). Second, these higher serum phosphate levels in albuminuric patients appear to depend on FGF-23 resistance in the kidneys (16). The fact that albumin directly decreases kidney Klotho by an epigenetic mechanism may explain how albuminuria decreases kidney Klotho and causes FGF-23 resistance and also why RAS blockade fails to protect renal function despite improving albuminuria, since epigenetic changes may be perpetuated in the same cell and even be transmitted to daughter cells (35). We hypothesize that Klotho downregulation itself may be a driving force for CKD progression and accelerated cardiovascular aging under these circumstances (5,9,11). The loss of Klotho in response to albuminuria coupled to a negative impact of Klotho deficiency on kidney disease may potentially generate a vicious circle where kidney injury results in low kidney Klotho and Klotho downregulation favors progression of kidney injury (8). Either preventing Klotho downregulation or supplementing the missing Klotho may interrupt the vicious circle (8). Thus, exogenous soluble Klotho restored the expression of endogenous Klotho in injured kidneys (8). In this regard, animal models of AKI and CKD have uniformly shown decreased kidney Klotho as well as a nephroprotective role of Klotho (6–13).

One important lesson from this and recent studies(16) is that the variable albuminuria may need to be taken into consideration when FGF23 is correlated with phosphaturia, phosphatemia, GFR or phosphate intake, since it may impact the renal response to FGF23 and dietary phosphate. In addition, an open question is to what extent the association of albuminuria with CKD progression or adverse cardiovascular and survival outcomes could be related to early Klotho deficiency and associated abnormalities of phosphate metabolism (36).

This study has several limitations. Thus, urinary Klotho was tested in a limited number of patients. However, this was the consequence of using a time-consuming technique, Western blot, given the substantial limitations of available Klotho ELISAs for humans and the need for fresh urine (37). In this regard, it is unclear whether in prior studies using ELISA to assess plasma Klotho in large CKD cohorts, these limitations may have contributed to the lack of association of plasma Klotho with phosphorus or FePi (38). In any case, urinary Klotho has direct, FGF23-independent phosphaturic effects dependent on degradation of NaPi-2a in the luminal tubular surface (32) that may underlie any potential differences between urinary and plasma Klotho. Circulating Klotho was not assessed. In addition to the low kidney levels of Klotho, we cannot exclude that lower urinary Klotho levels are influenced by altered shedding to the luminal side, or increased uptake of shed Klotho by proximal tubules.

In conclusion, we have shown that albumin directly decreases Klotho expression in tubular cells in culture through epigenetic mechanisms. This may explain or at least contribute to the experimental animal observation of decreased tubular cell Klotho in proteinuric nephropathies, the decrease in urinary Klotho excretion in human proteinuric kidney disease and the clinical observations of a correlation between serum phosphate levels and proteinuria as well as the evidence for FGF-23 resistance¹⁶ and the observation of an inverse correlation between serum Klotho and proteinuria (39). The hypothesis that this early albuminuria-driven decrease in Klotho expression may contribute to the higher risk of premature death and CKD progression in human CKD category G1/2 should be explored.

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TABLES

Table 1. Clinical characteristics of patients in the CKD cohort (n=351). Results are expressed as %, mean ± SD or median [inter-quartile (IQR) range].

Age (years)	67±14
Sex (male)	60%
DM (yes)	52%
25OH-Vitamin D (ng/dl)	20±10
sCr (mg/dl)	2.2±1.3
eGFR (ml/min/1.73 m ²)	40±24
sCa (mg/dL)	9.45±0.5
sP (mg/dL)	3.8±7.6
sMg (mg/dL)	1.95±0.35
iPTH (pg/ml)	94.3 [56.2-158.8]
Serum albumin (g/dl)	4.0±0.4
UACR (mg/g)	167 [22-588]
FE phosphate (%)	30±14

sCr: serum creatinine, eGFR: estimated glomerular filtration rate, sCa: serum calcium, sP: serum phosphate, sMg: serum magnesium, UACR: urinary albumin creatinine ratio, FE: fractional excretion.

Table 2. Univariate correlations of serum phosphate with quantitative variables.

	Correlation	p value
UACR (mg/g)	0.41	<0.0001
eGFR (ml/min/1.73 m ²)	-0.38	<0.0001
iPTH (pg/ml)	0.38	0.0006
sMg (mg/dL)	0.24	<0.0001
Age (years)	-0.06	0.19

eGFR: estimated glomerular filtration rate sMg: serum magnesium, UACR: urinary albumin creatinine ratio

Table 3. Multivariate model for predictors of serum phosphate (mg/dl) adjusted for age and sex

A. Without log UACR:eGFR interaction

	Coefficient (95% CI))	p value
Intercept	4.36 (3.88 to 4.84)	<0.0001
Age (years)	-0.009 (-0.014, -0.004)	<0.001
Log UACR (mg/g)	0.086 (0.049, 0.124)	<0.001
eGFR (ml/min/1.73 m ²)	-0.011 (-0.014, -0.008)	<0.001
Sex (female)	0.209 (0.062, 0.356)	0.006

R squared adjusted = 0.27

B. With log UACR:eGFR interaction

	Coefficient (95% CI))	p value
Intercept	3.78 (3.14, 4.42)	<0.0001
Age (years)	-0.007 (-0.013, -0.002)	0.005
Log UACR (mg/g)	0.175 (0.100, 0.249)	<0.001
eGFR (ml/min/1.73 m ²)	0.000 (-0.009, 0.009)	0.939
Sex (female)	0.200 (0.054, 0.346)	0.007
Log UACR:eGFR	-0.002 (-0.004, -0.001)	0.007

R squared adjusted = 0.23

FIGURE LEGENDS

Figure 1. Correlation between serum phosphate and proteinuria in human CKD. Data from 351 clinically stable outpatients. UACR: urinary albumin:creatinine ratio, $p < 0.0001$.

Figure 2. Decreased urinary Klotho expression in human proteinuric kidney disease. **A)** Urinary Klotho was assessed by Western blot in patients with different degrees of eGFR and albuminuria classified as 2012 KDIGO G and A categories (1). The full membrane is shown in supplementary figure 2. **B)** Quantification of Western blot results. * $p < 0.05$ vs G1-2/A1-2. The table below shows clinical data for the studied population. eGFR: estimated glomerular filtration rate (CKD-EPI), sP: serum phosphate, UACR: urinary albumin:creatinine ratio. Data presented as mean \pm SD or median (interquartile range).

Figure 3. Decreased urinary Klotho in human proteinuric kidney disease is associated with higher albuminuria and with decreased eGFR. Urinary Klotho protein results from figure 2 were plotted against CKD-EPI estimated glomerular filtration rate (eGFR). The size of the data points is proportional to the magnitude of urinary albumin excretion: the larger the size, the higher the UACR, as indicated in the figure. Each data point represents an individual patient. The graph shows that only individuals with normal eGFR and normal UACR had higher (normal) urinary Klotho levels and either pathological albuminuria or decreased eGFR was associated with lower urinary Klotho. The relationship between UACR and size of the points is indicated.

Figure 4. Experimental proteinuric kidney disease is associated to interstitial inflammation. **A)** Urinary protein before and after the injection of PAN or vehicle (control) in rats. * $p < 0.02$, # $p < 0.005$ vs control. **B)** Coomassie blue-stained urine protein gels showing increased urinary albumin in albumin overload-induced nephropathy induced by daily albumin ip administration for 7 days in mice. **C and D)** Whole kidney MCP1 mRNA expression was increased in **C)** PAN nephrosis and **D)** albumin overload-induced nephropathy. qRT-PCR results expressed as % change over control which was considered to be 100%. * $p < 0.006$ vs control. Mean \pm SD of 5 animals per group. **E)** Quantification and representative CD68 immunohistochemistry 10 days following PAN or vehicle injection. CD68+ macrophages are increased in PAN nephrosis. * $p < 0.001$ vs vehicle-injected control. **F)** Quantification and immunohistochemistry

image representative of F4/80 positive macrophages in albumin overload nephropathy at day 7. Original magnification x200. *p<0.001 vs vehicle-injected control.

Figure 5. Decreased kidney Klotho expression in experimental proteinuric kidney disease. A and D)

Decreased whole kidney Klotho mRNA expression in rats injected with PAN (A) and in mice with albumin overload-induced nephropathy (D), *p<0.0001 vs vehicle-injected control, **p<0.05 vs vehicle-injected control. qRT-PCR results expressed as % change over control which was considered to be 100%.

B and E) Klotho immunostaining. Monoclonal KM2076 antibody. Representative immunohistochemistry image in rats injected with PAN (B) and in albumin overload nephropathy (E). Original magnification x200. **C and F)** Quantification and representative western blot of Klotho expression in rats injected with PAN (C) and in mice with albumin overload-induced nephropathy (F)). Monoclonal KM2076 antibody. Mean±SD of 5 animals per group. *p<0.0001 vs vehicle-injected control.

Figure 6. Exposure to albumin decreases Klotho expression in murine cultured tubular cells. A)

Dose-response and time-course of Klotho mRNA expression, expressed as % change over control which was considered to be 100%. *p<0.005 vs control; ** p<0.05 vs albumin 10 mg/mL. **B)** Neither parthenolide nor SN50 modulate the downregulation of Klotho mRNA in response to albumin (10 mg/mL) for 3 hours. Both are NF-κB inhibitors previously shown to inhibit NF-κB-mediated responses at the same concentrations in this cell system (10). **C)** The HDAC inhibitor trichostatin A (TSA) prevents Klotho mRNA downregulation in response to albumin (10 mg/mL) for 3 hours. *p<0.05 vs control; #p<0.04 vs albumin alone. **D)** TSA prevents Klotho protein downregulation in response to albumin. Representative images for 3 independent experiments. When not otherwise specified, cells were incubated with 10 mg/mL albumin for 3 hours or vehicle control.

Supplementary figure 1. Correlation between serum phosphate and proteinuria in human CKD.

Data from 351 clinically stable outpatients who were categorized according to baseline eGFR. UACR: urinary albumin:creatinine ratio.

Supplementary figure 2. Full membrane and Ponceau red staining of the blot shown in figure 2.A.

Supplementary figure 3. Relationship between urinary Klotho and albuminuria (UACR in mg/g) in humans. Correlation= -0.378, $p = 0.076$. Note that above a certain magnitude of proteinuria, urinary Klotho levels fall. In addition, some patients with low UACR have low urinary Klotho. These correspond to patients with low GFR. To gain a wider perspective, figure 3 plots the three variables (UACR, GFR and urinary Klotho) in the same graph.

Supplementary figure 4. Representation of log urinary Klotho (arbitrary units) vs ratio of eGFR (ml/min/1.73 m²)/ log UACR (mg/g) in human individuals.

Supplementary figure 5. Relationship between kidney Klotho mRNA and proteinuria in rats with PAN nephrosis. Note that above a certain magnitude of proteinuria, kidney Klotho mRNA expression falls.

Supplementary figure 6. Decreased kidney Klotho expression in experimental proteinuric kidney disease. Polyclonal antibody. **A)** Quantification and representative western blot of Klotho expression in mice with albumin overload-induced nephropathy. Mean \pm SD of 5 animals per group. * $p<0.0001$ vs vehicle-injected control. Antibody used: Calbiochem. **B)** Klotho immunostaining. Representative immunohistochemistry image in albumin overload nephropathy. Original magnification x200. Antibody used: Calbiochem.

Supplementary figure 7. Exposure to albumin decreases Klotho expression in human cultured HK2 tubular cells. **A)** Dose-response and time-course of Klotho mRNA expression, expressed as % change over control which was considered to be 100%. * $p<0.05$ vs control. Neither parthenolide **(B)** nor SN50 **(C)** modulate the downregulation of Klotho mRNA in response to albumin (10 mg/mL) for 3 hours. The HDAC inhibitor trichostatin A (TSA) prevents Klotho mRNA **(D)** and protein **(E)** downregulation in response to albumin. Representative images and quantification for 3 independent experiments. Cells were incubated with 10 mg/mL albumin for 3 hours or vehicle control. * $p<0.0005$ vs control, # $p<0.05$ vs albumin.

Albumin downregulates Klotho in tubular cells

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DISCLOSURE

The authors report no conflict of interest

ABBREVIATIONS

AKI: Acute Kidney Injury

CKD: Chronic Kidney Disease

FBS: Fetal Bovine Serum

FGF-23: Fibroblast growth factor 23

GFR: Glomerular Filtration Rate

HDAC: Histone Deacetylases

KDIGO: Kidney disease improving global outcomes

MCP-1: Monocyte chemotactic protein 1

mRNA: Messenger Ribonucleic acid

NaPi2a: Sodium/phosphate cotransporter 2a

NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells

PAN: Puromycin Aminonucleoside

PTH: Parathyroid hormone

RAS: Renin-angiotensin System

TNF: Tumor necrosis factor

TWEAK: Tumor necrosis factor-like weak inducer of apoptosis

UACR: Urinary Albumin Creatinine Ratio

ABSTRACT

Kidney tubular cells are the main sources of Klotho, a protein with phosphaturic actions. Genetic Klotho deficiency causes premature cardiovascular aging in mice. Human chronic kidney disease (CKD) is characterized by acquired Klotho deficiency. Despite the lack of uremic toxin accumulation, category G1 CKD (normal glomerular filtration rate, GFR) is already associated with decreased Klotho and with premature cardiovascular aging. We have explored whether albuminuria, a criterion to diagnose CKD when GFR is normal, may directly decrease Klotho expression in human CKD, preclinical models and cultured tubular cells.

In a CKD cohort, albuminuria correlated with serum phosphate after adjustment for GFR, age and sex. In this regard, urinary Klotho was decreased in patients with pathological albuminuria but preserved glomerular filtration rate. Proteinuria induced in rats by puromycin aminonucleoside and in mice by albumin overload was associated with interstitial inflammation and reduced total kidney Klotho mRNA expression. Western blot disclosed reduced kidney Klotho protein in proteinuric rats and mice and immunohistochemistry localized the reduced kidney Klotho expression to tubular cells in proteinuric animals. In cultured murine and human tubular cells, albumin directly decreased Klotho mRNA and protein expression. This was inhibited by trichostatin A, an inhibitor of histone deacetylases (HDAC), but unlike cytokine-induced Klotho downregulation, not by NF- κ B inhibitors.

In conclusion, albumin directly decreases Klotho expression in cultured tubular cells. This may explain or at least contribute to decrease Klotho and to promote FGF-23 resistance in early CKD categories, as observed in preclinical and clinical proteinuric kidney disease.

Key words: aging, albuminuria, chronic kidney disease, inflammation, Klotho, phosphate

INTRODUCTION

The current definition and categorization of chronic kidney disease (CKD) reflects that below a certain threshold of glomerular filtration rate (GFR) and above a certain threshold of albuminuria there is an increased risk of CKD progression and of cardiovascular and all-cause death (1). Thus, CKD is associated with CKD progression and with accelerated cardiovascular aging. When GFR is decreased, accumulation of uremic toxins is thought to have a deleterious effect on cardiovascular aging. However, in early stage CKD, when GFR is still normal, uremic toxins do not accumulate and the pathogenic link between CKD and accelerated cardiovascular aging is unclear. In this regard, the loss of additional renal functions, beyond GFR, may account for the increased risk associated with category G1 CKD. One of the candidate functions is loss of Klotho expression. Klotho behaves as an anti-aging kidney-secreted hormone (2). Defective murine *klotho* gene expression results in a syndrome resembling human aging that includes hyperphosphatemia and accelerated cardiovascular disease and the phenotype is rescued by expression of a soluble Klotho transgene (3). Furthermore, kidney-specific Klotho deletion reproduced the accelerated aging phenotype (4). Indeed, kidney tubular cells are the main sites of Klotho expression. In addition, Klotho deficient mice develop renal failure, suggesting that Klotho deficiency is also deleterious for the kidney and could also contribute to CKD progression (5). In this regard, animal models of acute kidney injury (AKI) and CKD have uniformly shown decreased kidney Klotho as well as a nephroprotective role of Klotho (6–13).

Decreased Klotho has been reported in human category G1 CKD (9). However, such an early decrease in urinary Klotho is not expected to result from the loss of Klotho-producing cells, since at this stage the parenchymal kidney cell mass is essentially preserved. The observation of reduced Klotho already in category G1 human CKD suggests that there are factors that reduce Klotho in tubular epithelium beyond the loss of Klotho-producing cells. Some factors that suppress Klotho expression have been identified. Systemic or local inflammation may be one such factor. TNF superfamily inflammatory cytokines, TGF- β 1 or angiotensin II decreased Klotho expression in cultured tubular cells (6–8,10,11). Systemic delivery of TWEAK or angiotensin II decreased kidney Klotho levels and targeting of TWEAK, TNF or the renin-angiotensin system (RAS) prevented kidney Klotho downregulation in animal models of kidney injury or systemic inflammation (7,10). These tubular cell stressors could decrease Klotho synthesis through modulation of common downstream transcription factors (NF- κ B or Smad-3) or epigenetic modulation of Klotho expression (8,10). However, there is no information on the regulation of

Klotho expression by albuminuria, the main diagnostic criterion for CKD category G1. Albumin is a tubular cell stressor that promotes an inflammatory response and lethality in tubular cells (14) and, thus, is a candidate Klotho regulator.

A key anti-aging function of Klotho is to protect from excess dietary phosphate both by being a necessary co-receptor for the phosphaturic hormone FGF-23 and by directly inhibiting tubular phosphate reabsorption by the sodium-phosphate cotransporter NaPi2a (5–7). In this regard, higher serum phosphate levels were associated with a decreased nephroprotective response to RAS targeting in clinical trials (15), suggesting a potential involvement of Klotho deficiency in clinical CKD progression. More recently, albuminuria was found to predict higher serum phosphate levels independently from GFR, albuminuric patients displayed higher plasma FGF-23 and experimental glomerular proteinuria was associated with higher renal NaPi2a expression and decreased phosphorylation of FGF receptor substrate 2 α , a marker of FGF-23 signal transduction, suggesting renal FGF-23 resistance in proteinuric CKD (16). Interestingly, as in previous reports, experimental kidney injury was associated with lower renal Klotho protein expression (16). However, in vitro, albumin did not directly alter spontaneous or PTH-stimulated phosphate uptake in cultured proximal tubular cells (16) suggesting that albumin did not directly compete with NaPi-2a for endocytosis. Thus, additional molecular mechanism linking albuminuria to in vivo low Klotho levels and FGF-23 resistance should be explored. These include the proinflammatory effects of pathological albuminuria, promoting a local inflammatory response that leads to decreased Klotho expression (7) or a direct effect of albumin on tubular cells Klotho expression. This later hypothesis remains unexplored.

We have now explored the hypothesis that albuminuria directly decreases tubular cell Klotho, thus contributing to the observed FGF-23 resistance in proteinuric kidney disease. We report that albumin directly decreases Klotho in cultured tubular cells through epigenetic mechanisms, and this may have clinical impact, since urinary Klotho was low in patients with pathological albuminuria despite normal GFR. In this regard, albuminuria directly correlated with serum phosphate in human CKD and kidney Klotho was decreased in experimental proteinuric kidney disease.

METHODS

Cells and reagents

The murine proximal tubular epithelial (MCT) cell line was cultured in RPMI 1640 (GIBCO, Grand Island, NY, USA), 10% decomplexed fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml

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3 penicillin and 100 µg/ml streptomycin, in 5% CO₂ at 37 °C (17,18). Penicillin and streptomycin were
4 from BioWhittaker (Waltham, Massachusetts) and FBS from Life Technologies (Carlsbad, California).
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6 Exposure of cultured tubular cells to bovine serum albumin (Sigma, St. Louis, MO) was used as a
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8 surrogate for the in vivo exposure of tubular cells to albumin in proteinuric nephropathies (14). Cells were
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10 cultured in serum-free media 24 hours prior to the addition of the stimuli and throughout the experiment.
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12 The NF-κB inhibitors parthenolide (10 µM, Sigma) and SN50 (0.1 µM, Merck, Millipore), and the
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14 HDAC inhibitor trichostatin A (TSA, 100 ng/ml) (Upstate Biotechnology, Millipore) were added 1 hour
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16 before albumin: Doses were derived from prior experience in the lab (10,19). Additional studies were
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18 performed in HK2 human proximal tubular epithelial cells, cultured as previously described (20).
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20 21 **Animal models**

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23 Studies were conducted in accord with the European Union normative and NIH Guide for the
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25 Care and Use of Laboratory Animals. For experimental murine protein-overload nephropathy, C57/BL6
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27 (n=5/group) mice weighing 20 g were intraperitoneally injected daily with 0.2 g bovine serum albumin or
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29 saline for 7 days (21). Urinary albumin excretion was assessed by conventional Coomassie blue stains.
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31 This assay will detect both endogenous murine albumin and exogenous albumin.

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33 In 10-week-old Wistar Kyoto rats (Criffa, Barcelona, Spain) nephrosis was induced by a single
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35 i.v. injection of 150 mg/Kg puromycin aminonucleoside (PAN, Sigma) or vehicle (saline) (n=5/group)
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37 and rats were euthanized 2 and 10 days later, following a 24-h urine collection to assess proteinuria
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39 (14,21,22). Under general anesthesia, kidneys were perfused in situ with cold saline before removal. One
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41 kidney was snap-frozen in liquid nitrogen for RNA and protein studies and the other fixed and paraffin
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43 embedded and used for immunohistochemistry (22).
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45 46 **Immunohistochemistry**

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48 Immunohistochemistry was carried out as previously described in paraffin-embedded tissue
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50 sections 5 µm thick (23). Primary antibodies were rabbit polyclonal anti-Klotho (1:100, Calbiochem, La
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52 Jolla, California, Merck Millipore #423500) (10,24,25) or anti-human Klotho monoclonal antibody
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54 (1:500, Clone KM2076, Hölzel Diagnostika), rat polyclonal anti-F4/80 antigen (1:50, Serotec, Oxford,
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56 UK) for murine macrophages and goat polyclonal anti-CD68 (1:100, Santa Cruz Biotechnology, Santa
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58 Cruz, California) for rat macrophages. Sections were counterstained with Carazzi's hematoxylin.
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Negative controls included incubation with a non-specific immunoglobulin of the same isotype as the primary antibody. The total number of F4/80 positive macrophages and mouse monoclonal CD68 (1:100, Serotec) was quantitated in 20 randomly chosen fields (x40) using Image-Pro Plus software (Media cybernetics, MD). Samples were examined in a blinded manner.

Quantitative reverse transcription-polymerase chain reaction

One µg RNA isolated with Trizol (Invitrogen, Carlsbad, CA) was reverse transcribed with High Capacity cDNA Archive Kit and real-time PCR was performed on a ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA) using the DeltaDelta Ct method (26). Expression levels are given as ratios to GAPDH. Pre-developed primer and probe assays were obtained for murine GAPDH and Klotho (Applied Biosystems).

Western blot in cells samples and tissues

Cell samples or tissue were homogenized in lysis buffer (50 mM TrisHCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP-40, 0.1 mM PMSF, and 1 µg/ml pepstatin A) and then separated by 10% SDS-PAGE under reducing conditions (26). After electrophoresis, samples were transferred to PVDF membranes (Millipore, Bedford, Massachusetts), blocked with 5% skimmed milk in PBS/0.5% vol/vol Tween 20 for 1 h, washed with PBS/Tween, and incubated with rabbit polyclonal anti-Klotho (1:500, Calbiochem, La Jolla, California, Merck Millipore #423500) (10,24,25) or anti-human Klotho monoclonal antibody (1:500, Clone KM2076, Hölzel Diagnostika) diluted in 5% milk PBS/Tween. Blots were washed with PBS/Tween and incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:2000, Amersham, Aylesbury, UK). After washing with PBS/Tween blots were developed with the chemiluminescence method (ECL, Amersham) and then probed with mouse monoclonal anti-α-tubulin antibody (1:2000, Sigma). Levels of expression were corrected for minor differences in loading. The 130 kDa Klotho band was assessed to be consistent with human data described below.

Urinary Klotho protein

The IIS-Foundation Jimenez Diaz Ethics Committee approved the protocol. Patients signed an informed consent according to the European Union directive and Spanish Law. Urinary samples were

obtained from four groups of patients donating to the IIS-Fundacion Jimenez Diaz biobank according to eGFR and urinary albumin creatinine ratio (UACR) categories following KDIGO categories as follows (1): Group 1 (n=6), G1-2 (eGFR >60 ml/min/1.73 m²) A1-2 (UACR <300 mg/g); Group 2 (n=6), G1-2 (eGFR>60 ml/min/1.73 m²) A3 (UACR >300 mg/g); Group 3 (n=5), G3-5 (eGFR <60 ml/min/1.73 m²) A1-2 (UACR <300 mg/g); Group 4 (n=6), G3-5 (eGFR <60 ml/min/1.73 m²) A3 (UACR >300 mg/g). Patients were selected to represent four different combinations of eGFR and UACR, and extreme eUACR values were favored in order to increase the chances of observing differences despite the low number of patients. In this regard, patient selection was not meant to represent the actual prevalence of the different combinations in a general CKD population, since two key combinations (high eGFR/high UACR and low eGFR/low UACR) would have been underrepresented. Key patient characteristics are shown in supplementary table 1. To assess urinary Klotho by Western blot, second morning, fresh human urine was immediately processed and aliquots containing the same amount of creatinine per sample were concentrated to 0.2 ml through Amicon Ultra-4 filters with a 100 kD cutoff (Millipore), and 50 µl concentrated urine was separated by 8% SDS-PAGE, transferred to nitrocellulose membrane (Biorad) and incubated with anti-human Klotho monoclonal antibody (1:500, Clone KM2076, Hölzel Diagnostika) and anti-rat antibody conjugated with horseradish peroxidase (1:2000) (6). Specific signal was visualized using the ECL chemiluminescence kit (Amersham). Since a 100 kD filter was used, we focused on the 130 kDa Klotho band as described by Hu et al (9).

Serum phosphate and albuminuria

In addition, a potential correlation between serum phosphate and albuminuria was assessed in a cohort of 351 patients from the Fundación Jimenez Diaz Nephrology Outpatient clinic database. These were all-comers and stable outpatient. In-patients were excluded. Main clinical characteristics of the cohort are shown in **Table 1**. Serum and spot urine samples were collected in the morning after a 12 h fast and used for routine biochemistry in an automatic workstation ADVIA Centaur XP-Siemens Heathineers Global. UACR was measured using routine immunoassay-based measurement and eGFR was calculated from serum creatinine by the CKD-EPI equation (27).

Statistics

Statistical analysis was performed using SPSS 11.0 statistical software. Results are expressed as mean \pm SD or as median [inter-quartile (IQR) range]. Significance at the $p<0.05$ level was assessed by Student's t test for two groups of data and ANOVA for three or more groups. Associations between quantitative variables were assessed using Spearman correlation coefficient. In order to identify potential predictors of quantitative outcomes, multivariable linear regression models were fitted. UACR was log transformed to meet a normal distribution, as assessed by the Kolmogorov-Smirnov test. Models were built using forward stepwise procedures in order to maximize R-Squared with the smallest number of predictor variables. Age, sex and variables with statistically significant association in the univariate analysis were used. The statistical significance of variables in the models was assessed by ANOVA test.

RESULTS

Albuminuria correlated with serum phosphate in human CKD

A recent report has suggested that human proteinuric kidney disease is associated with FGF23 resistance, but the molecular underlying mechanisms are unclear (16). In a cohort of 351 CKD patients, a direct correlation was found between serum phosphate and UACR in univariate analysis (**Table 2, Figure 1**). Serum phosphate also correlated with serum magnesium and PTH and inversely with eGFR (**Table 2**). In multivariate analysis, eGFR and UACR were independent predictors of serum phosphate when adjusted for age and sex (**Table 3.A**). These data are consistent with previous reports of the presence of FGF23 resistance in human proteinuric kidney disease (16). In the multivariable model, a significant interaction between eGFR and UACR was found. Including the interaction term modified the results: when entering the eGFR \times UACR interaction term, the variable eGFR is no longer significant (**Table 3.B**): the effect that UACR has on serum phosphate depends on the value taken by eGFR, or, in other words eGFR modifies the relationship between UACR and serum phosphate. This is consistent with greater ability of kidneys with higher eGFR to excrete higher amounts of phosphate. In this regard, categorization by baseline eGFR disclosed that the impact of UACR on serum phosphate levels was more apparent at baseline eGFR <30 ml/min/1.73 m² (**Suppl figure 1**).

Urinary Klotho is decreased in patients with severe albuminuria

Following the observation that albuminuria correlates with serum phosphate independently of eGFR, we set out to study the mechanisms of this link and, specifically, explored the hypothesis that albuminuria may decrease Klotho to promote FGF-23 resistance. As a first step, the impact of pathological albuminuria on urinary Klotho excretion was assessed in human CKD. Urinary Klotho was reported to be decreased in category G1 CKD in humans (9). While category G1 CKD usually implies the presence of pathological albuminuria, it is possible to have category G1 CKD with normoalbuminuria if abnormal kidney imaging or histology are present (1). Thus, we focused on the relationship between albuminuria and urinary Klotho in individuals with diverse degrees of GFR impairment and of severity of albuminuria, expanding G categories G1 through G5 and albuminuria categories A1 through A3. Urinary Klotho levels were highest in individuals with preserved eGFR and minimal albuminuria, but either the presence of severe albuminuria or decreased GFR resulted in a dramatic decrease in urinary Klotho (**Figure 2.A,B**). **Suppl figure 2** shows the full blot and Ponceau red staining. Despite the low number of patients a trend was observed for higher phosphate levels in patients with higher UACR (**Figure 2.B**).

Patients with UACR above a certain threshold had suppressed urinary Klotho and there was a trend toward a negative correlation between UACR and urinary Klotho (Correlation= -0.378, $p = 0.076$)(**Suppl fig. 3**). In addition, some patients with low UACR also had low urinary Klotho: those with low GFR. When urinary Klotho was plotted against eGFR and the magnitude of UACR incorporated into the graph as the size of the dots for individual patients, a pattern emerged that either high UACR or low eGFR was associated with low urinary Klotho values (**Figure 3**). **Suppl fig. 4** represents urinary Klotho vs the ratio eGFR/log UACR. These results suggest that an inverse relationship between albuminuria and Klotho is present in human CKD and becomes more apparent when renal function is still preserved, since when renal function and mass are lost, the decreased tubular cell mass may already account for lower Klotho levels.

Experimental proteinuric kidney disease is associated with Klotho downregulation

Since albuminuria was associated with decreased urinary Klotho in human CKD, the relationship between albuminuria and Klotho expression was explored in two animal models of pathological albuminuria in the presence of preserved global renal function.

In rats albuminuria was induced by a single injection of the podocyte toxin PAN (**Figure 4.A**) while in mice it was induced by albumin overload (**Figure 4.B**). PAN nephrosis is a classical model of

minimal change nephrotic syndrome while albumin overload results in massive albuminuria (28). In these animals, renal function was preserved as assessed by serum creatinine (control mice 0.26 ± 0.05 mg/dl, albumin overload mice 0.26 ± 0.05 mg/dl; control rats day 10, 0.48 ± 0.10 mg/dl, PAN rats day 10, 0.52 ± 0.11 mg/dl, ns).

In both animal models pathological albuminuria was associated with interstitial inflammation characterized by increased kidney MCP-1 mRNA levels (**Figure 4.C,D**) and interstitial infiltration by macrophages (**Figure 4.E,F**). Reduced total Klotho mRNA expression was observed both in rat PAN nephrosis (**Figure 5.A**) and in murine albumin overload proteinuria (**Figure 5.D**). A correlation between proteinuria and Klotho mRNA was observed in rats: above a certain threshold of proteinuria, Klotho mRNA decreased (**Suppl fig. 5**). This was similar to the observation in humans (**Suppl fig. 3**), although in rats renal function was homogeneous.

Further characterization of Klotho protein levels was carried out in the mice and rats. Western blot using the monoclonal KM2076 antibody confirmed the reduced total kidney Klotho protein expression in rat PAN nephrosis (**Figure 5.C**) and in albumin-overloaded mice (**Figure 5.F**). Immunohistochemistry using the monoclonal KM2076 antibody localized Klotho expression to tubular cells and confirmed reduced tubular cell Klotho expression in proteinuric animals (**Figure 5.B and 5.E**). **Supplementary figure 6** shows results obtained using the polyclonal antibody.

These results suggest that pathological albuminuria decreases Klotho expression in experimental proteinuric kidney disease with preserved renal function. Both local inflammation and albumin itself could drive this response.

Albumin directly decreases Klotho expression in cultured renal tubular cells

Since pathological albuminuria was associated with decreased kidney or urinary Klotho in both human and experimental proteinuric kidney disease, and both local inflammation or a direct albumin effect could explain the association, we explored the second alternative, whether albumin had direct effects on Klotho expression in cultured tubular cells since inflammation induced Klotho downregulation is already well characterized. Murine tubular cells were cultured in the presence of albumin to simulate exposure to albumin when the glomerular permeability to protein is increased. Albumin dose-dependently decreased Klotho mRNA expression (**Figure 6.A**). Other stressors had previously been reported to decrease Klotho expression in tubular cells. Thus, both inflammatory cytokines present in the injured

1 kidney environment, such as TWEAK, and activation of the transcription factor NF- κ B decreased Klotho
2 expression by tubular cells (10). Indeed, TWEAK decreases Klotho mRNA through NF- κ B activation and
3 promotion of histone deacetylation (10). However, the molecular mechanism of albumin-induced Klotho
4 downregulation differed from that elicited by TWEAK. Thus, unlike cytokine-induced Klotho
5 downregulation, two structurally different NF- κ B inhibitors did not prevent Klotho downregulation in
6 response to albumin (**Figure 6.B**). By contrast the HDAC inhibitor trichostatin A prevented the decrease
7 in Klotho mRNA and protein induced by albumin (**Figure 6.C,D**). Albumin also decreased Klotho
8 expression in human proximal tubular cells, although only a trend towards a dose-response was observed
9 at 24h (**Suppl figure 7**). In these cells, trichostatin A also prevented the decrease in Klotho protein
10 induced by albumin. These results suggest that albumin directly represses Klotho expression in tubular
11 cells through epigenetic mechanisms.

12 DISCUSSION

13 The main finding is that exposure of proximal tubular cells to albumin in culture, a model that
14 reproduces the exposure of proximal tubular cells to albumin in proteinuric nephropathies, resulted in
15 decreased Klotho expression. This may be clinically significant since tubular Klotho was decreased in
16 experimental proteinuric nephropathies, urinary Klotho was decreased in human proteinuric disease and
17 proteinuria correlated with serum phosphate, a potential indication of FGF23 resistance, in human CKD.

18 Acquired Klotho deficiency has been described in human CKD and found to be present already
19 in category G1 CKD (9). Category G1 CKD means that eGFR is normal, but there is evidence of kidney
20 injury (1). The most frequent evidence of kidney injury in the clinic is pathological albuminuria or
21 proteinuria. However, there are other criteria to define CKD when eGFR is normal. These include urine
22 sediment abnormalities, electrolyte and other abnormalities due to tubular disorders, abnormalities
23 detected by histology and structural abnormalities detected by imaging (1). Thus, while decreased urinary
24 Klotho in category G1 CKD had already been described, this is, to our knowledge, the first study that
25 addresses specifically the association between albuminuria and urinary Klotho and found an inverse
26 relationship in humans and in preclinical models of kidney disease.

27 Albuminuria may theoretically decrease kidney Klotho by direct or indirect effects. In this
28 regard, albuminuria is toxic to proximal tubular cells and may result in tubular cell death or in a sublethal
29 stress response characterized by the secretion of inflammatory mediators and inflammation driven by

secretion of chemokines such as MCP-1 (14,29–31). Systemic or local inflammation reduces kidney Klotho and may account for low Klotho levels in patients with pathological albuminuria (7,10). In fact, we observed that in preclinical models of proteinuric kidney disease, increased MCP-1 mRNA expression and interstitial inflammation is a prominent feature, confirming prior observations. However, we have now shown that albuminuria also has a direct effect on kidney Klotho levels and that this effect is not mediated by the transcription factor NFκB, thus, arguing against autocrine activation of inflammatory cytokines such as TWEAK. Indeed, TWEAK- and TNF-induced downregulation of Klotho expression in tubular epithelium is mediated by NFκB (10). By contrast, cytokines and albumin share epigenetic mechanisms to downregulate Klotho expression. The HDAC inhibitor trichostatin A prevented Klotho downregulation induced either by TWEAK (10) or, as shown here, by albumin. Thus, HDAC inhibition may be an approach to preserve Klotho expression that protects against both the direct and the indirect (inflammation-mediated) effects of albuminuria on Klotho expression. The preclinical finding may be clinically relevant since in humans a decreased urinary Klotho excretion, generally accepted to represent kidney Klotho, was found in the presence of either albuminuria or decreased GFR. These findings confirm and extend the prior observation, using the same Western blot technique, of decreased urinary Klotho in category G1 CKD (9) and pinpoint pathological albuminuria as a factor associated with decreased urinary Klotho when GFR is normal. Unfortunately, Western blot is a time-consuming technique that is not well suited to study a large number of samples. In addition, in a cohort study we confirmed a recent observation of a direct correlation between albuminuria and serum phosphate (16). These authors identified FGF23 resistance as a potential driver of the relationship. We now pinpoint the problem to acquired kidney Klotho deficiency probably resulting from direct and indirect effects of albumin on tubular cells. Genetic Klotho deficiency has been previously shown to result in FGF-23 resistance and high circulating FGF-23 levels (4). In addition, urinary Klotho has direct phosphaturic effects on the proximal tubule, dependent on its enzymatic glycosidase activity that inactivates the NaPi2a phosphate transporters in proximal tubules (32). It is theoretically possible that albuminuria-induced FGF23 resistance may help preserve serum 1,25(OH)₂D₃ levels. This in turn may contribute to higher serum phosphate levels that may not be excreted as required, given the loss of the direct effect of Klotho on phosphate transporters in proximal tubules and on phosphaturia.

Current therapy for proteinuric kidney disease is based on renin angiotensin system (RAS) blockade (33). However, residual albuminuria despite RAS blockade is a key prognostic factor. The

present result suggests that in addition to generating kidney inflammation, residual albuminuria may directly decrease kidney Klotho expression, thus potentially favoring CKD progression and accelerated cardiovascular aging despite normal GFR and no accumulation of uremic toxins. The sensitivity of this response to HDAC inhibitors, such as trichostatin A, suggests the involvement of epigenetic mechanisms, and, thus, the potential for chronification of Klotho downregulation driven by albuminuria. In this regard, epigenetic downmodulation of RASAL1 in fibroblasts is a key contributor to the AKI-to-CKD transition (34). Further characterization of intracellular signaling pathways that lead to a direct effect of albuminuria on tubular cell Klotho expression may identify novel therapeutic approaches aimed at preserving Klotho expression despite persistence of albuminuria. In this regard, two recent observations help illustrate the clinical relevance of our findings. First, RAS blockade does not efficiently prevent CKD progression in patients with higher serum phosphate levels (15). Second, these higher serum phosphate levels in albuminuric patients appear to depend on FGF-23 resistance in the kidneys (16). The fact that albumin directly decreases kidney Klotho by an epigenetic mechanism may explain how albuminuria decreases kidney Klotho and causes FGF-23 resistance and also why RAS blockade fails to protect renal function despite improving albuminuria, since epigenetic changes may be perpetuated in the same cell and even be transmitted to daughter cells (35). We hypothesize that Klotho downregulation itself may be a driving force for CKD progression and accelerated cardiovascular aging under these circumstances (5,9,11). The loss of Klotho in response to albuminuria coupled to a negative impact of Klotho deficiency on kidney disease may potentially generate a vicious circle where kidney injury results in low kidney Klotho and Klotho downregulation favors progression of kidney injury (8). Either preventing Klotho downregulation or supplementing the missing Klotho may interrupt the vicious circle (8). Thus, exogenous soluble Klotho restored the expression of endogenous Klotho in injured kidneys (8). In this regard, animal models of AKI and CKD have uniformly shown decreased kidney Klotho as well as a nephroprotective role of Klotho (6–13).

One important lesson from this and recent studies(16) is that the variable albuminuria may need to be taken into consideration when FGF23 is correlated with phosphaturia, phosphatemia, GFR or phosphate intake, since it may impact the renal response to FGF23 and dietary phosphate. In addition, an open question is to what extent the association of albuminuria with CKD progression or adverse cardiovascular and survival outcomes could be related to early Klotho deficiency and associated abnormalities of phosphate metabolism (36).

This study has several limitations. Thus, urinary Klotho was tested in a limited number of patients. However, this was the consequence of using a time-consuming technique, Western blot, given the substantial limitations of available Klotho ELISAs for humans and the need for fresh urine (37). In this regard, it is unclear whether in prior studies using ELISA to assess plasma Klotho in large CKD cohorts, these limitations may have contributed to the lack of association of plasma Klotho with phosphorus or FePi (38). In any case, urinary Klotho has direct, FGF23-independent phosphaturic effects dependent on degradation of NaPi-2a in the luminal tubular surface (32) that may underlie any potential differences between urinary and plasma Klotho. Circulating Klotho was not assessed. In addition to the low kidney levels of Klotho, we cannot exclude that lower urinary Klotho levels are influenced by altered shedding to the luminal side, or increased uptake of shed Klotho by proximal tubules.

In conclusion, we have shown that albumin directly decreases Klotho expression in tubular cells in culture through epigenetic mechanisms. This may explain or at least contribute to the experimental animal observation of decreased tubular cell Klotho in proteinuric nephropathies, the decrease in urinary Klotho excretion in human proteinuric kidney disease and the clinical observations of a correlation between serum phosphate levels and proteinuria as well as the evidence for FGF-23 resistance¹⁶ and the observation of an inverse correlation between serum Klotho and proteinuria (39). The hypothesis that this early albuminuria-driven decrease in Klotho expression may contribute to the higher risk of premature death and CKD progression in human CKD category G1/2 should be explored.

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TABLES

Table 1. Clinical characteristics of patients in the CKD cohort (n=351). Results are expressed as %, mean \pm SD or median [inter-quartile (IQR) range].

Age (years)	67 \pm 14
Sex (male)	60%
DM (yes)	52%
25OH-Vitamin D (ng/dl)	20 \pm 10
sCr (mg/dl)	2.2 \pm 1.3
eGFR (ml/min/1.73 m ²)	40 \pm 24
sCa (mg/dL)	9.45 \pm 0.5
sP (mg/dL)	3.8 \pm 7.6
sMg (mg/dL)	1.95 \pm 0.35
iPTH (pg/ml)	94.3 [56.2-158.8]
Serum albumin (g/dl)	4.0 \pm 0.4
UACR (mg/g)	167 [22-588]
FE phosphate (%)	30 \pm 14

sCr: serum creatinine, eGFR: estimated glomerular filtration rate, sCa: serum calcium, sP: serum phosphate, sMg: serum magnesium, UACR: urinary albumin creatinine ratio, FE: fractional excretion.

Table 2. Univariate correlations of serum phosphate with quantitative variables.

	Correlation	p value
UACR (mg/g)	0.41	<0.0001
eGFR (ml/min/1.73 m ²)	-0.38	<0.0001
iPTH (pg/ml)	0.38	0.0006
sMg (mg/dL)	0.24	<0.0001
Age (years)	-0.06	0.19

eGFR: estimated glomerular filtration rate sMg: serum magnesium, UACR: urinary albumin creatinine ratio

Table 3. Multivariate model for predictors of serum phosphate (mg/dl) adjusted for age and sex**A. Without log UACR:eGFR interaction**

	Coefficient (95% CI))	p value
Intercept	4.36 (3.88 to 4.84)	<0.0001
Age (years)	-0.009 (-0.014, -0.004)	<0.001
Log UACR (mg/g)	0.086 (0.049, 0.124)	<0.001
eGFR (ml/min/1.73 m ²)	-0.011 (-0.014, -0.008)	<0.001
Sex (female)	0.209 (0.062, 0.356)	0.006

R squared adjusted = 0.27

B. With log UACR:eGFR interaction

	Coefficient (95% CI))	p value
Intercept	3.78 (3.14, 4.42)	<0.0001
Age (years)	-0.007 (-0.013, -0.002)	0.005
Log UACR (mg/g)	0.175 (0.100, 0.249)	<0.001
eGFR (ml/min/1.73 m ²)	0.000 (-0.009, 0.009)	0.939
Sex (female)	0.200 (0.054, 0.346)	0.007
Log UACR:eGFR	-0.002 (-0.004, -0.001)	0.007

R squared adjusted = 0.23

FIGURE LEGENDS

Figure 1. Correlation between serum phosphate and proteinuria in human CKD. Data from 351 clinically stable outpatients. UACR: urinary albumin:creatinine ratio, $p<0.0001$.

Figure 2. Decreased urinary Klotho expression in human proteinuric kidney disease. **A)** Urinary Klotho was assessed by Western blot in patients with different degrees of eGFR and albuminuria classified as 2012 KDIGO G and A categories (1). The full membrane is shown in supplementary figure 2. **B)** Quantification of Western blot results. * $p<0.05$ vs G1-2/A1-2. The table below shows clinical data for the studied population. eGFR: estimated glomerular filtration rate (CKD-EPI), sP: serum phosphate, UACR: urinary albumin:creatinine ratio. Data presented as mean \pm SD or median (interquartile range).

Figure 3. Decreased urinary Klotho in human proteinuric kidney disease is associated with higher albuminuria and with decreased eGFR. Urinary Klotho protein results from figure 2 were plotted against CKD-EPI estimated glomerular filtration rate (eGFR). The size of the data points is proportional to the magnitude of urinary albumin excretion: the larger the size, the higher the UACR, as indicated in the figure. Each data point represents an individual patient. The graph shows that only individuals with normal eGFR and normal UACR had higher (normal) urinary Klotho levels and either pathological albuminuria or decreased eGFR was associated with lower urinary Klotho. The relationship between UACR and size of the points is indicated.

Figure 4. Experimental proteinuric kidney disease is associated to interstitial inflammation. **A)** Urinary protein before and after the injection of PAN or vehicle (control) in rats. * $p<0.02$, # $p<0.005$ vs control. **B)** Coomassie blue-stained urine protein gels showing increased urinary albumin in albumin overload-induced nephropathy induced by daily albumin ip administration for 7 days in mice. **C and D)** Whole kidney MCP1 mRNA expression was increased in **C)** PAN nephrosis and **D)** albumin overload-induced nephropathy. qRT-PCR results expressed as % change over control which was considered to be 100%. * $p<0.006$ vs control. Mean \pm SD of 5 animals per group. **E)** Quantification and representative CD68 immunohistochemistry 10 days following PAN or vehicle injection. CD68+ macrophages are increased in PAN nephrosis. * $p<0.001$ vs vehicle-injected control. **F)** Quantification and immunohistochemistry

image representative of F4/80 positive macrophages in albumin overload nephropathy at day 7. Original magnification x200. * $p < 0.001$ vs vehicle-injected control.

Figure 5. Decreased kidney Klotho expression in experimental proteinuric kidney disease. A and D)

Decreased whole kidney Klotho mRNA expression in rats injected with PAN (A) and in mice with albumin overload-induced nephropathy (D), * $p < 0.0001$ vs vehicle-injected control, ** $p < 0.05$ vs vehicle-injected control. qRT-PCR results expressed as % change over control which was considered to be 100%.

B and E) Klotho immunostaining. Monoclonal KM2076 antibody. Representative immunohistochemistry image in rats injected with PAN (B) and in albumin overload nephropathy (E). Original magnification x200. **C and F)** Quantification and representative western blot of Klotho expression in rats injected with PAN (C) and in mice with albumin overload-induced nephropathy (F)). Monoclonal KM2076 antibody. Mean \pm SD of 5 animals per group. * $p < 0.0001$ vs vehicle-injected control.

Figure 6. Exposure to albumin decreases Klotho expression in murine cultured tubular cells. A)

Dose-response and time-course of Klotho mRNA expression, expressed as % change over control which was considered to be 100%. * $p < 0.005$ vs control; ** $p < 0.05$ vs albumin 10 mg/mL. **B)** Neither parthenolide nor SN50 modulate the downregulation of Klotho mRNA in response to albumin (10 mg/mL) for 3 hours. Both are NF- κ B inhibitors previously shown to inhibit NF- κ B-mediated responses at the same concentrations in this cell system (10). **C)** The HDAC inhibitor trichostatin A (TSA) prevents Klotho mRNA downregulation in response to albumin (10 mg/mL) for 3 hours. * $p < 0.05$ vs control; # $p < 0.04$ vs albumin alone. **D)** TSA prevents Klotho protein downregulation in response to albumin. Representative images for 3 independent experiments. When not otherwise specified, cells were incubated with 10 mg/mL albumin for 3 hours or vehicle control.

Supplementary figure 1. Correlation between serum phosphate and proteinuria in human CKD.

Data from 351 clinically stable outpatients who were categorized according to baseline eGFR. UACR: urinary albumin:creatinine ratio.

Supplementary figure 2. Full membrane and Ponceau red staining of the blot shown in figure 2.A.

Supplementary figure 3. Relationship between urinary Klotho and albuminuria (UACR in mg/g) in humans. Correlation= -0.378, p = 0.076. Note that above a certain magnitude of proteinuria, urinary Klotho levels fall. In addition, some patients with low UACR have low urinary Klotho. These correspond to patients with low GFR. To gain a wider perspective, figure 3 plots the three variables (UACR, GFR and urinary Klotho) in the same graph.

Supplementary figure 4. Representation of log urinary Klotho (arbitrary units) vs ratio of eGFR (ml/min/1.73 m²)/ log UACR (mg/g) in human individuals.

Supplementary figure 5. Relationship between kidney Klotho mRNA and proteinuria in rats with PAN nephrosis. Note that above a certain magnitude of proteinuria, kidney Klotho mRNA expression falls.

Supplementary figure 6. Decreased kidney Klotho expression in experimental proteinuric kidney disease. Polyclonal antibody. **A)** Quantification and representative western blot of Klotho expression in mice with albumin overload-induced nephropathy. Mean±SD of 5 animals per group. *p<0.0001 vs vehicle-injected control. Antibody used: Calbiochem. **B)** Klotho immunostaining. Representative immunohistochemistry image in albumin overload nephropathy. Original magnification x200. Antibody used: Calbiochem.

Supplementary figure 7. Exposure to albumin decreases Klotho expression in human cultured HK2 tubular cells. **A)** Dose-response and time-course of Klotho mRNA expression, expressed as % change over control which was considered to be 100%. *p<0.05 vs control. Neither parthenolide **(B)** nor SN50 **(C)** modulate the downregulation of Klotho mRNA in response to albumin (10 mg/mL) for 3 hours. The HDAC inhibitor trichostatin A (TSA) prevents Klotho mRNA **(D)** and protein **(E)** downregulation in response to albumin. Representative images and quantification for 3 independent experiments. Cells were incubated with 10 mg/mL albumin for 3 hours or vehicle control. *p<0.0005 vs control, #p<0.05 vs albumin.

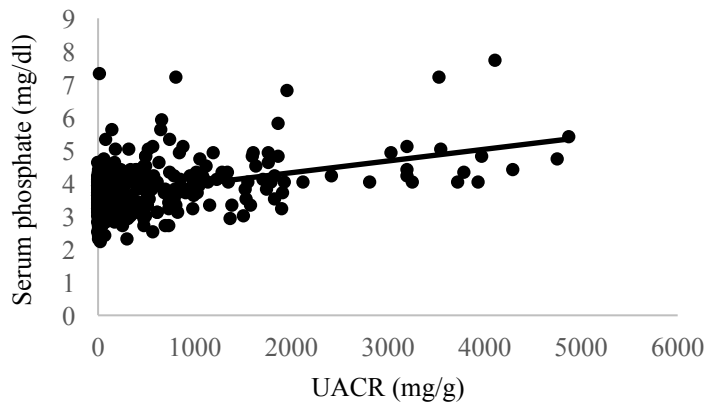
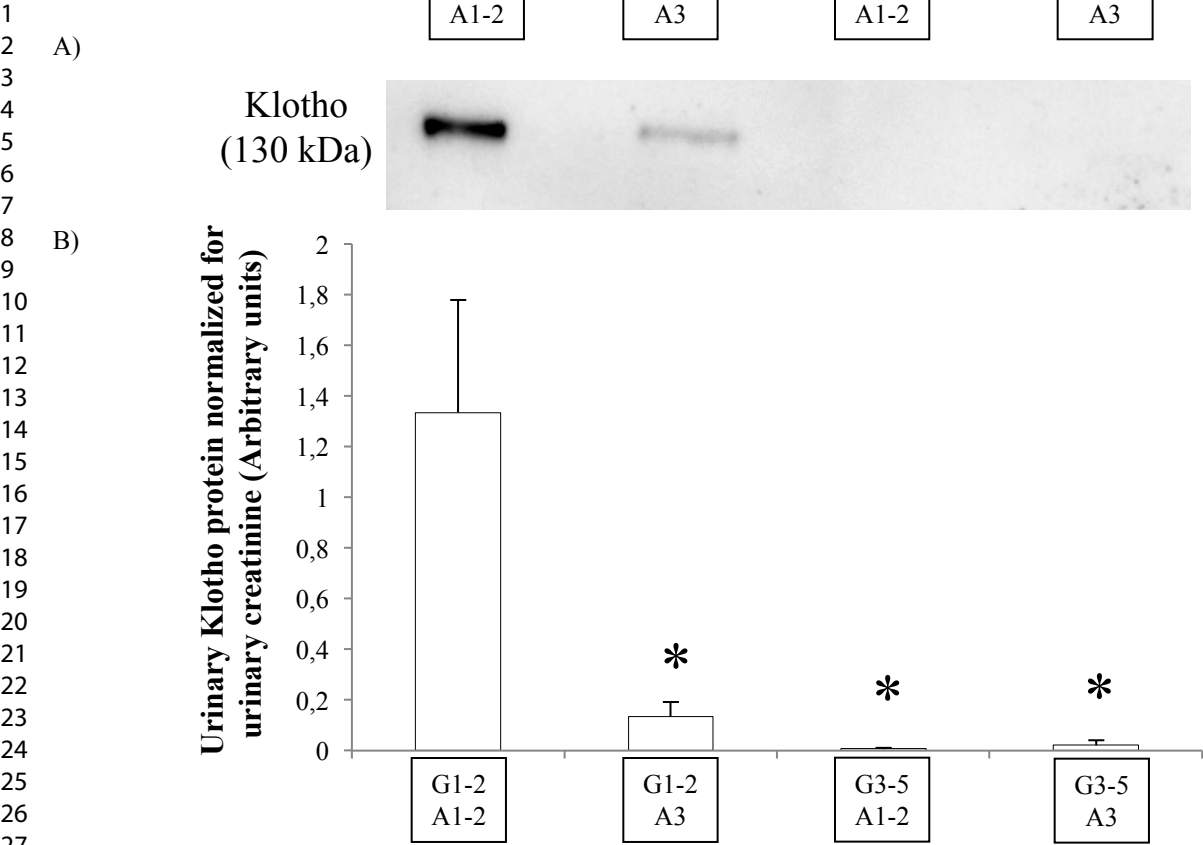
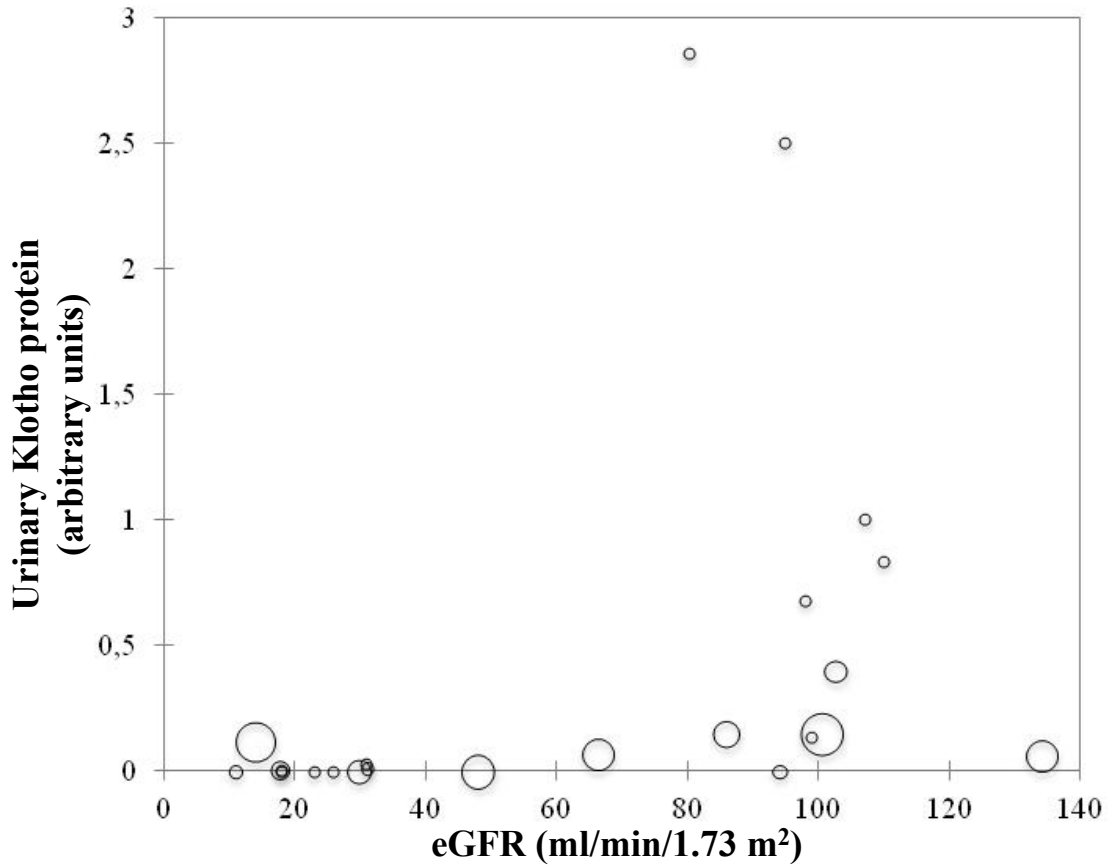
Figure 1

Figure 2



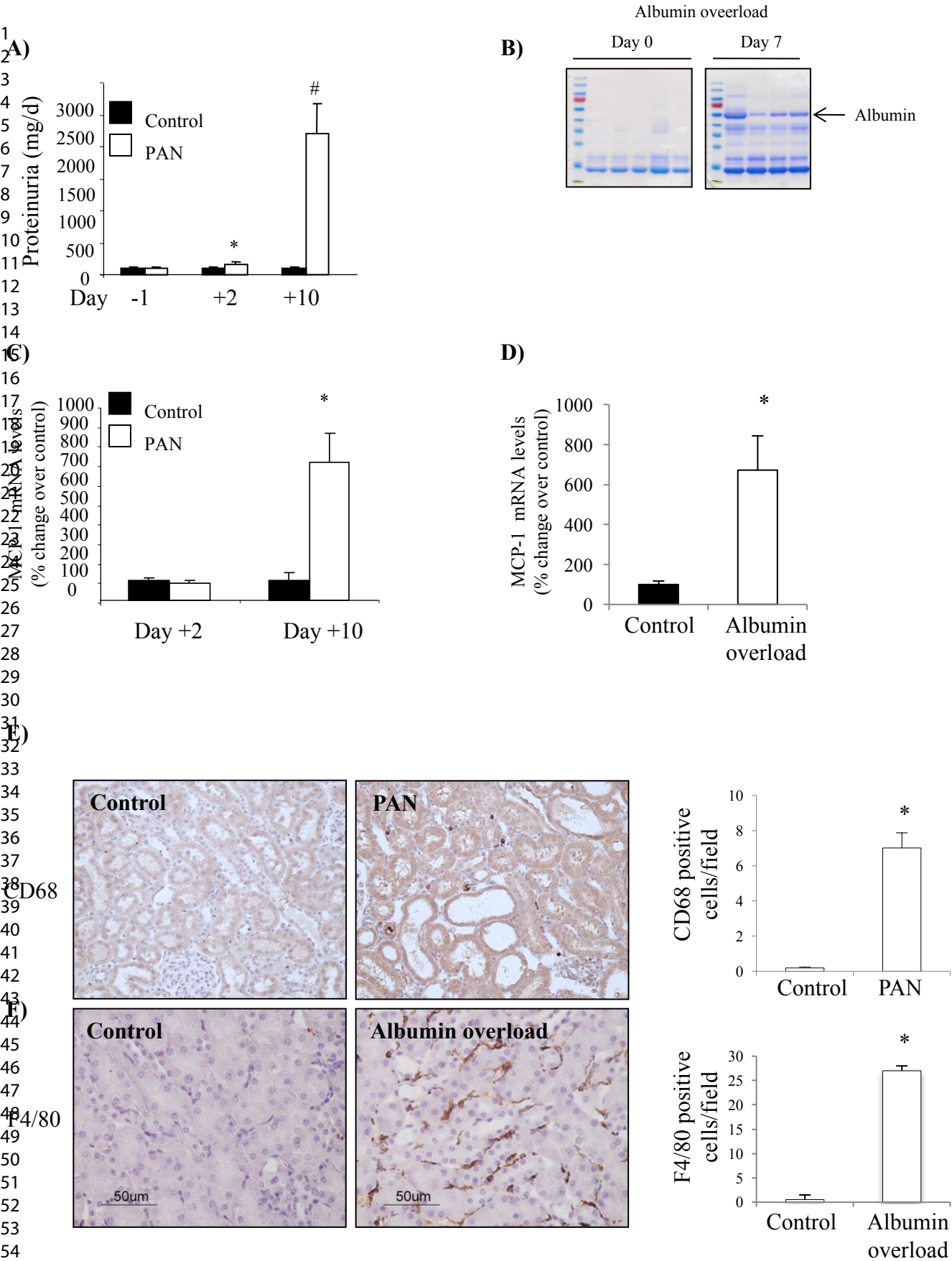
Male (%)	66	83	60	50
Age (years)	50±14	48±17	61±15	57±13
eGFR (ml/min/1.73m²)	102±17	97±21	22±5	25±14
UACR (mg/g)	3.0 [3.0-5.2]	4343 [2775-5005]	59 [37-106]	2182 [988-4639]
sP (mg/dl)	3.33±0.14	3.63±0.99	3.94±0.63	4.50±0.55

Figure 3



UACR ○ >6000 mg/g ○ 2500-4500 mg/g ○ 500-1500 mg/g
 ○ 4500-6000 mg/g ○ 1500-2500 mg/g ○ <500 mg/g

Figure 4



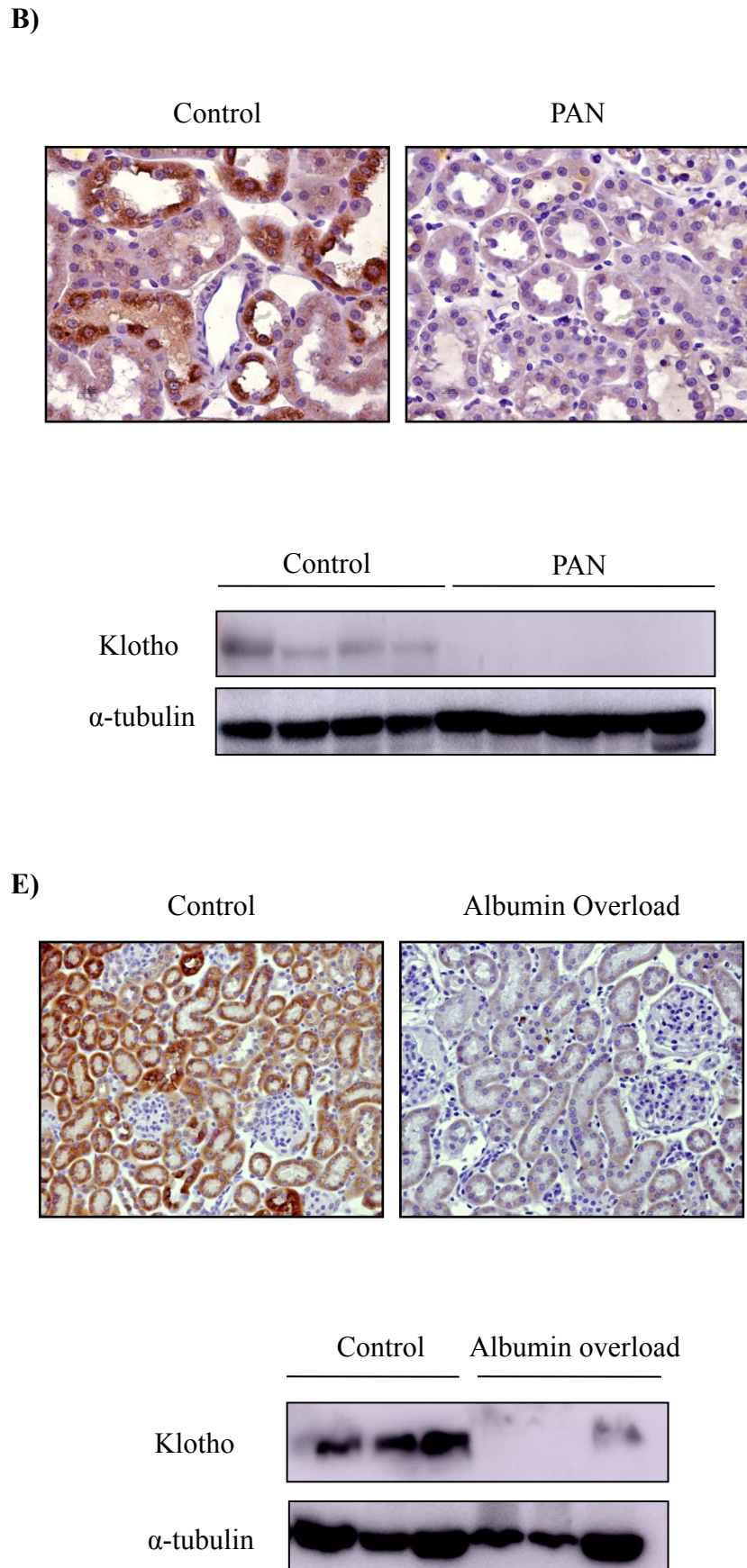
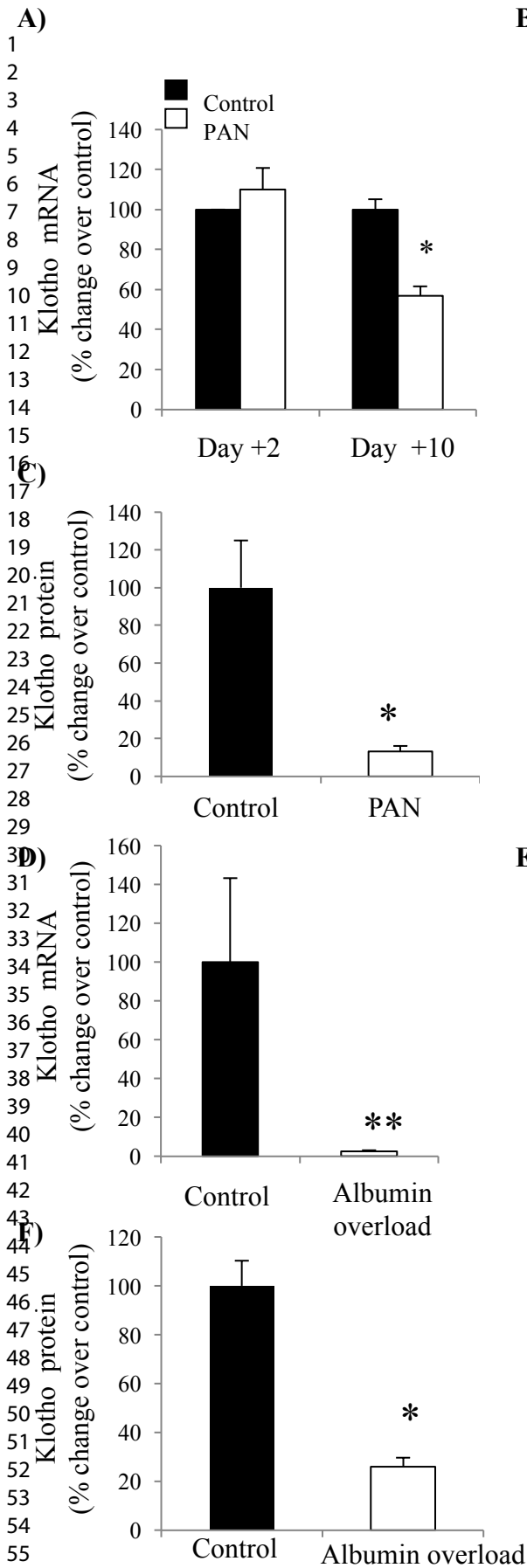
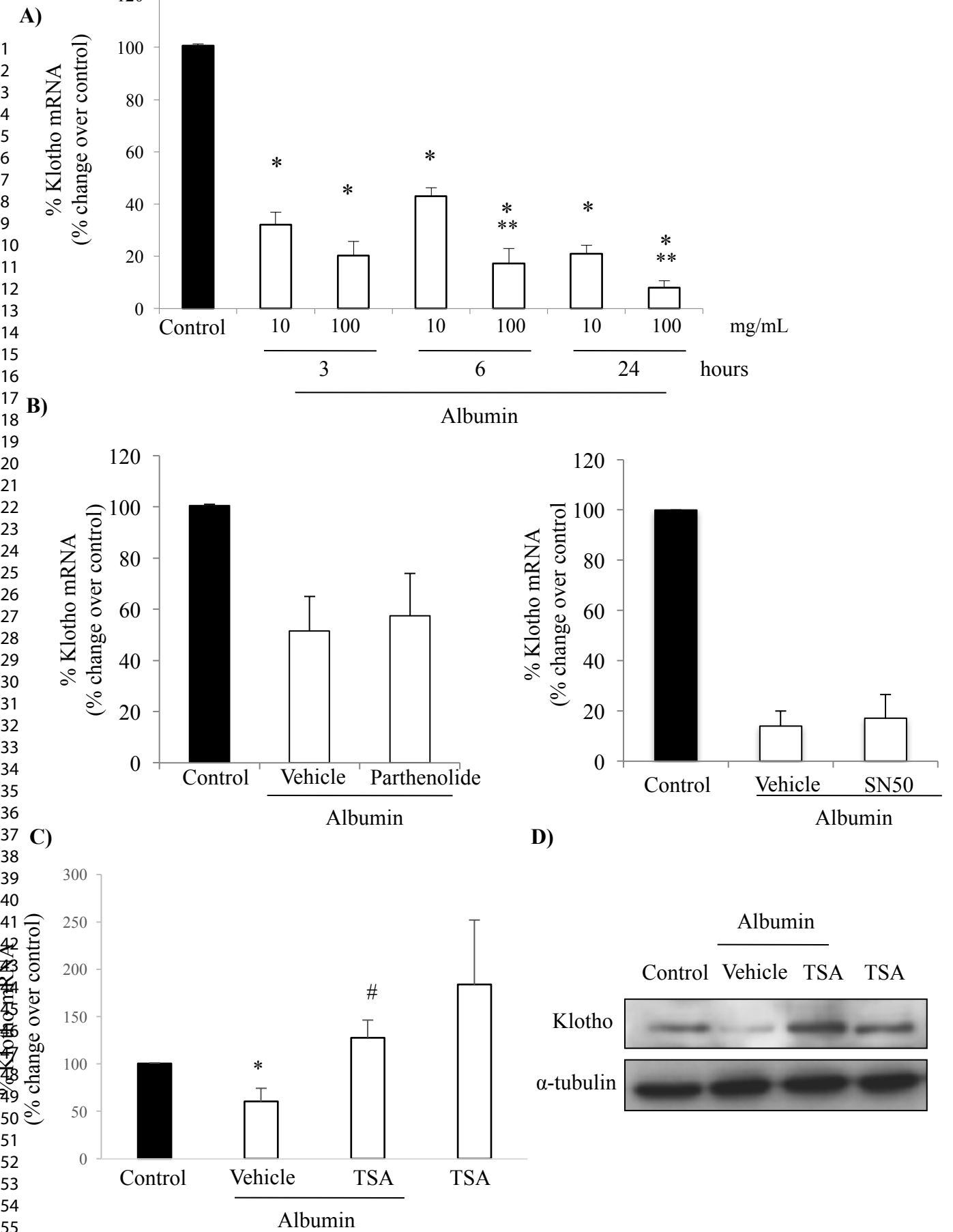
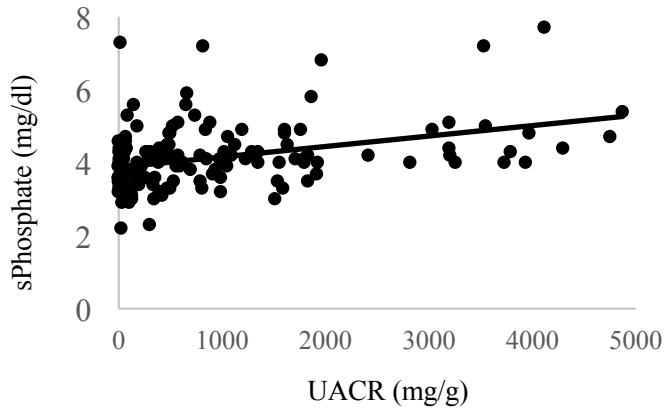


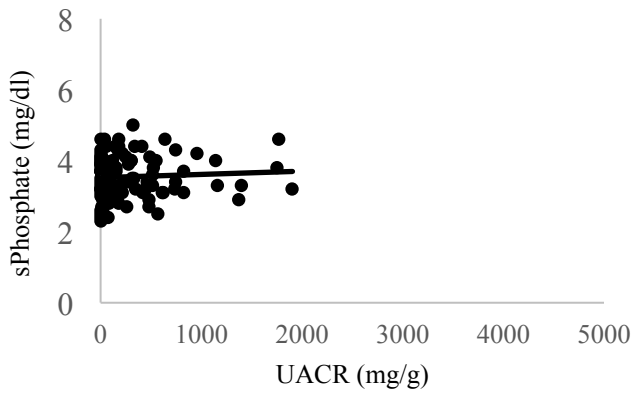
Figure 6



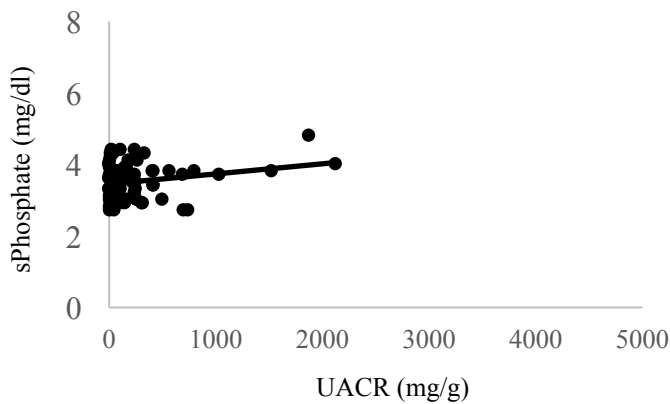
A) eGFR <30 ml/min/1.73 m²

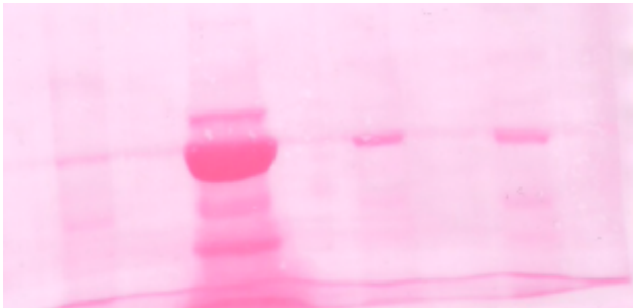
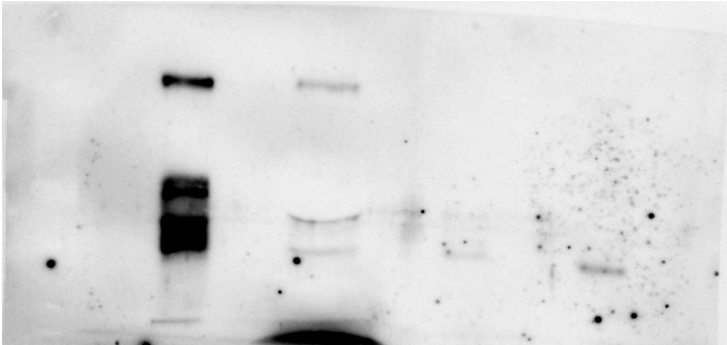


B) eGFR 30-60 ml/min/1.73 m²

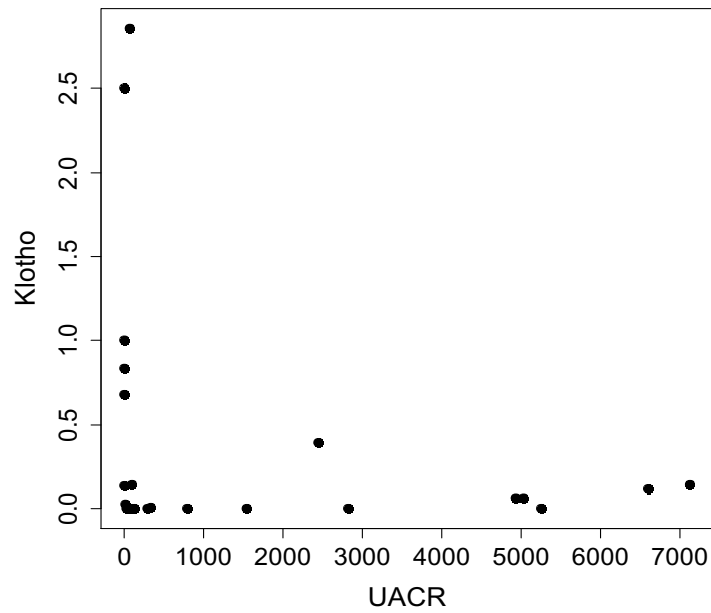


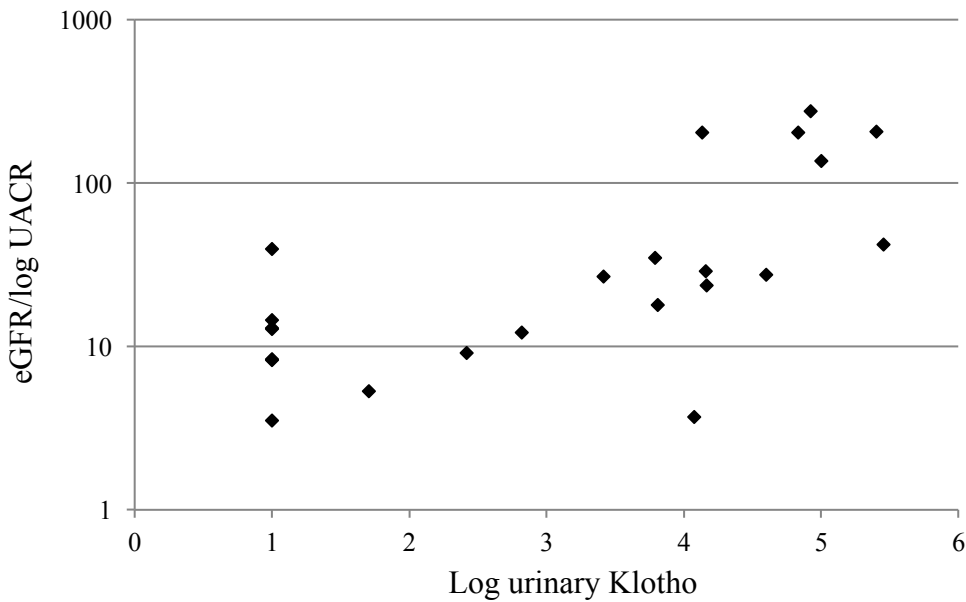
C) eGFR ≥60 ml/min/1.73 m²



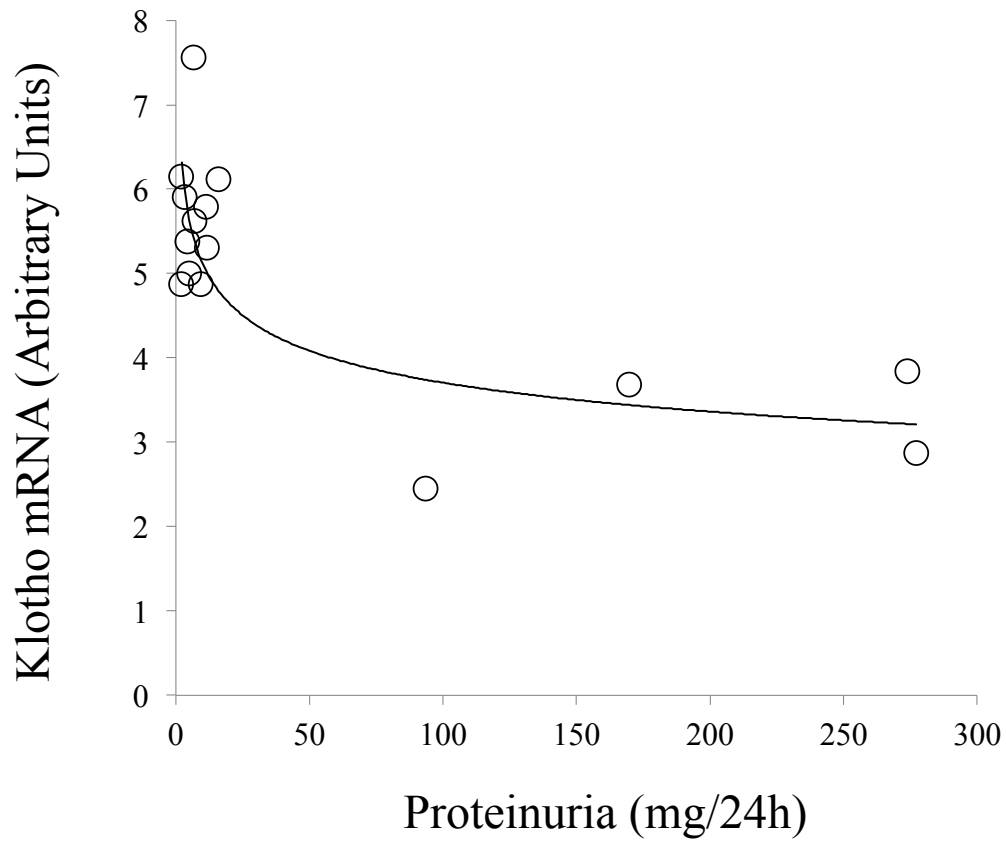


Supplementary figure 3



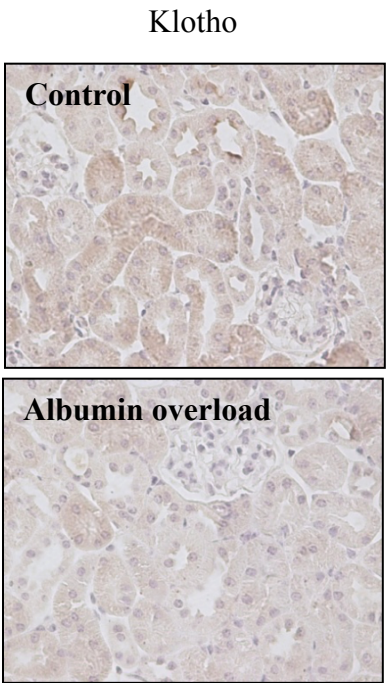
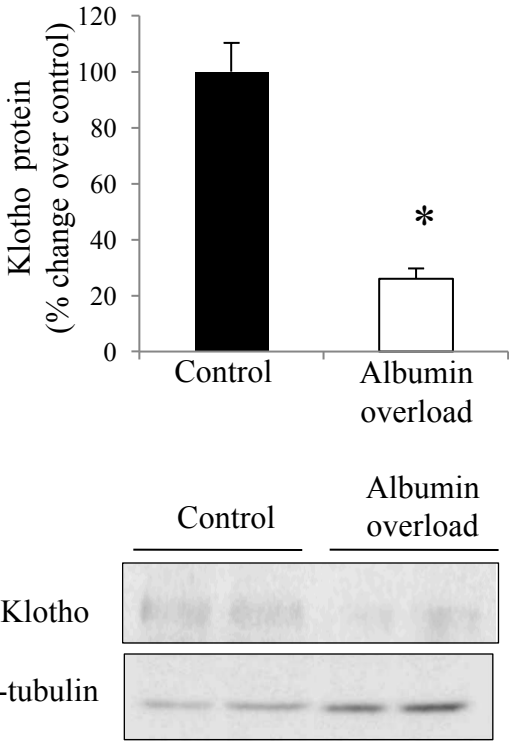


Supplementary figure 5



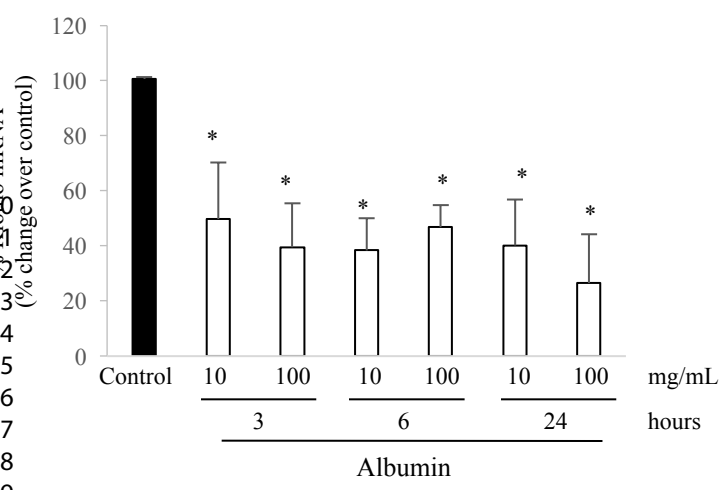
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A) **B)**

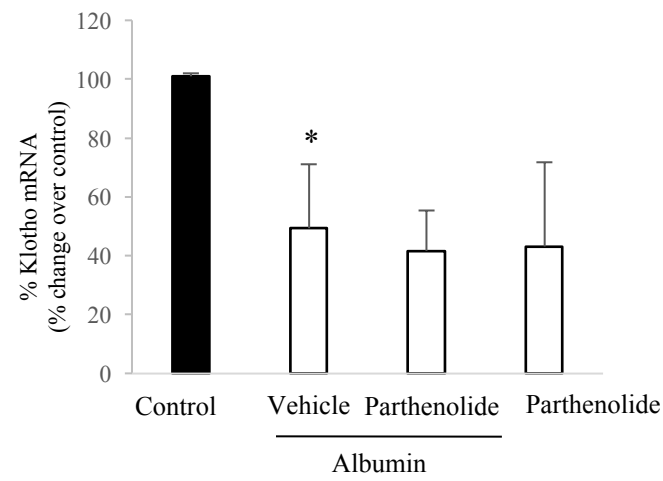


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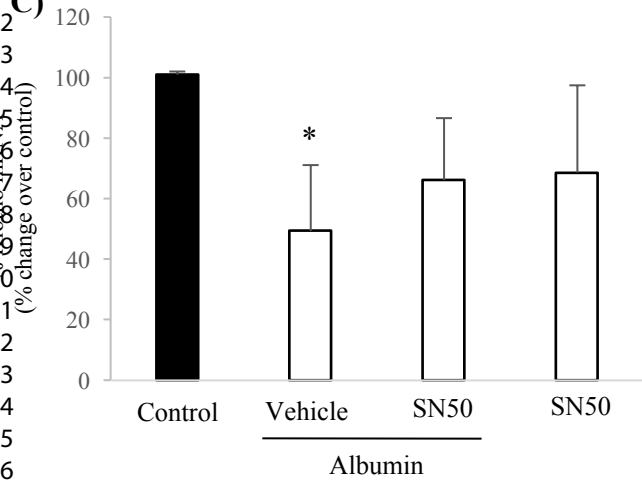
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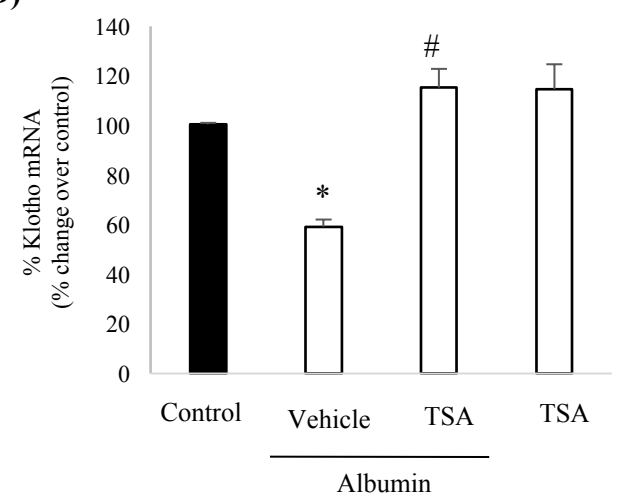
B)



C)



D)



E)

