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ORIGINAL ARTICLE

Albumin downregulates Klotho in tubular cells

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ABSTRACT

Background. Kidney tubular cells are the main sources of Klotho, a protein with phosphaturic action. 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.

Methods. 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.

Results. 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 GFR. Proteinuria induced in rats by puromycin aminonucleoside and in mice by albumin overload was associated with interstitial inflammation and reduced total kidney Klotho messenger ribonucleic acid (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, but unlike cytokine-induced Klotho downregulation, not by inhibitors of nuclear factor kappa-light-chain-enhancer of activated B cells.

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

Keywords: aging, albuminuria, chronic kidney disease, 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]. 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 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 Category G1 human 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. Tumour necrosis factor (TNF) superfamily inflammatory cytokines, transforming growth factor β1 or angiotensin II decreased Klotho expression in cultured tubular cells [6–8, 10, 11]. Systemic delivery of TNF-like weak inducer of apoptosis (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 nuclear factor kappa-light-chain-enhancer of activated B cells (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 coreceptor for the phosphaturic hormone fibroblast growth factor 23 (FGF-23) and by directly inhibiting tubular phosphate reabsorption by sodium/phosphate cotransporter 2a (NaPi-2a) [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 NaPi-2a expression and decreased phosphorylation of FGF receptor substrate 2α, 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 parathyroid hormone (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 mechanisms linking albuminuria to *in vivo* low Klotho levels and FGF-23 resistance should be explored. These include the pro-inflammatory effects of pathological albuminuria, promoting a local inflammatory response that leads to decreased Klotho expression [7] or a direct effect of albumin on the Klotho expression of tubular cells. 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 a 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.

MATERIALS AND METHODS

Cells and reagents

The mouse cortical tubule cells line was cultured in Roswell Park Memorial Institute medium 1640 (Gibco, Grand Island, NY, USA), 10% decomplemented foetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in 5% carbon dioxide at 37°C [17, 18]. Penicillin and streptomycin were from BioWhittaker (Waltham, MA, USA) and FBS from Life Technologies (Carlsbad, CA, USA). Exposure of cultured tubular cells to bovine serum albumin (Sigma, St. Louis, MO, USA) 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 h 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, Billerica, MA, USA), and the histone deacetylase (HDAC) inhibitor trichostatin A (TSA; 100 ng/mL; Upstate Biotechnology, Millipore) were added 1 h before albumin: doses were derived from prior experience in the laboratory [10, 19]. Additional studies were performed in HK2 human proximal tubular epithelial cells, cultured as previously described [20].

Animal models

Studies were conducted in accordance with the European Union normative and National Institutes of Health 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 intravenous injection of 150 mg/kg puromycin aminonucleoside (PAN) 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 anaesthesia, 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, CA, USA, Merck Millipore #423500) [10, 24, 25] or anti-human Klotho monoclonal antibody

(1: 500; Clone KM2076, Hölzel Diagnostika Köln, Germany), rat polyclonal anti-F4/80 antigen (1:50; Serotec, Oxford, UK) for murine macrophages and goat polyclonal anti-CD68 (1:100; Santa Cruz Biotechnology, Dallas, TX, USA) for rat macrophages. Sections were counterstained with Carazzi's haematoxylin. 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 (×40) using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA). Samples were examined in a blinded manner.

Quantitative real-time polymerase chain reaction (PCR)

RNA of 1 μg isolated with Trizol (Invitrogen, Carlsbad, CA, USA) was reverse transcribed with the High-Capacity cDNA Archive Kit and real-time PCR was performed on a ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA, USA) using the $\Delta\Delta C_T$ method [26]. Expression levels are given as ratios to glyceraldehyde 3-phosphate dehydrogenase (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 Tris-HCl, 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% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions [26]. After electrophoresis, samples were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), 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, 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 [enhanced chemiluminescence (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 estimated glomerular filtration rate (eGFR) and urinary albumin:creatinine ratio (UACR) categories following Kidney Disease: Improving Global Outcomes (KDIGO) categories as follows [1]: Group 1 ($n = 6$), G1–2 (eGFR >60 mL/min/1.73 m^2), A1–2 (UACR <300 mg/g); Group 2 ($n = 6$), G1–2 (eGFR >60 mL/min/1.73 m^2), A3 (UACR >300 mg/g); Group 3 ($n = 5$), G3–5 (eGFR <60 mL/min/1.73 m^2), A1–2 (UACR <300 mg/g); Group 4 ($n = 6$), G3–5 (eGFR <60 mL/min/1.73 m^2), A3 (UACR >300 mg/g). Patients were selected to represent four different combinations of eGFR and UACR and extreme UACR values were favored in order to increase the chances of observing differences despite the small 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 data, Table S1. 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 cut-off (Millipore), and 50 μL concentrated urine was separated by 8% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) 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 chemiluminiscence kit (Amersham). Since a 100-kD filter was used, we focused on the 130-kDa Klotho band as described by Hu *et al.* [9].

Table 1. Clinical characteristics of patients in the CKD cohort ($n = 351$)

Characteristic	Value
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/1.73m^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), median (IQR)	94.3 (56.2–158.8)
Serum albumin (g/dL)	4.0 ± 0.4
UACR (mg/g), median (IQR)	167 (22–588)
FE phosphate, %	30 ± 14

Results are expressed as mean ± SD unless stated otherwise.
SCr, serum creatinine; SCa, serum calcium; SP, serum phosphate; SMg, serum magnesium; iPTH, intact parathyroid hormone; FE, fractional excretion.

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 Díaz Nephrology Outpatient clinic database. These were all-comers and stable outpatients. Inpatients were excluded. The 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, Erlangen, Germany). UACR was measured using routine immunoassay-based measurement and eGFR was calculated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [27].

Table 2. Univariate correlations of serum phosphate with quantitative variables

Variable	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

iPTH, intact parathyroid hormone; SMg, serum magnesium.

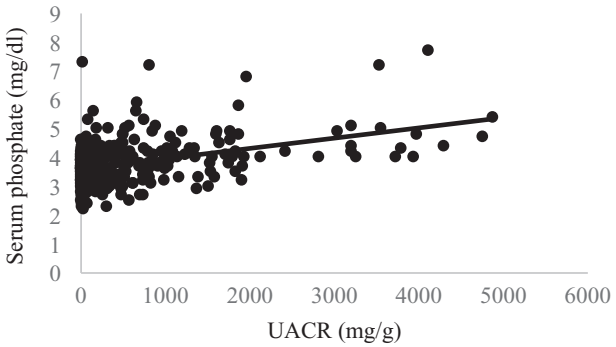


FIGURE 1: Correlation between serum phosphate and proteinuria in human CKD. Data from 351 clinically stable outpatients. $P < 0.0001$.

Statistics

Statistical analysis was performed using SPSS 11.0 statistical software (SPSS, Chicago, IL, USA). Results are expressed as mean ± standard deviation (SD) or as median [interquartile range (IQR)]. Significance at the $P < 0.05$ level was assessed by Student's *t*-test for two groups of data and analysis of variance (ANOVA) for three of more groups. Associations between quantitative variables were assessed using Spearman's 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^2 with the smallest number of predictor variables. Age, sex and variables with a statistically significant association in the univariate analysis were used. The statistical significance of variables in the models was assessed by ANOVA.

RESULTS

Albuminuria correlated with serum phosphate in human CKD

A recent report has suggested that human proteinuric kidney disease is associated with FGF-23 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 3A). These

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 to −0.004)	<0.001
Log UACR (mg/g)	0.086 (0.049 to 0.124)	<0.001
eGFR (mL/min/1.73 m^2)	−0.011 (−0.014 to −0.008)	<0.001
Sex (female)	0.209 (0.062 to 0.356)	0.006

R^2 adjusted = 0.27. CI, confidence interval.

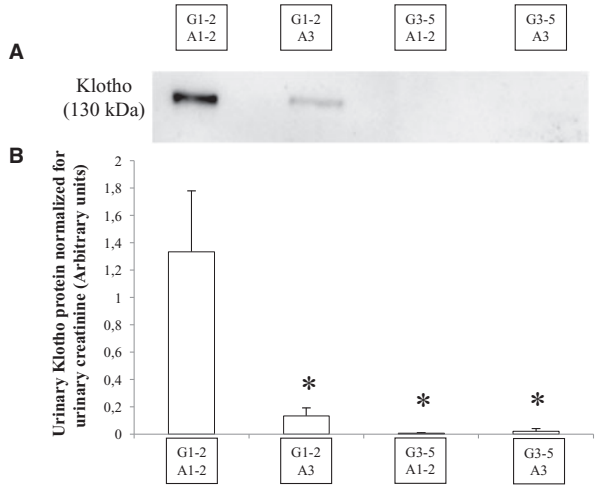
B. With log UACR × eGFR interaction		
	Coefficient (95% CI)	P-value
Intercept	3.78 (3.14–4.42)	<0.0001
Age (years)	−0.007 (−0.013 to −0.002)	0.005
Log UACR (mg/g)	0.175 (0.100–0.249)	<0.001
eGFR (mL/min/1.73 m^2)	0.000 (−0.009–0.009)	0.939
Sex (female)	0.200 (0.054–0.346)	0.007
Log UACR: eGFR	−0.002 (−0.004 to −0.001)	0.007

R^2 adjusted = 0.23. CI, confidence interval.

data are consistent with previous reports of the presence of FGF-23 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 × UACR interaction term, the variable eGFR is no longer significant (Table 3B). 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 the greater ability of kidneys with a higher eGFR to excrete greater amounts of phosphate. In this regard, categorization by baseline eGFR disclosed that the impact of UACR on serum phosphate levels was more apparent at a baseline eGFR <30 mL/min/1.73m^2 (Supplementary data, Figure S1).

Urinary Klotho is decreased in patients with severe albuminuria

Following the observation that albuminuria correlates with serum phosphate independent 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 is present [1]. Thus we focused on the relationship between albuminuria and urinary Klotho in individuals with diverse degrees of GFR impairment and severity of albuminuria, expanding G Categories G1–G5 and albuminuria Categories A1–A3. Urinary Klotho levels were highest in individuals with preserved eGFR and minimal albuminuria, but



Male (%)	66	83	60	50
Age (years)	50±14	48±17	61±15	57±13
eGFR (ml/min/1.73m^2)	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 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 data, Figure 2. **(B)** Quantification of western blot results. *$P < 0.05$ versus G1–2/A1–2. The table shows clinical data for the studied population. sP, serum phosphate. Data presented as mean ± SD or median [IQR].

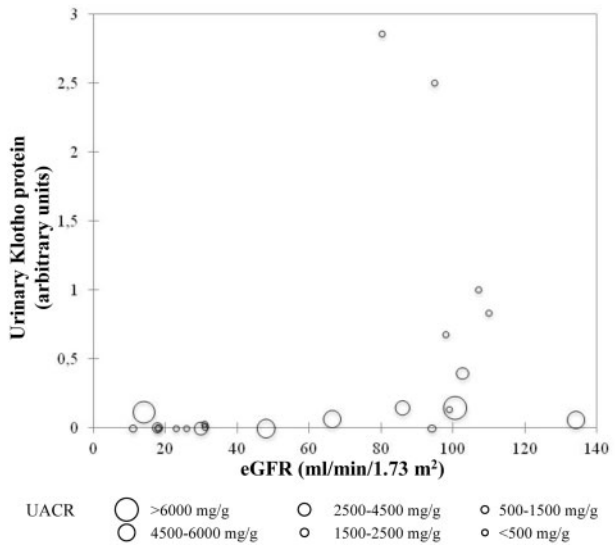


FIGURE 3: Decreased urinary Klotho in human proteinuric kidney disease is associated with higher albuminuria and with decreased eGFR. Urinary Klotho protein results from Figure 2 were plotted against CKD-EPI eGFR. The size of the data points is proportional to the magnitude of urinary albumin excretion: 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 the size of the points is indicated.

either the presence of severe albuminuria or decreased GFR resulted in a dramatic decrease in urinary Klotho (Figure 2A and B). Supplementary data, Figure S2 shows the full blot and Ponceau red staining. Despite the small number of patients, a trend was observed for higher phosphate levels in patients with higher UACR (Figure 2B).

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, $P = 0.076$) (Supplementary data, Figure S3). 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). Supplementary data, Figure S4 represents urinary Klotho versus 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 4A), while in mice it was induced by albumin overload (Figure 4B). PAN nephrosis is a classic 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; Day 10: control rats 0.48 ± 0.10 mg/dL, PAN rats 0.52 ± 0.11 mg/dL, not significant).

In both animal models, pathological albuminuria was associated with interstitial inflammation characterized by increased kidney monocyte chemotactic protein 1 (MCP-1) mRNA levels (Figure 4C and D) and interstitial infiltration by macrophages (Figure 4E and F). Reduced total Klotho mRNA expression was observed both in rat PAN nephrosis (Figure 5A) and in murine albumin overload proteinuria (Figure 5D). A correlation between proteinuria and Klotho mRNA was observed in rats: above a certain threshold of proteinuria, Klotho mRNA decreased (Supplementary data, Figure S5). This was similar to the observation in humans (Supplementary data, Figure S3), although renal function was homogeneous in rats.

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 5C) and in albumin-overloaded mice (Figure 5F). Immunohistochemistry using the monoclonal KM2076 antibody localized Klotho expression to

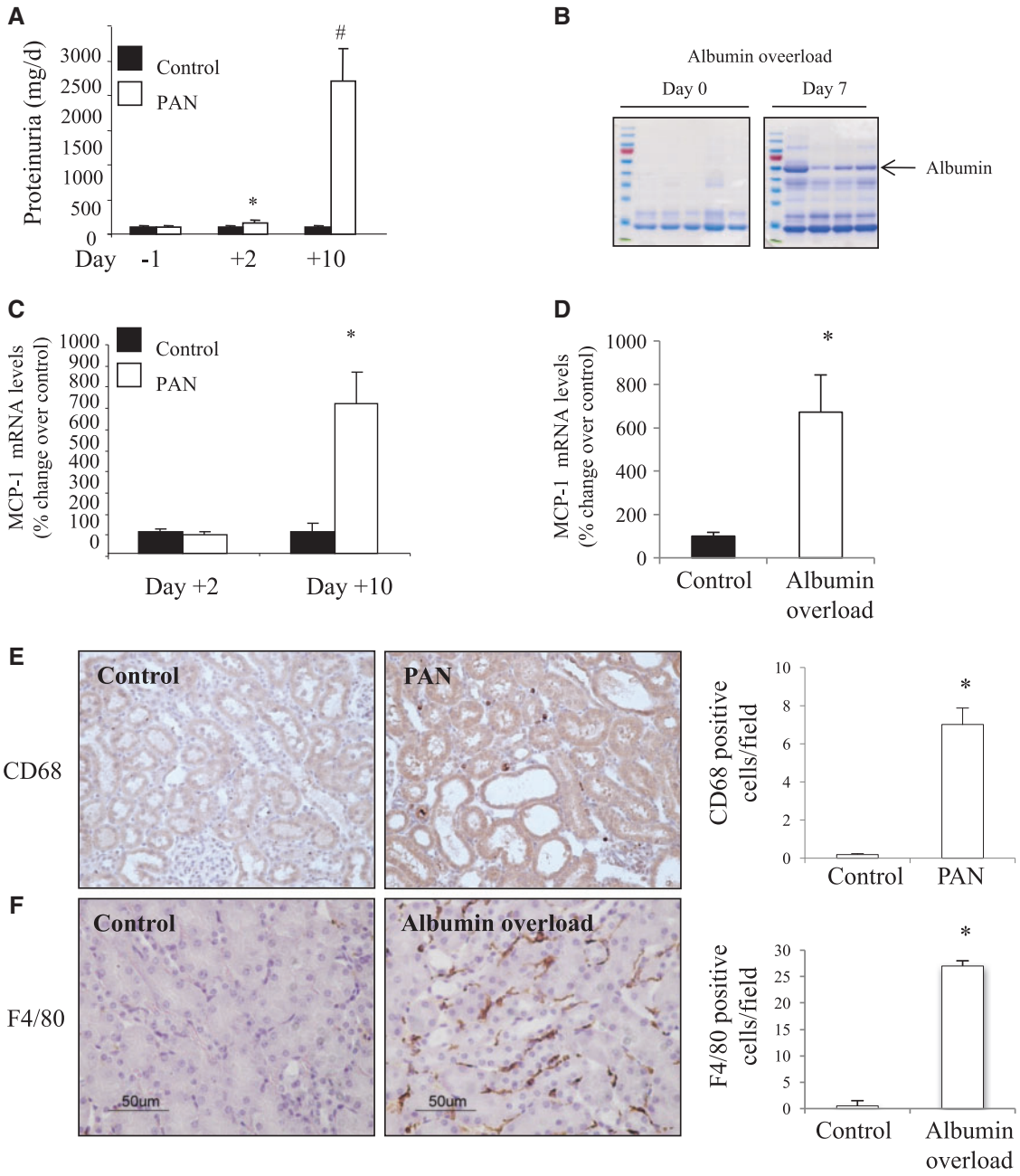


FIGURE 4: Experimental proteinuric kidney disease is associated to interstitial inflammation. **(A)** Urinary protein before and after the injection of PAN or vehicle (control) in rats. *$P < 0.02$, #$P < 0.005$ versus control. **(B)** Coomassie blue–stained urine protein gels showing increased urinary albumin in albumin overload–induced nephropathy induced by daily intraperitoneal albumin administration for 7 days in mice. Whole kidney MCP-1 mRNA expression was increased in **(C)** PAN nephrosis and **(D)** albumin overload–induced nephropathy. Real-Time Quantitative Reverse Transcription polymerase chain reaction (qRT-PCR) results expressed as percent change over control, which was considered to be 100%. *$P < 0.006$ versus control. Mean ± SD of five animals per group. **(E)** Quantification and representative CD68 immunohistochemistry 10 days following PAN or vehicle injection. $CD68^+$ macrophages are increased in PAN nephrosis. *$P < 0.001$ versus vehicle-injected control. **(F)** Quantification and immunohistochemistry image representative of F4/80-positive macrophages in albumin overload nephropathy at Day 7. Original magnification ×200. *$P < 0.001$ versus vehicle-injected control.

tubular cells and confirmed reduced tubular cell Klotho expression in proteinuric animals (Figure 5B and E). Supplementary data, Figure S6 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 and a direct albumin effect could explain the association, we explored the second alternative, whether albumin had direct

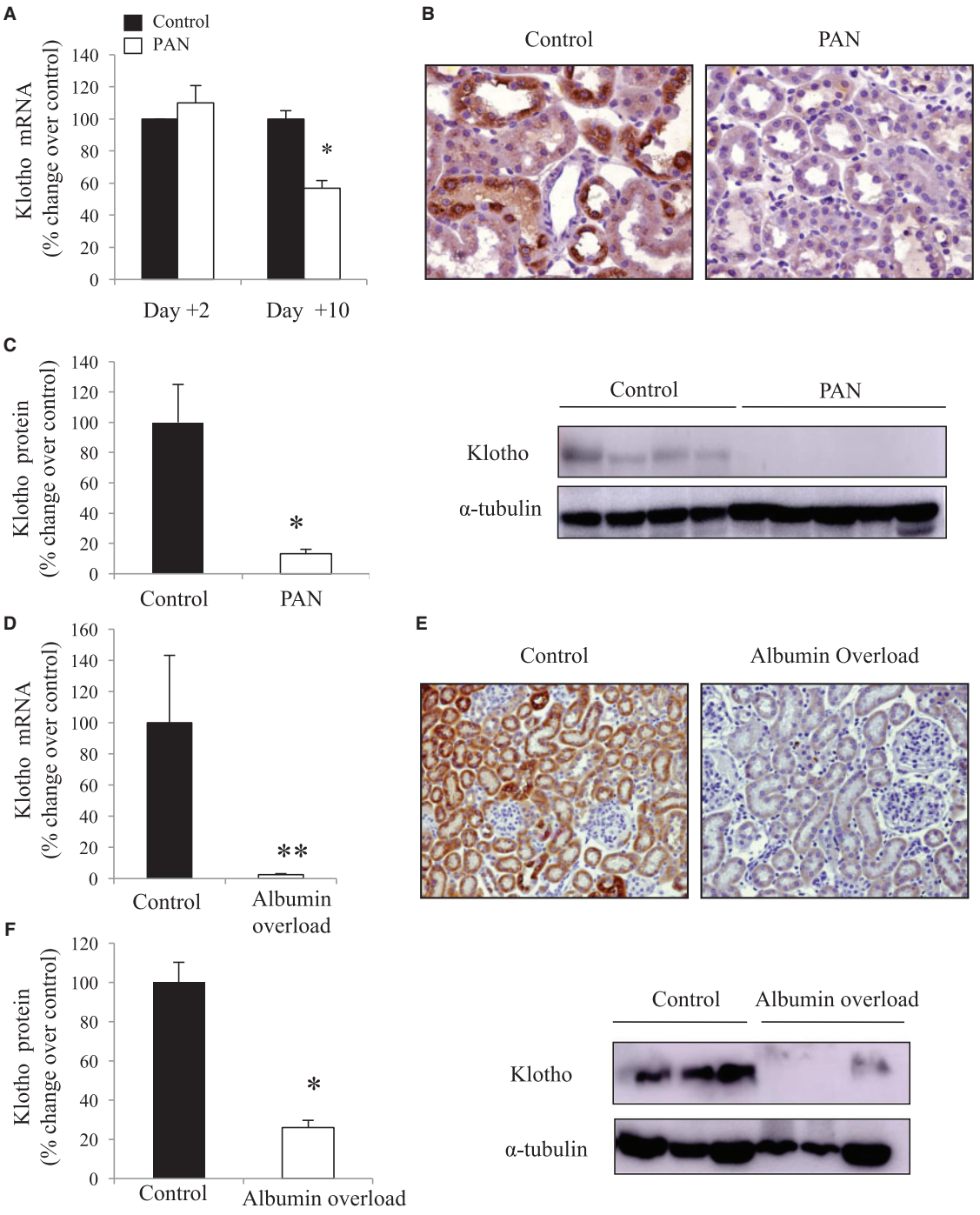


FIGURE 5: Decreased kidney Klotho expression in experimental proteinuric kidney disease. (**A** and **D**) Decreased whole kidney Klotho mRNA expression **(A)** in rats injected with PAN and **(D)** in mice with albumin overload–induced nephropathy; *$P < 0.0001$ versus vehicle-injected control, **$P < 0.05$ versus vehicle-injected control. Quantitative real-time PCR results expressed as percent change over control, which was considered to be 100%. (**B** and **E**) Klotho immunostaining. Monoclonal KM2076 antibody. Representative immunohistochemistry image **(B)** in rats injected with PAN and **(E)** in albumin overload nephropathy. Original magnification ×200. **(C** and **F)** Quantification and representative western blot of Klotho expression **(C)** in rats injected with PAN and **(F)** in mice with albumin overload–induced nephropathy. Monoclonal KM2076 antibody. Mean ± SD of five animals per group. *$P < 0.0001$ versus vehicle-injected control.

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 6A). 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

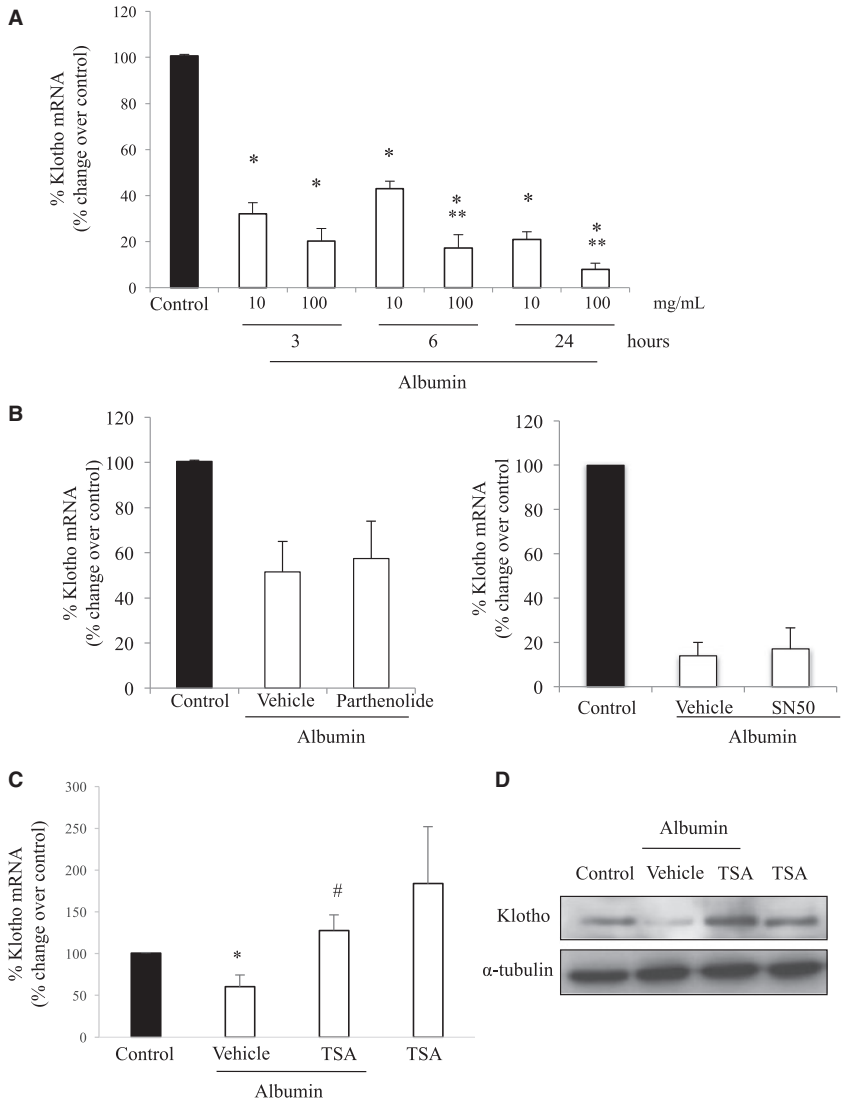


FIGURE 6: Exposure to albumin decreases Klotho expression in murine cultured tubular cells. **(A)** Dose response and time course of Klotho mRNA expression, expressed as percent change over control, which was considered to be 100%. *P < 0.005 versus control; **P < 0.05 versus albumin 10 mg/mL. **(B)** Neither parthenolide nor SN50 modulate the downregulation of Klotho mRNA in response to albumin (10 mg/mL) for 3 h. 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 TSA prevents Klotho mRNA downregulation in response to albumin (10 mg/mL) for 3 h. *P < 0.05 versus control; #P < 0.04 versus albumin alone. **(D)** TSA prevents Klotho protein downregulation in response to albumin. Representative images for three independent experiments. When not otherwise specified, cells were incubated with 10 mg/mL albumin for 3 h or vehicle control.

NF-κB decreased Klotho expression by tubular cells [10]. Indeed, TWEAK decreases Klotho mRNA through NF-κB 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-κB inhibitors did not prevent Klotho downregulation in response to albumin (Figure 6B). In contrast, the HDAC inhibitor TSA prevented the decrease in Klotho mRNA and protein induced by albumin (Figure 6C and D). Albumin also decreased Klotho expression in human proximal tubular cells, although only a trend toward a dose response was observed at 24 h (Supplementary data, Figure S7). In these cells, TSA 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 FGF-23 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 has 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 decrease 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 are prominent features, 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-κB, 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-κB [10]. In contrast, cytokines and albumin share epigenetic mechanisms to downregulate Klotho expression. The HDAC inhibitor TSA 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 decreased urinary Klotho excretion in humans, 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 point to 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 for studying 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 FGF-23 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 NaPi-2a phosphate transporters in proximal tubules [32]. It is theoretically possible that albuminuria-induced FGF-23 resistance may help preserve serum 1,25-dihydroxyvitamin 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 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 TSA 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 the 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 FGF-23 is correlated with phosphaturia, phosphatemia, GFR or phosphate intake, since it may impact the renal response to FGF-23 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. First, 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 enzyme-linked immunosorbent assays (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 urinary fractional phosphate excretion [38]. In any case, urinary Klotho has direct, FGF-23-independent phosphaturic effects dependent on the 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 resistance [16] 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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SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

CONFLICT OF INTEREST STATEMENT

None declared.

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Fibroblast growth factor 23 is induced by an activated renin–angiotensin–aldosterone system in cardiac myocytes and promotes the pro-fibrotic crosstalk between cardiac myocytes and fibroblasts

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ABSTRACT

Background. Fibroblast growth factor 23 (FGF23) is discussed as a new biomarker of cardiac hypertrophy and mortality in patients with and without chronic kidney disease (CKD). We previously demonstrated that FGF23 is expressed by cardiac myocytes, enhanced in CKD and induces cardiac hypertrophy via activation of FGF receptor 4 independent of its co-receptor klotho. The impact of FGF23 on cardiac fibrosis is largely unknown.

Methods. By conducting a retrospective case–control study including myocardial autopsy samples from 24 patients with end-stage CKD and *in vitro* studies in cardiac fibroblasts and myocytes, we investigated the pro-fibrotic properties of FGF23.

Results. The accumulation of fibrillar collagens I and III was increased in myocardial tissue of CKD patients and correlated with dialysis vintage, klotho deficiency and enhanced cardiac angiotensinogen (AGT) expression. Using human fibrosis RT^2 Profiler PCR array analysis, transforming growth factor (TGF)-β and its related TGF-β receptor/Smad complexes, extracellular matrix remodeling enzymes and pro-fibrotic growth factors were upregulated in myocardial tissue of CKD patients. FGF23 stimulated cell proliferation, migration, pro-fibrotic TGF-β receptor/Smad complexes and collagen synthesis in cultured cardiac fibroblasts. In isolated cardiac myocytes, FGF23 enhanced collagen remodeling, expression of pro-inflammatory genes and pro-survival pathways and induced pro-hypertrophic genes. FGF23 stimulated AGT expression in cardiac myocytes and angiotensin II and aldosterone, as components of the renin–angiotensin–aldosterone system (RAAS), induced FGF23 in cardiac myocytes.