Direct Blood Pressure-Independent Anti-Fibrotic Effects by the Selective Nonsteroidal Mineralocorticoid Receptor Antagonist Finerenone in Progressive Models of Kidney Fibrosis

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Keywords

Mineralocorticoid receptor antagonist · Sodium-glucose cotransporter-2 inhibitor · Kidney fibrosis · Anti-fibrotic effects

Abstract

Introduction: The nonsteroidal mineralocorticoid receptor (MR) antagonist finerenone and sodium-glucose cotransporter-2 (SGLT2) inhibitors have demonstrated clinical benefits in chronic kidney disease patients with type 2 diabetes. Precise molecular mechanisms responsible for these benefits are incompletely understood. Here, we investigated potential direct anti-fibrotic effects and mechanisms of nonsteroidal MR antagonism by finerenone or SGLT2 inhibition by empagliflozin in 2 relevant mouse kidney fibrosis models: unilateral ureter obstruction and sub-chronic ischemia reperfusion injury. **Methods:** Kidney fibrosis was induced in mice via unilateral ureteral obstruction or ischemia. In a series of experiments, mice were treated orally with the MR antagonist finerenone (3 or 10 mg/kg), the SGLT2 inhibitor empagliflozin (10 or 30 mg/kg), or in a direct comparison of both drugs. Interstitial myofibroblast accumulation was quantified via alpha-smooth muscle actin and interstitial col-

lagen deposition via Sirius Red/Fast Green staining in both models. Secondary analyses included the assessment of inflammatory cells, kidney mRNA expression of fibrotic markers as well as functional parameters (serum creatinine and albuminuria) in the ischemic model. Blood pressure was measured via telemetry in healthy conscious compound-treated animals. **Results:** Finerenone dose-dependently decreased pathological myofibroblast accumulation and collagen deposition with no effects on systemic blood pressure and inflammatory markers in the tested dose range. Reduced kidney fibrosis was paralleled by reduced kidney plasminogen activator inhibitor-1 (PAI-1) and naked cuticle 2 (NKD2) expression in finerenone-treated mice. In contrast, treatment with empagliflozin strongly increased urinary glucose excretion in both models and reduced ischemia-induced albuminuria but had no effects on kidney myofibroblasts or collagen deposition. **Discussion/Conclusion:** Finerenone has direct anti-fibrotic properties resulting in reduced myofibroblast and collagen deposition accompanied by a reduction in renal PAI-1 and NKD2 expression in mouse models of progressive kidney fibrosis at blood pressure-independent dosages.

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Introduction

Chronic kidney disease (CKD) is a major global health problem with significant growing prevalence rates for progression to end-stage renal disease (ESRD) [1]. CKD patients experience a high number of cardiovascular events due to myocardial infarction, stroke, or hospitalization for heart failure during progression to ESRD [2]. Hypertension and type 2 diabetes (T2D) are the most common causes for ESRD, and current pharmacotherapy is accordingly aiming to reduce the rate of progression of kidney disease by optimal control of glycaemia, blood pressure, and blood lipids [3, 4]. Renin-angiotensin system blockade with either angiotensin-converting enzyme inhibitors or angiotensin-receptor blocker was introduced as blood pressure controlling mode of action also for the treatment of CKD >2 decades ago [5, 6].

Most recently, inhibitors of the sodium-glucose co-transporter-2 (SGLT2) were introduced as a novel pharmacological treatment option for blood glucose control in T2D. In the EMPA-REG OUTCOME trial, the SGLT2 inhibitor empagliflozin reduced the risk of major adverse CV events in patients with T2D at high risk for CV events [7] and was associated with slower progression of kidney disease and lower rates of clinically relevant renal events in comparison to placebo [8]. The SGLT2 inhibitor canagliflozin when added to renin-angiotensin system blockade demonstrated a significant reduction of the kidney-specific composite endpoint of ESRD in CKD patients with T2D [9]. SGLT2 inhibition in the proximal renal tubule is believed to confer primarily metabolic and hemodynamic benefits, for example, glucosuria and lowering glomerular hyperfiltration by activation of the tubular glomerular feedback [10]. Other described pharmacodynamic effects of SGLT2 inhibitors including weight loss, systemic blood pressure reduction, ketone generation, increase in insulin sensitivity, and even attenuating inflammation [11]. However, renal anti-fibrotic effects mediated by the SGLT2 inhibitor empagliflozin are ambiguous, might depend on the presence of T2D, and/or are indirect consequences of the primary metabolic/hemodynamic action in the long term.

It is well documented that mineralocorticoid receptor (MR) antagonists (MRAs) have kidney-protective effects in preclinical models including reduction of albuminuria/proteinuria which is a relevant marker for renal outcome in clinical trials [12]. Conditions like elevated aldosterone release or MR expression, high salt load, increased generation of reactive oxygen species, and ligand-independent activation by the small GTPase rac-1 may cause

a status of the so-called MR overactivation with subsequent expression of pro-inflammatory and pro-fibrotic proteins in different cell types such as vascular smooth muscle cells, fibroblasts, and monocytes and macrophages [13]. These pro-inflammatory/pro-fibrotic proteins include osteopontin-1 [14] and the prothrombotic protein plasminogen activator inhibitor-1 (PAI-1) [15], which is involved in a complex cascade of collagen deposition and degradation processes leading to interstitial fibrosis.

Finerenone is a novel, selective, and nonsteroidal MRA which blocks the binding of the MR ligands aldosterone and cortisol more potently than spironolactone and eplerenone [16]. Finerenone effectively inhibits recruitment of transcriptional co-modulators involved in hypertrophic and pro-fibrotic gene expression [17]. Most recently, the FIDELIO-DKD phase III study that investigated the efficacy and safety of finerenone in comparison to placebo in addition to optimized standard of care on the reduction of kidney failure and kidney disease progression in 5,734 patients with CKD and T2D met both its composite primary renal endpoint and its composite key secondary CV endpoint [18]. However, since the direct impact of both finerenone and SGLT2 inhibition on renal myofibroblast transformation and collagen deposition is unknown, we compared potential direct anti-fibrotic effects of finerenone with the SGLT2 inhibitor empagliflozin in 2 relevant mouse kidney fibrosis models [19, 20].

Materials and Methods

Animals

All procedures conformed to European Community directives and national legislation (German law for the protection of animals) for the use of animals for scientific purposes and were approved by the competent regional authority. Experimental studies were performed in C57BL/6J mice (age: 7–8 weeks) that were obtained from Charles River. Animals were housed with free access to chow and water and maintained on a light/dark cycle at 22–24°C.

Arterial Blood Pressure Measurement via Implanted Transmitter in Conscious Mice

Telemetry transmitters (PA-C10; Data Sciences) were implanted surgically under isoflurane anesthesia in the carotid artery. In brief, the neck was opened in the area of the hyoid bone up to the sternum in the median line. A subcutaneous pocket for the transmitter housing was prepared. The right carotid artery was then bluntly exposed and clamped. The sender was pushed caudally into the carotid artery through an incision. The catheter was fixed with a monofilament prolene thread, which also closes the incision. The vessel was also ligated cranially, and the clamp was then

removed. The transmitter housing was pushed subcutaneously. For arterial blood pressure measurements, animals were kept in a 12-h/12-h day/night cycle in a suitable room for at least 1 day before the first measurement. Compound or placebo was applied by p.o. gavage in the morning after baseline measurement and followed up for 8 consecutive days. Telemetric measurements were performed on day 8 and lasted over 24 h. The averaged data (1–10-min grid) were displayed graphically.

Unilateral Ureter Obstruction

Mice were randomly assigned into the following 4 groups with 9–12 mice in each group: (1) sham-operated mice (sham); (2) unilateral ureter obstruction (UUO) mice with vehicle treatment (placebo); and (3 + 4) UUO mice treated with either finerenone or empagliflozin. Mice were anesthetized with continuous isoflurane inhalation; the left ureter was exposed via a mid-abdominal incision and was obstructed completely near the renal pelvis using a 4-0 silk tie suture at 2 points. sham-operated mice ($n = 6$) underwent the same procedure, except for the obstruction of the left ureter. The number of mice in each experiment is reported in the respective figures.

Sub-Chronic Ischemia/Reperfusion Injury

Mice were randomly assigned into the following 4 groups with 12 mice in each group: (1) sham-operated mice (sham); (2) sub-chronic ischemia/reperfusion injury (sci/RI) mice with vehicle treatment (placebo); and (3 + 4) sci/RI mice treated with either finerenone or empagliflozin. Mice were anesthetized with continuous isoflurane inhalation; the left kidney was exposed through flank incision and subjected to ischemia by clamping the renal pedicle with nontraumatic microaneurysm clamps (Braun Aesculap; YASARGIL® Aneurysm Clip 9 mm) for 25 min. After clamp removal, return of blood flow was confirmed and the incision closed. After 7 days of reperfusion, contralateral nephrectomy was performed via right flank incision. Sham-operated mice ($n = 6$) were exposed to the same surgery without pedicle clamping but underwent delayed contralateral nephrectomy. The number of mice in each experiment is reported in the respective figures and tables.

Compounds and Treatment

Finerenone was synthesized, as previously described [21], and empagliflozin was synthesized at WuXi AppTec, China. Finerenone and empagliflozin were dissolved in 40% Kolliphor, 10% ethanol, and 50% water and administered to mice by oral gavage at dosages of 3 or 10 mg/kg for finerenone and 10 or 30 mg/kg for empagliflozin once daily starting with the first dose 2 days before UUO or sci/RI.

Serum and Urine Collection and Analysis

Urine samples were collected in the third UUO study and in sci/RI from mice placed in metabolic cages at day 6 post-UUO and at day 7 post-ischemia. Urinary creatinine and glucose concentrations were quantified by using an enzymatic method using the ABX PENTRA 400 analyzer (Horiba Medical). For the assessment of urinary albumin, a mouse-specific Albumin Kit (FUJIFILM Wako Shibayagi Corporation) was used within the ABX PENTRA 400 analyzer. Both urinary creatinine and albumin were used to determine albuminuria (albumin/creatinine ratio). Upon sacrifice, blood samples were obtained under terminal anesthesia from

sci/RI animals. After centrifugation of the blood samples, serum was isolated. Serum creatinine and urea were measured via an ABX PENTRA 400 analyzer.

Tissue Collection

Mice were euthanized, and kidneys were harvested at day 3 post-UUO for the assessment of inflammatory cells. Kidney was harvested and divided into 2 parts 10 days post-UUO or 8 days post-ischemia surgery. One part was snap-frozen in liquid nitrogen for RNA analysis. The other part was a transversally cut slice that was fixed in Davidson's fixative processed and embedded in paraffin.

Histological Analysis

Paraffin sections were prepared at 4 μ m and stained with Sirius Red/Fast Green (SR/FG) for the detection of collagen fibers. From additional paraffin sections, an immunochemical stain for myofibroblast via α -smooth muscle actin (α SMA) was performed using a monoclonal antibody (clone 1A4, Mu128UC; BioGenex). Subsequent steps were conducted with Dako Envision (K4001; Dako) and 3,3'-diaminobenzidine as chromogen. Hematoxylin was used as counterstaining. Quantitative measurements of α SMA-positive as well as SR (collagen fibers)-positive areas were obtained by computer image analysis of stained, full transversal kidney sections using the Axio Scan Z1 (Carl Zeiss AG) microscope and the Zen software.

Flow Cytometry

Flow cytometry was performed, as described before [22]. In brief, single-cell suspensions of pre-weighted kidney were made by digesting kidney tissue with the Multi Tissue Dissociation Kit 1 (Miltenyi Biotec) according to the user manual. Following filtration through a 70- μ m mesh, erythrocytes were lysed using BD Pharm Lyse (BD Biosciences) and washed with autoMACS Running Buffer (Miltenyi Biotec). Fc receptors were blocked with CD16/CD32 (BD Biosciences) for 10 min, and extracellular surface markers were stained with an antibody cocktail containing FITC anti-mouse CD86 (clone GL-1; BioLegend, San Diego, CA, USA), PE anti-mouse F4/80 (clone T45-2342; BD Biosciences, Franklin Lakes, NJ, USA), APC anti-mouse Ly-6G (clone 1A8; BD), APC-Cy7 anti-mouse CD45 (clone 30-F11; BD), PerCP-Cy5.5 anti-mouse CD11b (clone M1/70; BD), BV421 anti-mouse TCR β Chain (clone H57-597; BD), and PerCP-Cy5.5 anti-mouse CD19 (clone 1D3; BD), each at a 1:100 dilution. For intracellular CD206 staining, cells were fixated for 10 min with Leucoperm reagent A and after a washing step permeabilized with Leucoperm reagent B (Bio-Rad, Hercules, CA, USA) containing 1:100 BV421 anti-mouse CD206 (clone C068C2; BioLegend). After washing, cells were analyzed on a BD FACSVerser Flow Cytometer (BD Biosciences). Data analysis was performed using FlowJo 7.6 software (Treestar, Ashland, OR, USA). Cell numbers are given as cells per mg kidney calculated by following formula: (cell count/kidney weight) $\times$ (total sample volume/measured sample volume).

Quantitative PCR

Frozen kidney tissue was lysed using the soft tissue homogenizing kit (Qiagen) with ceramic beads on the Precellys 24 homogenizer (Precellys; Bertin Instruments). RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA amount and purity were quantified on the NanoDrop

Fig. 1. Mean arterial pressure (a) and heart rate (b) determined on day 8 of once daily treatments with 10 mg/kg finerenone or 30 mg/kg empagliflozin. Telemetric measurements lasted for 24 h. Data are mean $\pm$ SEM. Placebo $n = 4$, all other groups $n = 5$. SEM, standard error of mean.

(Thermo Fisher). RNA preparation underwent DNase digestion to eliminate genomic DNA remnants by incubation with DNase I amplification grade (Thermo Fisher) for 15 min at room temperature followed by 15 min at 65°C and chilling on ice. RNA was then reverse transcribed with the Improm-II kit (Promega) using random primers. Transcript-specific FAM/TAMRA-labeled probes and forward/reverse primer pairs were used to run quantitative TaqMan PCR on the 7900HT Fast Real-Time PCR System (Thermo Fisher). The relative expression level was measured in relation to the housekeeping gene RPL32 and calculated as $2^{(20-\Delta Ct)}$, with $\Delta Ct = Ct (\text{target gene}) - Ct (\text{RPL32})$.

Statistics

All results are expressed as means. Error bars are given as the standard deviation, except for the telemetry data where error bars indicate the standard error of mean. Statistical analysis was performed using GraphPad Prism Software v8.02 (La Jolla). One-way ANOVA followed by Dunnett's multiple comparisons test was used to determine statistical differences versus placebo group. A probability value of $p < 0.05$ was considered significantly different.

Results

Telemetric measurements were carried out in conscious implanted mice treated once daily for 8 days with 10 mg/kg finerenone or 30 mg/kg empagliflozin. Dosages of 10 and 30 mg/kg empagliflozin have previously been described as efficacious glycosuric dosages in rodents

[22]. Figure 1a and b shows that neither the mean arterial pressure nor heart rate was modulated in healthy conscious mice after 8 days of finerenone or empagliflozin treatment.

We evaluated potential effects of finerenone treatment on fibrosis, by subjecting C57BL/6 mice to UUO. Obstructed kidneys from placebo-treated mice showed an 11-fold increase in interstitial myofibroblast accumulation (α SMA immunohistochemistry) and 7-fold enhanced interstitial collagen fiber deposition (SR/FG staining) as compared to sham animals (shown in Fig. 2a, b). Treatment with finerenone resulted in a dose-dependent reduction of both kidney fibrosis parameters. Although the effect of the low dose of 3 mg/kg did not reach statistical significance, application of 10 mg/kg resulted in a significant reduction of myofibroblast accumulation (-22% at 3 mg/kg, $p = 0.1$; -41% at 10 mg/kg, $p = 0.002$; shown in Fig. 2a) as well as collagen fiber deposition (-22% at 3 mg/kg, $p = 0.1$; -44% at 10 mg/kg, $p = 0.001$; shown in Fig. 2b).

Then, we explored anti-fibrotic effect of empagliflozin to clarify whether SGLT2 inhibition has direct effects on fibrotic responses in the UUO model. Therefore, obstructed mice were treated with 10 and 30 mg/kg of empagliflozin. Similar to the independent UUO study with finerenone, a significant increase in myofibroblast accu-

Fig. 2. Renal fibrosis in UUO model. Finerenone treatment decreased interstitial myofibroblast accumulation (a) and collagen (b) deposition in UUO mice. Empagliflozin treatment showed no anti-fibrotic effects as assessed by α SMA for the detection of myofibroblast (c) and SR/FG staining for the detection of collagen fibers (d). Morphometric α SMA (e) and SR/FG quantification (f) after direct comparison of finerenone and empagliflozin treatment in the mouse UUO model. Representative α SMA (g) and SR/FG staining (h) images are shown for the sham, UUO, and UUO + finerenone and UUO + empagliflozin groups. Results are mean $\pm$ SD, sham $n = 5$, placebo groups $n = 10$, finerenone groups $n = 10$, and empagliflozin groups $n = 9$. Head to head comparison: $n = 6$, all other groups $n = 12$. $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ versus placebo. SR/FG, Sirius Red/Fast Green; UUO, unilateral ureter obstruction; α SMA, alpha-smooth muscle actin; SD, standard deviation.

Table 1. Kidney weights normalized for body weights and body weights from both models

	Sham	Placebo	Finerenone 10 mg/kg	Empagliflozin 30 mg/kg
UUO model (day 10)				
Kidney weight/bw (obstructed ureter), mg/g	5.2±0.2****	19.2±3.3	18.2±3.7	17.8±2.8
Kidney weight/bw (released ureter), mg/g	5.1±0.2*	6.2±0.8	5.8±1.0	5.9±0.5
Body weight, g	24.7±0.7	23.3±1.5	22.8±1.3	23.3±0.8
scI/RI model (day 8)				
Kidney weight/bw	6.9±0.6	7.3±0.7	7.1±0.6	7.4±0.6
Body weight, g	21.8±0.9	21.6±1.0	21.5±0.9	21.7±1.0

Sham *n* = 6 per group; all other groups *n* = 12; data are mean ± SD, one-way ANOVA with Dunnett's corrections for multiple comparisons. bw, body weight; UUO, unilateral ureter obstruction; scI/RI, sub-chronic ischemia/reperfusion injury; SD, standard deviation. Statistical significance was defined as *p* ≤ 0.05.

mulation (αSMA staining) and SR/FG collagen fiber staining shown in Figure 2c and d was observed in placebo control-treated mice of the second UUO study. However, treatment with empagliflozin had significant effects neither on kidney myofibroblasts (0% at 10 mg/kg, *p* = 0.7; −10% at 30 mg/kg, *p* > 0.99; shown in Fig. 2c) nor on collagen fiber deposition (−6% at 10 mg/kg, *p* = 0.9; −9% at 30 mg/kg, *p* = 0.8; shown in Fig. 2d).

We next compared the effects of both compounds directly within one UUO experiment. Male C57BL/6 mice were again subjected to UUO and were either treated with 10 mg/kg of finerenone or with 30 mg/kg of empagliflozin. After 10 days of UUO, no significant differences in body weights between sham-operated and UUO mice were observed. There was a significant increase in kidney weight of obstructed kidneys in comparison to sham, with no significant effect of any treatment regimen (shown in Table 1). An 11-fold increase in αSMA protein levels and 7-fold higher collagen fiber content access via SR/FG staining were observed in control animals in comparison with sham (shown in Fig. 2e, f). Finerenone-treated mice showed a significant reduction of myofibroblast accumulation (31%, *p* = 0.0007; shown in Fig. 2e) and collagen deposition (−34%, *p* = 0.0006; shown in Fig. 2f). In contrast, treatment with empagliflozin strongly increased urinary glucose excretion (395-fold in comparison with placebo, *p* < 0.0001; data not shown) but had no anti-fibrotic effects. Both αSMA and collagen remained almost unchanged after treatment with empagliflozin with a slight reduction of −3% and −2% (*p* = 0.9 for both), respectively, as shown in Figure 2e and f. Representative pictures of UUO kidneys after treatment with finerenone and empagliflozin are shown in Figure 2g and h.

Finally, we conducted a second model, where fibrosis is not induced by ureter obstruction but via ischemia. Unilateral ischemia/reperfusion injury followed by delayed contralateral nephrectomy at day 7 was performed in male C57BL/6 mice. Mice experienced no differences in their body weights, and there was also no statistical difference between the groups with respect to kidney weight (as shown in Table 1). Placebo-treated animals showed an 8-fold increase in interstitial myofibroblast accumulation (αSMA IHC) and 4-fold elevated interstitial collagen content (SR/FG staining) as compared to sham animals at day 8 post-ischemia (shown in Fig. 3a, b). Figure 3a and b shows that treatment with finerenone significantly decreased αSMA and SR/FG staining by 33% and 28%, respectively (*p* = 0.006 and *p* = 0.003), while empagliflozin administration resulted in only slightly reduced myofibroblast numbers (−7%, *p* = 0.8) and collagen deposition (−5%, *p* = 0.8). Representative pictures of fibrotic kidneys after treatment with finerenone and empagliflozin are shown in Figure 3c and d. Delayed contralateral nephrectomy increased serum creatinine levels and albuminuria in ischemic kidneys shown in Figure 3c and d. There was a 9-fold increase in serum creatinine and 14-fold increase in albuminuria in placebo-treated animals in comparison with sham mice. Finerenone and empagliflozin revealed no effects on serum creatinine (0%, *p* = 0.9 and −1%, *p* = 0.9; shown in Fig. 3c) but efficacious albuminuria reduction (−68%, *p* = 0.03 and −72%, *p* = 0.002; shown in Fig. 3d). Empagliflozin strongly acted glycosuric (386-fold in comparison with placebo, *p* < 0.0001; data not shown).

To evaluate whether interfering with inflammation precedes the anti-fibrotic effect of finerenone, the infiltration of inflammatory cells was assessed via flow cytometry

Fig. 3. Renal fibrosis in mouse scI/RI model. Finerenone significantly reduced morphometrically measured myofibroblast numbers (**a**, αSMA staining) as well as the content of collagen fibers (**b**, SR/FG staining). Representative αSMA (**c**) and SR/FG staining (**d**) images are shown for the sham, scI/RI, and scI/RI + finerenone and scI/RI + empagliflozin groups. Both treatments had no effect on serum creatinine (**e**) but resulted in significant reduction of

urinary albumin excretion (**f**). Data are mean ± SD, sham $n = 6$, all other groups $n = 12$. Values for urinary albumin normalized with urinary creatinine: sham $n = 6$, all other values $n = 10$, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ versus placebo. scI/RI, sub-chronic ischemia/reperfusion injury; SR/FG, Sirius Red/Fast Green; αSMA, alpha-smooth muscle actin; SD, standard deviation.

Table 2. Number of inflammatory cells per mg kidney tissue in the 3-day UUO model analyzed via flow cytometry

Cell subset	Sham	Placebo	Finerenone 10 mg/kg	Empagliflozin 30 mg/kg
Leucocytes $\times 10^3$	0.54 $\pm$ 0.22***	6.14 $\pm$ 1.52	6.05 $\pm$ 3.49	5.87 $\pm$ 2.38
Neutrophils $\times 10^3$	0.03 $\pm$ 0.01**	0.38 $\pm$ 0.09	0.48 $\pm$ 0.22	0.59 $\pm$ 0.27*
Macrophages $\times 10^3$	0.04 $\pm$ 0.02***	1.89 $\pm$ 0.58	1.87 $\pm$ 1.05	1.92 $\pm$ 1.11
M1 macrophages $\times 10^3$	0.09 $\pm$ 0.03****	1.50 $\pm$ 0.52	1.60 $\pm$ 0.58	1.56 $\pm$ 0.63
M2 macrophages $\times 10^3$	0.09 $\pm$ 0.11***	2.00 $\pm$ 0.89	1.86 $\pm$ 0.83	2.24 $\pm$ 1.23
T cells $\times 10^3$	0.06 $\pm$ 0.02**	0.87 $\pm$ 0.32	0.98 $\pm$ 0.64	1.02 $\pm$ 0.34
B cells $\times 10^3$	0.16 $\pm$ 0.06*	0.39 $\pm$ 0.11	0.37 $\pm$ 0.11	0.39 $\pm$ 0.10

Sham $n = 6$, all other groups $n = 12$; data are expressed as means $\pm$ SD percentage values calculated versus placebo. Differences between placebo groups were analyzed by one-way ANOVA with Dunnett's corrections for multiple comparisons. Surface markers used for quantification of cell subsets: CD45⁺ for leucocytes, Ly6G⁺ for neutrophils, CD45⁺F4/80⁺ for macrophages, CD86⁺MHCII⁺CD206⁻ for M1, CD206⁺ for M2 macrophages, TCR⁺ for T cells, and CD19⁺ for B cells. Statistical significance was defined as $p \leq 0.05$. UUO, unilateral ureter obstruction; SD, standard deviation.

Table 3. Renal RNA expression of pro-fibrotic markers in UUO model normalized for placebo-treated, ureteral-obstructed mice

Marker	Sham	Placebo	Finerenone 10 mg/kg	Empagliflozin 30 mg/kg
α SMA	22.8 $\pm$ 2.8****	100.0 $\pm$ 15.1	62.6 $\pm$ 12.3**	99.9 $\pm$ 37.9
Col1 α 1	3.3 $\pm$ 0.8****	100.0 $\pm$ 10.1	74.8 $\pm$ 11.5**	96.8 $\pm$ 30.1
Col3 α 1	5.1 $\pm$ 1.1****	100.0 $\pm$ 15.8	68.6 $\pm$ 20.2**	87.2 $\pm$ 26.4
Col4 α 1	28.0 $\pm$ 1.5****	100.0 $\pm$ 33.3	69.0 $\pm$ 14.9**	109.8 $\pm$ 28.0
CTGF	14.2 $\pm$ 2.6****	100.0 $\pm$ 21.0	79.7 $\pm$ 30.0	93.2 $\pm$ 17.8
TGF- β	17.8 $\pm$ 0.5****	100.0 $\pm$ 8.3	87.4 $\pm$ 29.4	99.3 $\pm$ 13.4
PAI-1	1.5 $\pm$ 0.2****	100.0 $\pm$ 19.2	72.5 $\pm$ 25.9**	97.9 $\pm$ 16.2
NKD2	1.8 $\pm$ 0.3****	100.0 $\pm$ 23.3	67.1 $\pm$ 24.3**	88.7 $\pm$ 17.0
KIM-1	0.4 $\pm$ 0.2****	100.0 $\pm$ 18.7	81.1 $\pm$ 31.1	94.6 $\pm$ 22.3
NGAL	1.4 $\pm$ 0.2****	100.0 $\pm$ 23.8	90.8 $\pm$ 30.7	96.5 $\pm$ 20.9

Sham $n = 6$, all other groups $n = 12$; data are expressed as means $\pm$ SD percentage values calculated versus placebo. Differences between placebo group were analyzed by one-way ANOVA with Dunnett's corrections for multiple comparisons. α SMA, alpha-smooth muscle actin; Col1 α 1, alpha-1 type I collagen; Col3 α 1, alpha-1 type III collagen; Col4 α 1, alpha-1 type IV collagen; CTGF, connective tissue growth factor; TGF- β , transforming growth factor beta; PAI-1, plasminogen activator inhibitor-1; NKD2, naked cuticle 2; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; UUO, unilateral ureter obstruction; SD, standard deviation. Statistical significance was defined as $p \leq 0.05$.

in a short-term UUO (3 days) study. The gating strategy and representative blots of the results are depicted in online supplementary Figures 1 and 2 (for all online suppl. material, see www.karger.com/doi/10.1159/000518254). The number of all investigated monocytes and lymphocytes was significantly increased in renal tissue from placebo-treated mice following 3 days UUO in comparison to sham-operated animals. However, neither finerenone

nor empagliflozin influenced the immune cell infiltration or changed the polarization of macrophages (as shown in Table 2).

Next, we decided to investigate the expression of pro-fibrotic markers in both models. The obstructed kidneys from UUO mice receiving vehicle treatment showed a strong upregulation of all investigated mRNA markers, as shown in Table 3. Confirming the histological findings

Fig. 4. Renal mRNA expression. Reduced expression of PAI-1 in the UUO (a) and scI/RI (b) model in finerenone-treated mice. Reduced kidney fibrosis was also paralleled by reduced kidney naked cuticle homologue 2 (NKD2) expression in the UUO model (c) as well as in the scI/RI model (d). Data are mean $\pm$ SD, percentage

values normalized to placebo. sham $n = 6$, all other groups $n = 12$. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ versus placebo. PAI-1, plasminogen activator inhibitor type-1; UUO, unilateral ureter obstruction; scI/RI, sub-chronic ischemia/reperfusion injury; SD, standard deviation.

on the transcription level, finerenone treatment also reduced the mRNA expression of α SMA and collagen types I, III, and IV. Analyzing molecular markers of pro-fibrotic pathways, particularly PAI-1 (-28% ; $p = 0.006$, shown in Fig. 4a) and naked cuticle 2 (NKD2; -33% ; $p = 0.008$, shown in Fig. 4c) were downregulated in finerenone-treated UUO mice, while no changes were detected in the expression of connective tissue growth factor (CTGF), transforming growth factor beta (TGF- β), kidney injury molecule-1, or neutrophil gelatinase-associated lipocalin. Treatment with empagliflozin had no significant effect on the expression of pro-fibrotic mRNA markers. The expressions of the 2 fibrosis regulators PAI-1 and NKD2 were also reduced in finerenone-treated

mice in the scI/RI model, as shown in Figure 4b and d. NKD2 mRNA expression was reduced by 43% ($p = 0.03$; shown in Fig. 4d), and PAI-1 mRNA expression was reduced by 30% after finerenone treatment; however, this effect did not reach statistical significance ($p = 0.2$; shown in Fig. 4b).

Taken together, finerenone treatment resulted in blood pressure-independent reduction of fibrosis in 2 mouse models of kidney fibrosis. In both models, reduced fibrosis was paralleled by reduced mRNA expression of 2 key fibrosis regulators. In contrast, treatment with empagliflozin strongly increased urinary glucose excretion and reduced albuminuria in the scI/RI model but showed no direct anti-fibrotic effects.

Discussion/Conclusion

CKD has a high prevalence and is associated with a tremendous economic burden. Diabetes and hypertension are 2 principal causes of CKD. The pathogenesis of CKD is characterized by a progressive decline of kidney function and accumulation of extracellular matrix proteins leading to glomerulosclerosis and tubulointerstitial fibrosis [23]. Kidney fibrosis is the common end stage of all CKDs independent of the underlying etiology. Thus, inhibition of renal fibrosis appears to be an ultimate goal of future therapies. Among the recently developed drugs, SGLT2 inhibitors and finerenone, a novel, selective, and nonsteroidal MRA, have demonstrated clinical benefits in CKD patients with T2D [9, 24]. However, cellular and molecular mechanisms responsible for these benefits are incompletely understood. Therefore, we wanted to evaluate possible direct anti-fibrotic effects of both compounds in 2 mouse models of progressive kidney fibrosis.

Our data demonstrate direct anti-fibrotic effects after treatment with the nonsteroidal MRA finerenone in 2 models of kidney fibrosis. Finerenone treatment resulted in a reduction of myofibroblasts and collagen deposition in both fibrosis models in mice. As assessed via telemetric measurements in conscious mice, finerenone treatment had no effects on blood pressure. The administration of SGLT2 inhibitor empagliflozin was also blood pressure neutral but also did not reveal anti-fibrotic effects.

Anti-fibrotic effects of MR antagonism have been demonstrated before in preclinical kidney disease models. Spironolactone and eplerenone treatment attenuated renal fibrosis in UUO models in mice and rats [25–27]. Moreover, beneficial effects were also demonstrated after spironolactone and finerenone treatment in AKI-to-CKD transition models performed in rats and pigs [28–31]. MR is expressed not only in infiltrating cells, including macrophages, T cells, and renal fibroblasts, but also in intrinsic tubular, glomerular, and vascular cells [12, 32]. Thus, MR blockade not only reduced fibrosis but also decreased serum creatine levels and proteinuria in these models [28, 30]. The data obtained in our study support these previous findings. Besides anti-fibrotic effects, treatment with finerenone also decreased albuminuria in the scI/RI model. Contrary to previous findings, serum creatine levels were not affected by finerenone in our model. This is most likely due to the short duration of our scI/RI model. A longer treatment duration might have attenuated the decline in renal function as demonstrated by Lattenist et al. [28] in a study with a significantly longer duration of 28 days. Previous UUO studies demonstrated

that MR antagonism inhibited renal inflammation, interstitial cell proliferation, and reduced oxidative stress after UUO. Chen et al. [26] demonstrated decreased macrophage infiltration and monocyte chemoattractant protein 1 levels after eplerenone treatment in UUO rats. In ischemia-induced CKD model reduction of inflammation, increased M2 macrophage polarization and interleukin-4 receptor signaling contributed to improved outcomes [30]. However, in our study, neither finerenone nor empagliflozin reduced cytokine levels or renal expression of MCP-1 or murine homolog of the EGF-like module-containing mucin-like hormone receptor-like 1, F4/80, in both models (shown in online suppl. Tables 1–3). Moreover, inflammatory cell infiltration and macrophage polarization were also unaffected by finerenone treatment 3 days after UUO. Despite the fact that we were not able to identify effects of finerenone on central mechanisms of inflammation in our studies, we cannot completely rule out its impact on inflammatory processes in kidney diseases. Our animal model and inflammatory marker selection as well as the time point of inflammation evaluation post-injury differ from previous studies and might be a reason for the different outcomes. Although finerenone did not influence inflammation in our studies, we noticed that the reduction of renal fibrosis after finerenone treatment was associated with significantly reduced renal PAI-1 and NKD2 expression.

It is well-described in the literature that increased levels of PAI-1 are associated with tissue fibrosis [33]. PAI-1 is responsible for the inhibition of tissue-type plasminogen activator and urokinase-type plasminogen activator. Both proteins degrade ECM proteins and activate enzymes that degrade collagens [34, 35]. Decreased PA and increased PAI-1 levels have been reported in many experimental and human renal fibrotic diseases [36]. In addition, multiple studies using models of liver, lung, and kidney fibrosis suggest that PAI-1 deficiency or inhibition of PAI-1 activity attenuates fibrosis [37–40]. It was previously demonstrated in several cell types that aldosterone directly increases PAI-1 expression [15, 41–43]. Moreover, aldosterone and TGF- β synergistically increased PAI-1 and decreased matrix degradation, in rat renal mesangial and fibroblast cells [44]. PAI-1 deficient mice are protected not only from UUO but also against aldosterone-induced glomerular injury [45, 46]. Supporting this finding, aldosterone contributed not only to glomerulosclerosis but also to injury-induced increases in PAI-1 expression in different models of kidney injury in rats. MR antagonism with spironolactone or eplerenone decreased both glomerular sclerosis and PAI-1 expres-

sion [47, 48]. Finerenone was previously shown to block dose-dependently the induction of renal PAI-1 mRNA expression in a chronic rat model of hypertension-induced cardiorenal end-organ damage and that this reduction in renal PAI-1 gene expression was more pronounced than a maximum dose of eplerenone [49]. Our current study fully confirms the earlier generated data.

In finerenone-treated mice, reduced kidney fibrosis was also paralleled by reduced kidney NKD2 expression. NKD2 regulates both canonical and noncanonical Wnt/ β -catenin and epidermal growth factor receptor signaling via its function in transforming growth factor alpha delivery [50, 51]. Its role within the kidney is poorly characterized. However, it seems that NKD2 plays a crucial role in kidney fibrosis. NKD2 is strongly upregulated in medullary myofibroblast during different experimental models of kidney fibrosis [52]. In addition, overexpression of NKD2 induced ECM production in human cells, whereas its knockdown resulted in decreased levels of collagen production in human fibroblasts and in iPS cell-derived kidney organoids [53]. Thus, myofibroblast-specific NKD2 seems to be a key regulator of human kidney fibrosis. Finerenone-treated mice showed a significant reduction of NKD2 expression in both fibrosis models in our study. Our data point toward an involvement of the MR signaling in the regulation of NKD2 expression. However, further research is needed to confirm this novel finding.

Another goal of our study was to investigate potential direct anti-fibrotic effects of empagliflozin. SGLT2 inhibitors demonstrated kidney-protective effects in several models of diabetic kidney diseases [54], but experimental studies examining its role in nondiabetic kidney diseases are limited. Nevertheless, Abbas et al. [55] described anti-fibrotic effects after treatment with 10 mg/kg empagliflozin in a UUO model in rats. The authors demonstrated that both prophylactic and immediate treatments with empagliflozin exert anti-fibrotic effects 14 or 21 days post-UUO in rats. They found that empagliflozin-treated rats showed reduced protein levels of TGF- β 1, NF- κ B, TLR4, α SMA, fibronectin, and CTGF. It should be mentioned at this point that the evaluation of almost all described markers was performed via ELISA from kidney tissue lysates. Only CTGF was stained on kidney sections. Moreover, an assessment of collagen deposition via immunohistochemical investigation of collagen subtypes or histological staining (e.g., SR, periodic acid-Schiff and Masson's trichrome) is missing in this study. Most published UUO studies use histological staining techniques or immunohistochemistry for the assessment of myofi-

broblasts and tissue scarring being key readout parameter [19, 23]. Even though CTGF represents a valid fibrosis marker, it makes the comparison of data with other UUO studies difficult [56].

In our hands, empagliflozin strongly increased urinary glucose excretion but had no anti-fibrotic effects in the UUO model. Given that SGLT2 inhibitors have a diuretic action [57] and that the injury in the UUO model is driven by postrenal obstruction, there is a hypothetical risk that the application of empagliflozin might increase renal urinary pressure, leading to more severe renal damage. Intending to exclude such effects, we conducted a second model where fibrosis was induced via ischemia. Empagliflozin treatment reduced albuminuria in the scI/RI model, but had no direct anti-fibrotic effects as assessed by myofibroblast staining or collagen deposition. In our study, empagliflozin treatment had also no effect on the mRNA expression of pro-fibrotic markers including CTGF in kidneys. One possible explanation for this discrepancy is that the study described by Abbas et al. [55] was performed in rats, whereas our study was conducted in mice. Treatment with empagliflozin prevented the development of renal fibrosis also in an angiotensin II-induced model of hypertensive nephropathy in rats [58]. Furthermore, there are differences in the treatment duration between these studies. Rats were treated with empagliflozin for 14 days in the angiotensin II study and for 14 or even 21 days in the UUO study. In contrast, the therapy in our studies lasted for 8 or 10 days. It is quite possible that a longer treatment duration with empagliflozin might also result in delayed, indirect anti-fibrotic effects mediated by decreased hyperfiltration. Indirect anti-fibrotic effects are well-described in models of diabetic kidney diseases, where the administration of SGLT2 inhibitors improves hyperglycemia-induced kidney damage including fibrosis [59, 60]. These findings were confirmed in a study, where the genetic deletion of SGLT2 in diabetic mice prevented streptozotocin-induced glomerular hyperfiltration but had no direct effect on the induction of fibrosis [61]. A similar conclusion was drawn from an empagliflozin treatment study in Akita mice. SGLT2 inhibition lowered blood glucose and thereby indirectly attenuated albuminuria, kidney growth, and inflammation in the early diabetic kidney [62]. However, the situation seems to be less clear and more complex in hyperglycemia-independent models. Nevertheless, the reduction of albuminuria in the scI/RI model is most likely the result of decreased hyperfiltration by SGLT2 inhibition.

We used 2 different models of kidney fibrosis for our study. While the UUO model is a frequently used, very

popular experimental model of kidney fibrosis, the scI/RI model allows the investigation of chronic consequences of ischemia leading to fibrosis [63]. The early phase of injury in both models is characterized by tubulointerstitial hypoxia, oxidative stress, endothelial injury, and complement activation. This very early injury phase is then followed by extensive inflammation mainly driven by infiltrating cells such as neutrophils and macrophages leading to pronounced myofibroblast accumulation and collagen deposition. They have the advantages of good reproducibility, relative short duration, and easy performance, making it useful to examine mechanisms of tubulointerstitial fibrosis in vivo [19, 20, 63–65]. However, both models are rather simple, do not allow for a proper assessment of renal function, and are therefore of limited relevance for human CKD representing the main limitation of our study. Studies in more complex, longer lasting fibrosis models in diabetic and hypertensive animals or in renal mass reduction models might have allowed for a better backtranslation of human findings. Another, more technical limitation is the assessment of kidney fibrosis at only on time point. Evaluation of anti-fibrotic effects at earlier time points and a longer study duration may lead to a more precise differentiation of underlying mechanisms of both treatments. It also needs to be pointed out that blood pressure was measured in healthy mice treated with compounds over a period of 8 days. Therefore, it cannot be excluded completely that the drugs were unable to lower blood pressure in the used models of fibrosis.

In the present work, we were able to demonstrate direct anti-fibrotic effects in 2 mouse models of kidney fibrosis after treatment with the novel, selective, and non-steroidal MRA finerenone. Treatment with finerenone resulted in reduced myofibroblast numbers and collagen deposition in mouse kidneys. Finerenone exerts its anti-fibrotic efficacy at least in part through the direct modu-

lation of PAI-1. Moreover, finerenone treatment regulates the expression of NKD2, a novel regulator of kidney fibrosis. Further research might be warranted in order to elucidate the role of the MR signaling in the regulation of NKD2 expression.

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Statement of Ethics

All procedures conformed to European Community directives and national legislation (German law for the protection of animals) for the use of animals for scientific purposes and were approved by the competent regional authority.

Conflict of Interest Statement

All authors are employees of Bayer AG. Peter Kolkhof is co-inventor of finerenone and holds patents for finerenone.

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Author Contributions

L.G., J.N., M.P., E.H., and M.G. performed experiments and prepared figures. K.D., P.K., J.K., and M.G. designed experiments, analyzed data, and interpreted results of experiments. K.D., F.E., and P.K. drafted the manuscript. P.K., K.D., M.G., M.P., J.N., L.G., J.K., E.H., and F.E. approved the final version of the manuscript.

References

- 1 Liyanage T, Ninomiya T, Jha V, Neal B, Patrice HM, Okpechi I, et al. Worldwide access to treatment for end-stage kidney disease: a systematic review. *Lancet*. 2015;385(9981):1975–82.
- 2 Herzog CA, Asinger RW, Berger AK, Charytan DM, Díez J, Hart RG, et al. Cardiovascular disease in chronic kidney disease: A clinical update from kidney disease: improving global outcomes (KDIGO). *Kidney Int*. 2011;80(6):572–86.
- 3 Bakris GL. Protecting renal function in the hypertensive patient: clinical guidelines. *Am J Hypertens*. 2005;18(4 Pt 2):112S–9S.
- 4 Ferro CJ, Mark PB, Kanbay M, Sarafidis P, Heine GH, Rossignol P, et al. Lipid management in patients with chronic kidney disease. *Nat Rev Nephrol*. 2018;14(12):727–49.
- 5 Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. *N Engl J Med*. 1993;329(20):1456–62.
- 6 Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, et al. Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med*. 2001;345(12):851–60.
- 7 Zinman B, Lachin JM, Inzucchi SE. Empagliflozin, cardiovascular outcomes, and mortality in type 2 diabetes. *N Engl J Med*. 2016;374(11):1094.
- 8 Wanner C, Inzucchi SE, Lachin JM, Fitchett D, von Eynatten M, Mattheus M, et al. Empagliflozin and progression of kidney disease in type 2 diabetes. *N Engl J Med*. 2016;375(18):323–34.
- 9 Perkovic V, Jardine MJ, Neal B, Bompoint S, Heerspink HJL, Charytan DM, et al. Canagliflozin and renal outcomes in type 2 diabetes and nephropathy. *N Engl J Med*. 2019;380(24):2295–306.

- 10 Vallon V. The mechanisms and therapeutic potential of SGLT2 inhibitors in diabetes mellitus. *Annu Rev Med*. 2015;66:255–70.
- 11 Wanner C, Marx N. SGLT2 inhibitors: the future for treatment of type 2 diabetes mellitus and other chronic diseases. *Diabetologia*. 2018;61(10):2134–9.
- 12 Barrera-Chimal J, Girerd S, Jaisser F. Mineralocorticoid receptor antagonists and kidney diseases: pathophysiological basis. *Kidney Int*. 2019;96(2):302–19.
- 13 Kolkhof P, Jaisser F, Kim SY, Filippatos G, Nowack C, Pitt B. Steroidal and novel non-steroidal mineralocorticoid receptor antagonists in heart failure and cardiorenal diseases: comparison at bench and bedside. *Handb Exp Pharmacol*. 2017;243:271–305.
- 14 Sugiyama T, Yoshimoto T, Hirono Y, Suzuki N, Sakurada M, Tsuchiya K, et al. Aldosterone increases osteopontin gene expression in rat endothelial cells. *Biochem Biophys Res Commun*. 2005;336(1):163–7.
- 15 Chun TY, Pratt JH. Aldosterone increases plasminogen activator inhibitor-1 synthesis in rat cardiomyocytes. *Mol Cell Endocrinol*. 2005;239(1–2):55–61.
- 16 Kolkhof P, Bärfacker L. 30 Years of the mineralocorticoid receptor: mineralocorticoid receptor antagonists: 60 years of research and development. *J Endocrinol*. 2017;234(1):T125–40.
- 17 Grune J, Beyhoff N, Smeir E, Chudek R, Blumrich A, Ban Z, et al. Selective mineralocorticoid receptor cofactor modulation as molecular basis for finerenone's antifibrotic activity. *Hypertension*. 2018;71(4):599–608.
- 18 Bakris GL, Agarwal R, Anker SD, Pitt B, Ruilope LM, Rossing P, et al. Effect of finerenone on chronic kidney disease outcomes in type 2 diabetes. *N Engl J Med*. 2020;383(23):2219–29.
- 19 Chevalier RL, Forbes MS, Thornhill BA. Ureteral obstruction as a model of renal interstitial fibrosis and obstructive nephropathy. *Kidney Int*. 2009;75(11):1145–52.
- 20 Skrypnik NI, Harris RC, de Caestecker MP. Ischemia-reperfusion model of acute kidney injury and post injury fibrosis in mice. *J Vis Exp*. 2013;78:50495.
- 21 Barfacker L, Kuhl A, Hillisch A, Grosser R, Figueroa-Pérez S, Heckroth H, et al. Discovery of BAY 94-8862: a nonsteroidal antagonist of the mineralocorticoid receptor for the treatment of cardiorenal diseases. *ChemMedChem*. 2012;7(8):1385–403.
- 22 Nordlohne J, Hulsman I, Schwafertz S, Zgrajek J, Grundmann M, von Vietinghoff S, et al. A flow cytometry approach reveals heterogeneity in conventional subsets of murine renal mononuclear phagocytes. *Sci Rep*. 2021 Jun 24;11(1):13251.
- 23 Eddy AA. Molecular basis of renal fibrosis. *Pediatr Nephrol*. 2000;15(3–4):290–301.
- 24 Bakris GL, Agarwal R, Chan JC, Cooper ME, Gansevoort RT, Haller H, et al. Effect of finerenone on albuminuria in patients with diabetic nephropathy: a randomized clinical trial. *JAMA*. 2015;314(9):884–94.
- 25 Trachtman H, Weiser AC, Valderrama E, Morgado M, Palmer LS. Prevention of renal fibrosis by spironolactone in mice with complete unilateral ureteral obstruction. *J Urol*. 2004;172(4 Pt 2):1590–4.
- 26 Chen H, Sun F, Zhong X, Shao Y, Yoshimura A, Liu Y. Eplerenone-mediated aldosterone blockade prevents renal fibrosis by reducing renal inflammation, interstitial cell proliferation and oxidative stress. *Kidney Blood Press Res*. 2013;37(6):557–66.
- 27 Wang CH, Wang Z, Liang LJ, Wang XT, Ma XL, Liu BB, et al. The inhibitory effect of eplerenone on cell proliferation in the contralateral kidneys of rats with unilateral ureteral obstruction. *Nephron*. 2017;136(4):328–38.
- 28 Lattenist L, Lechner SM, Messaoudi S, Le Mercier A, El Moghrabi S, Prince S, et al. Non-steroidal mineralocorticoid receptor antagonist finerenone protects against acute kidney injury-mediated chronic kidney disease: role of oxidative stress. *Hypertension*. 2017;69(5):870–8.
- 29 Barrera-Chimal J, Rocha L, Amador-Martínez I, Pérez-Villalva R, González R, Cortés-González C, et al. Delayed spironolactone administration prevents the transition from acute kidney injury to chronic kidney disease through improving renal inflammation. *Nephrol Dial Transplant*. 2019;34(5):794–801.
- 30 Barrera-Chimal J, Estrela GR, Lechner SM, Giraud S, El Moghrabi S, Kaaki S, et al. The myeloid mineralocorticoid receptor controls inflammatory and fibrotic responses after renal injury via macrophage interleukin-4 receptor signaling. *Kidney Int*. 2018;93(6):1344–55.
- 31 Sanchez-Pozos K, Barrera-Chimal J, Garzon-Muvdi J, Pérez-Villalva R, Rodríguez-Romo R, Cruz C, et al. Recovery from ischemic acute kidney injury by spironolactone administration. *Nephrol Dial Transplant*. 2012;27(8):3160–9.
- 32 Tesch GH, Young MJ. Mineralocorticoid receptor signaling as a therapeutic target for renal and cardiac fibrosis. *Front Pharmacol*. 2017;8:313.
- 33 Ghosh AK, Vaughan DE. PAI-1 in tissue fibrosis. *J Cell Physiol*. 2012;227(2):493–507.
- 34 Flevaris P, Vaughan D. The role of plasminogen activator inhibitor type-1 in fibrosis. *Semin Thromb Hemost*. 2017;43(2):169–77.
- 35 Potempa J, Korzus E, Travis J. The serpin superfamily of proteinase inhibitors: structure, function, and regulation. *J Biol Chem*. 1994;269(23):15957–60.
- 36 Rerolle JP, Hertig A, Nguyen G, Sraer JD, Rondeau EP. Plasminogen activator inhibitor type 1 is a potential target in renal fibrogenesis. *Kidney Int*. 2000;58(5):1841–50.
- 37 Chuang-Tsai S, Sisson TH, Hattori N, Tsai CG, Subbotina NM, Hanson KE, et al. Reduction in fibrotic tissue formation in mice genetically deficient in plasminogen activator inhibitor-1. *Am J Pathol*. 2003;163(2):445–52.
- 38 Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, et al. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J Clin Invest*. 1996;97(1):232–7.
- 39 Krag S, Danielsen CC, Carmeliet P, Nyengaard J, Wogensen L. Plasminogen activator inhibitor-1 gene deficiency attenuates TGF-beta1-induced kidney disease. *Kidney Int*. 2005;68(6):2651–66.
- 40 Wang H, Zhang Y, Heuckeroth RO. PAI-1 deficiency reduces liver fibrosis after bile duct ligation in mice through activation of tPA. *FEBS Lett*. 2007;581(16):3098–104.
- 41 Brown NJ, Kim KS, Chen YQ, Blevins LS, Nadeau JH, Meranze SG, et al. Synergistic effect of adrenal steroids and angiotensin II on plasminogen activator inhibitor-1 production. *J Clin Endocrinol Metab*. 2000;85(1):336–44.
- 42 Calo LA, Zaghetto F, Pagnin E, Davis PA, De Mozzi P, Sartorato P, et al. Effect of aldosterone and glycyrrhetic acid on the protein expression of PAI-1 and p22(phox) in human mononuclear leukocytes. *J Clin Endocrinol Metab*. 2004;89(4):1973–6.
- 43 Yuan J, Jia R, Bao Y. Aldosterone up-regulates production of plasminogen activator inhibitor-1 by renal mesangial cells. *J Biochem Mol Biol*. 2007;40(2):180–8.
- 44 Huang W, Xu C, Kahng KW, Noble NA, Border WA, Huang Y. Aldosterone and TGF-beta1 synergistically increase PAI-1 and decrease matrix degradation in rat renal mesangial and fibroblast cells. *Am J Physiol Renal Physiol*. 2008;294(6):F1287–95.
- 45 Oda T, Jung YO, Kim HS, Cai X, López-Guisa JM, Ikeda Y, et al. PAI-1 deficiency attenuates the fibrogenic response to ureteral obstruction. *Kidney Int*. 2001;60(2):587–96.
- 46 Ma J, Weisberg A, Griffin JP, Vaughan DE, Fogo AB, Brown NJ. Plasminogen activator inhibitor-1 deficiency protects against aldosterone-induced glomerular injury. *Kidney Int*. 2006;69(6):1064–72.
- 47 Brown NJ, Nakamura S, Ma L, Nakamura I, Donnert E, Freeman M, et al. Aldosterone modulates plasminogen activator inhibitor-1 and glomerulosclerosis in vivo. *Kidney Int*. 2000;58(3):1219–27.
- 48 Sun QL, Li M, Rui HL, Chen YP. Inhibition of local aldosterone by eplerenone reduces renal structural damage in a novel model of chronic cyclosporine A nephrotoxicity. *J Renin Angiotensin Aldosterone Syst*. 2015;16(2):301–10.
- 49 Kolkhof P, Delbeck M, Kretschmer A, Steinke W, Hartmann E, Bärfacker L, et al. Finerenone, a novel selective nonsteroidal mineralocorticoid receptor antagonist protects from rat cardiorenal injury. *J Cardiovasc Pharmacol*. 2014;64(1):69–78.
- 50 Van Raay TJ, Coffey RJ, Solnica-Krezel L. Zebrafish Naked1 and Naked2 antagonize both canonical and non-canonical Wnt signaling. *Dev Biol*. 2007;309(2):151–68.

- 51 Li C, Franklin JL, Graves-Deal R, Jerome WG, Cao Z, Coffey RJ. Myristoylated Naked2 escorts transforming growth factor alpha to the basolateral plasma membrane of polarized epithelial cells. *Proc Natl Acad Sci U S A*. 2004;101(15):5571–6.
- 52 Grgic I, Krautzberger AM, Hofmeister A, Lalli M, DiRocco DP, Fleig SV, et al. Translational profiles of medullary myofibroblasts during kidney fibrosis. *J Am Soc Nephrol*. 2014; 25(9):1979–90.
- 53 Kuppe C, Ibrahim MM, Kranz J, Zhang X, Ziegler S, Perales-Patón J, et al. Decoding myofibroblast origins in human kidney fibrosis. *Nature*. 2021;589(7841):281–6.
- 54 Kawanami D, Matoba K, Takeda Y, Nagai Y, Akamine T, Yokota T, et al. SGLT2 inhibitors as a therapeutic option for diabetic nephropathy. *Int J Mol Sci*. 2017;18(5):1083.
- 55 Abbas NAT, El Salem A, Awad MM. Empagliflozin, SGLT2 inhibitor, attenuates renal fibrosis in rats exposed to unilateral ureteric obstruction: potential role of klotho expression. *Naunyn Schmiedeberg Arch Pharmacol*. 2018;391(12):1347–60.
- 56 Toda N, Mukoyama M, Yanagita M, Yokoi H. CTGF in kidney fibrosis and glomerulonephritis. *Inflamm Regen*. 2018;38:14.
- 57 Ansary TM, Nakano D, Nishiyama A. Diuretic effects of sodium glucose cotransporter 2 inhibitors and their influence on the renin-angiotensin system. *Int J Mol Sci*. 2019;20(3): 629.
- 58 Castoldi G, Carletti R, Ippolito S, Colzani M, Barzaghi F, Stella A, et al. Renal anti-fibrotic effect of sodium glucose cotransporter 2 inhibition in angiotensin II-dependent hypertension. *Am J Nephrol*. 2020;51(2):119–29.
- 59 Huang F, Zhao Y, Wang Q, Hillebrands JL, van den Born J, Ji L, et al. Dapagliflozin attenuates renal tubulointerstitial fibrosis associated with type 1 diabetes by regulating STAT1/TGFβ1 signaling. *Front Endocrinol*. 2019;10:441.
- 60 Tang L, Wu Y, Tian M, Sjöström CD, Johansson U, Peng XR, et al. Dapagliflozin slows the progression of the renal and liver fibrosis associated with type 2 diabetes. *Am J Physiol Endocrinol Metab*. 2017;313(5):E563–76.
- 61 Vallon V, Rose M, Gerasimova M, Satriano J, Platt KA, Koepsell H, et al. Knockout of Na-glucose transporter SGLT2 attenuates hyperglycemia and glomerular hyperfiltration but not kidney growth or injury in diabetes mellitus. *Am J Physiol Renal Physiol*. 2013; 304(2):F156–67.
- 62 Vallon V, Gerasimova M, Rose MA, Masuda T, Satriano J, Mayoux E, et al. SGLT2 inhibitor empagliflozin reduces renal growth and albuminuria in proportion to hyperglycemia and prevents glomerular hyperfiltration in diabetic Akita mice. *Am J Physiol Renal Physiol*. 2014;306(2):F194–204.
- 63 Le Clef N, Verhulst A, D’Haese PC, Vervaet BA. Unilateral renal ischemia-reperfusion as a robust model for acute to chronic kidney injury in mice. *PLoS One*. 2016;11(3):e0152153.
- 64 Eddy AA, López-Guisa JM, Okamura DM, Yamaguchi I. Investigating mechanisms of chronic kidney disease in mouse models. *Pediatr Nephrol*. 2012;27(8):1233–47.
- 65 Malek M, Nematbakhsh M. Renal ischemia/reperfusion injury; from pathophysiology to treatment. *J Renal Inj Prev*. 2015;4(2):20–7.