Original Article

Angiotensin–neprilysin inhibition confers renoprotection in rats with diabetes and hypertension by limiting podocyte injury

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Objectives: Combined angiotensin receptor–neprilysin inhibition (ARNI) reduces glomerulosclerosis better than single angiotensin receptor blockade (ARB) in diabetic, hypertensive rats. The renoprotective mechanism remains unknown, but may depend on superior blood pressure control, improved renal hemodynamics, suppressed renal inflammation or prevention of podocyte loss.

Methods: To address this, TGR(mREN2)27 rats (a model of angiotensin II-dependent hypertension) were made diabetic for 12 weeks and treated with vehicle ($n = 10$), valsartan (ARB; $n = 7$) or sacubitril/valsartan (ARNI; $n = 8$) for the final 3 weeks. Arterial pressure was measured via radiotelemetry.

Results: Sacubitril/valsartan lowered mean arterial pressure by -50 ± 4 mmHg and valsartan by -43 ± 4 mmHg ($P = 0.3$). Both treatments lowered albuminuria, but only sacubitril/valsartan maintained high urinary atrial natriuretic peptide, improved glycemic control and protected podocyte integrity, reflected by increased nephrin expression and suppression of transient receptor potential canonical 6 and regulator of calcineurin 1. This resulted in markedly reduced glomerulosclerosis ($P < 0.05$ vs. control and valsartan). Despite higher effective renal plasma flow and glomerular filtration rates, sacubitril/valsartan did neither improve filtration fraction nor renal immune cell infiltration.

Conclusion: Sacubitril/valsartan offers drug-class-specific renoprotection in a preclinical model of diabetes and hypertension. Renoprotection is unrelated to antihypertensive efficacy, renal hemodynamics or inflammation, but may be related to protective effects of natriuretic peptides on podocyte integrity.

Keywords: atrial natriuretic factor, chronic kidney disease, diabetes mellitus, hypertension, podocytes, sacubitril/valsartan, transient receptor potential canonical 6

Abbreviations: Ang, angiotensin; ANP, atrial natriuretic peptide; ARB, angiotensin receptor blockade; ARNI, angiotensin receptor–neprilysin inhibition; AT1R, Ang II type 1 receptor; CKD, chronic kidney disease; ERPF, effective renal plasma flow; FACS, fluorescent activated cell sorting; GC-A, guanylyl cyclase-A; GFR, glomerular filtration rate; MAP, mean arterial pressure; NEP, neutral endopeptidase; NFATc, nuclear factor of activated T cell; RAAS, renin–angiotensin–aldosterone system; RCAN1, regulator of calcineurin 1; TNFα, tumor necrosis factor alpha; TRPC6, transient receptor potential canonical 6

INTRODUCTION

Hypertension is highly prevalent among diabetic patients [1]. High blood pressure is a risk factor for the development of cardiovascular disease and chronic kidney disease (CKD), and the coexistence of diabetes mellitus further increases these risks [2]. Pharmacological inhibition of the renin–angiotensin–aldosterone system (RAAS) reduces albuminuria and delays the progression of diabetic kidney disease [3], but is insufficient to fully prevent the development of end-stage renal disease. Dual angiotensin receptor–neprilysin inhibition (ARNI) is a novel therapy, currently registered for the treatment of patients with heart failure [4], in whom studies showed a substantial reduction in mortality and hospitalization [5]. Sacubitril/valsartan (LCZ696) is the first-in-class ARNI. After ingestion, sacubitril is rapidly metabolized into sacubitrilat (AHU377), the active neprilysin (neutral endopeptidase; NEP) inhibitor. NEP degrades both vasodilators (e.g. natriuretic peptides, bradykinin, adrenomedullin) and vasoconstrictors [e.g. angiotensin (Ang) II, endothelin-1]. Therefore, the effect of single NEP inhibition is unpredictable, as it

Journal of Hypertension 2019, 37:000–000

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Received 29 July 2019 **Revised** 3 November 2019 **Accepted** 4 November 2019

DOI:10.1097/HJH.0000000000002326

depends on the dominance of either vasodilators or vasoconstrictors [6]. Dual therapy, however, guarantees the beneficial effects of increasing natriuretic peptides (e.g. increased diuresis, natriuresis, and vasodilation), as simultaneous blockade of the Ang II type 1 (AT1) receptor with valsartan counteracts the NEP-inhibitor-induced rise in Ang II.

Interestingly, sacubitril/valsartan may exert beneficial effects on kidney damage beyond its antihypertensive properties. When compared with conventional RAS inhibitors, sacubitril/valsartan slowed the rate at which renal function declined in patients with heart failure, both in those with and without concurrent CKD [7,8]. Unexpectedly, sacubitril/valsartan-treated patients displayed a slight increase in urinary albumin-to-creatinine ratio [7,8]. However, baseline albuminuria was very low in these studies, and sacubitril/valsartan may reduce proteinuria particularly in patients with macroalbuminuria [9]. In contrast, in the United Kingdom Heart and Renal Protection-III (UK HARP-III) trial, sacubitril/valsartan had similar effects to irbesartan on both measured glomerular filtration rate (GFR) and albuminuria in patients with moderate-to-severe CKD, even though blood pressure control and neurohumoral markers of cardiac dysfunction were improved superiorly [10]. However, in only a subset of participants was CKD caused by diabetes, whereas sacubitril/valsartan appears to be most effective at protecting renal function in this population [11]. Indeed, in a previous study in rats with severe diabetic, hypertensive kidney damage, we observed a greater reduction in proteinuria and glomerulosclerosis after ARNI, when compared with AT1 receptor blockade (ARB) [12]. Therefore, the aim of the current study was to investigate the mechanism through which sacubitril/valsartan confers this renoprotective effect. In addition to attributing this to potential differences in antihypertensive efficacy, we hypothesized that sacubitril/valsartan may protect podocyte integrity, as the ultrastructure of podocyte foot processes appeared improved in obese Zucker rats after combination treatment [13]. Furthermore, enhanced glomerular hemodynamics may result in a lower filtration fraction. In addition to reducing albuminuria, this could also prevent renal damage mediated by inflammatory cells, which are attracted by tubular endocytosis of filtered proteins [14]. To address this, we compared the effects of ARNI (sacubitril/valsartan), ARB (valsartan) and placebo in rats with kidney damage caused by diabetes and hypertension [15–17]. Collectively, the data indicate that sacubitril/valsartan-dependent renoprotection is unrelated to antihypertensive efficacy, renal hemodynamics or inflammation, but may be related to recently characterized protective effects of natriuretic peptides on podocyte integrity.

MATERIALS AND METHODS

Animal studies

All studies were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC (protocol number 3392, 118-14-02). Male, 12-week old heterozygous rats were bred by crossing homozygous TGR(mREN2)27 with Sprague–Dawley rats. Rats were maintained on a 12-h light–dark cycle with access to standard rat chow and water ad libitum. Diabetes mellitus was induced by administering streptozotocin i.p. (55 mg/kg, Merck Millipore, Amsterdam, The Netherlands). Diabetic rats (nonfasting blood glucose levels $\geq$15 mmol/l; Precision Xceed, Abbott, Zwolle, The Netherlands) received 3–5 units insulin subcutaneously per day (Levemir, Novo Nordisk, Copenhagen, Denmark), based on nonfasting blood glucose and β-ketone levels (Precision Xceed) measured every other week. Diabetic rats were studied for 12 weeks. During the final 3 weeks, rats were treated with vehicle ($n = 10$), valsartan (31 mg/kg per day; $n = 7$) or sacubitril/valsartan (68 mg/kg per day; $n = 8$) in drinking water (both drugs a kind gift of Novartis, Arnhem, The Netherlands). Ethanol (600 μl/kg per day) was added to solubilize valsartan. Dosages were recommended by the manufacturer and based on equivalent blockade of the AT1 receptor. Water intake was monitored every other day, and drug concentrations were adjusted to achieve the daily doses. Blood pressure, heart rate, and activity were recorded continuously via radiotelemetry transmitters (HD-S10, Data Sciences International, St. Paul, Minnesota, USA), implanted 2 weeks before the induction of diabetes. Twenty-four hours' urine was collected in metabolic cages prior to the induction of diabetes mellitus (baseline), and at weeks 9 and 12 of diabetes mellitus (pretreatment and posttreatment, respectively). After 3 weeks of treatment, rats were anaesthetized by inhalation of isoflurane for measurement of GFR and effective renal plasma flow (ERPF; see below). Subsequently, rats were killed by exsanguination, and kidneys, spleen, and heart were rapidly excised, weighed, and snap-frozen.

Kidney function

Intravenous infusion of a single bolus injection of 50 mg inulin and 10 mg para-aminohippuric acid (PAH) dissolved in 0.9% NaCl, was followed by continuous infusion of 2.5% inulin, 1.25% PAH, and 12% mannitol in 0.9% NaCl at a rate of 27 μl/min for 150 min (all Sigma Aldrich, Zwijndrecht, The Netherlands). Prior to infusion, we collected serum from the carotid artery and emptied the bladder to obtain baseline samples. After a 1-h equilibration period, serum and urine were collected simultaneously at 30-min intervals, repeated three times. Clearances of inulin (GFR), PAH (ERPF), and filtration fraction were calculated by standard formulae.

Biochemical measurements

In 24-h urine, we measured albumin with a rat albumin ELISA kit (Abcam, Cambridge, UK). Creatinine, urea, and sodium in 24-h urine and creatinine, urea, and blood glucose in serum collected prior to kidney function assessment were measured at the clinical chemical laboratory of the Erasmus MC. Atrial natriuretic peptide (ANP) was assessed by radio-immunoassay (Phoenix Europe, Karlsruhe, Germany) in urine collected during the 1-h equilibration period applied before clearance measurements.

Quantitative PCR

Total RNA was isolated from snap-frozen kidney cortex using TRI Reagent (Sigma Aldrich) and reverse transcribed

into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). cDNA was amplified in 40 cycles (denaturation at 95 °C for 3 min; thermal cycling at 95 °C for 3 s, annealing/extension at 60 °C for 20 s) followed by a melt curve with a CFX384 (BioRad, Lunteren, The Netherlands) using Kapa SYBR Fast (Kapa Biosystems, Wilmington, Massachusetts, USA). The intron-spanning oligonucleotide primers were designed with NCBI (Primer-BLAST; Table S1, http://links.lww.com/HJH/B199). The ΔΔCT method was used for relative quantification of mRNA expression levels, using glyceraldehyde 3-phospate dehydrogenase (*Gapdh*) for *Rcan1,* and the geometric mean of hypoxanthine phosphoribosyl transferase-1 (*Hprt1*), β_2-microglobulin (*B2m*) and β-actin (*Actb*) for normalization of all other mRNAs.

Quantification of cytokines

Concentrations of interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and interleukin 1 beta (IL-1β) were quantified with a rat cytokine magnetic bead panel (RECYTMAG-65K, EMD-Millipore, Amsterdam, The Netherlands) in equal amounts of protein (3 μg/μl), extracted from one snap-frozen kidney quarter, homogenized on ice in RIPA buffer supplemented with protease inhibitors. After subsequent incubation steps with magnetic beads [2 h; room temperature (RT)], detection antibodies (1 h; RT) and streptavidin–phycoerythrin (30 min; RT), samples were analyzed with a Luminex 100/200 (Luminex Corp, 's-Hertogenbosch, The Netherlands) multiplex reader. Data were analyzed using Bio-Rad manager software.

Histology, immunofluorescence, and immunohistochemistry

Kidney segments, fixed in 4% paraformaldehyde (PFA), were dehydrated and paraffin-embedded. Deparaffinized kidney sections (2 μm) were stained with periodic acid–Schiff (PAS) and scored semiquantitatively in a blinded fashion by a renal pathologist (M.C.C.v.G.) [18]. Focal segmental glomerulosclerosis (FSGS) was assessed in all glomeruli of one kidney section per rat, using an arbitrary scale wherein 0%, less than 25%, 25–50%, 50–75%, and greater than 75% of glomerular sclerosis were represented by grade zero (n_0), 1 (n_1), 2 (n_2), 3 (n_3), and 4 (n_4), respectively. The glomerulosclerosis index (GSI) was calculated with the formula: $[(1 \times n_1)+(2 \times n_2)+(3 \times n_3)+(4 \times n_4)]/[n_0 + n_1 + n_2 + n_3 + n_4]$. Tubular atrophy, interstitial fibrosis, and tubulointerstitial inflammation were scored in the same kidney section and summed to obtain the tubulointerstitial score (TIS). A score of 0–3 indicated that less than 25% of tubulointerstitial tissue was affected, a score of 4–6 indicated 25–50% and a score of 7–9 indicated more than 50%.

Semiquantitative scoring of immunofluorescence was used to determine glomerular abundance of transient receptor potential canonical 6 (TRPC6; Alomone Labs, Jerusalem, Israel) in 2-μm cryosections of rat kidney. Mean fluorescent intensity was scored independently by two investigators in 35–50 glomeruli per rat on blinded sections, as described previously [19].

For immunohistochemistry, kidney sections were stained with an antibody for either macrophages (ED1; 1 : 500; Abcam, Cambridge, UK) or for T lymphocytes ($CD3^+$; 1 : 200; DAKO, Amstelveen, The Netherlands) after heat antigen retrieval using citrate buffer (pH 6.0) and blocking with either bovine serum albumin (BSA; 2%) or avidin/biotin (Vector Laboratories, Burlingame, California, USA) and normal goat serum (NGS; 10%), respectively. Brightvision-horseradish peroxidase-conjugated antirabbit and antimouse antibodies (Immunologic) were used for a secondary incubation. Positive cells were visualized with Nova Red (Vector) and kidney sections were briefly counterstained with hematoxylin. ED1-postive and CD3-positive cells were counted in 50 separate glomeruli and 20 peritubular fields (magnification ×20).

Fluorescent-activated cell sorting

After mechanical dissociation of spleen and left kidney, and incubation (1 h; 37 °C; with agitation) of kidney samples with 0.1% collagenase NB 4G (Serva Electrophoresis GmbH, Heidelberg, Germany) in RPMI 1640 (Gibco, Life Technologies, Paisley, UK), followed by inactivation with 10% fetal bovine serum (FBS, Biowhittaker, Verviers, Belgium), splenic and kidney samples were passed through a 70 μm cell strainer (Greiner Bio-One, Alphen aan de Rijn, The Netherlands). Erythrocytes were removed with red blood cell lysis (30 min incubation; RT; 5 Prime Gmbh, Hamburg, Germany). Renal and splenic cells were stained (15 min, in the dark, RT) with BD Via-Probe live/dead viability stain (PerCP; BD Biosciences, Vianen. The Netherlands and fluorochrome-conjugated antibodies for surface markers including CD45 (clone OX-1; APC-Cy7; BD Biosciences), $CD3^+$ (clone 1F4; BV421; BD Biosciences), $CD4^+$ (clone W3/25; PE-Cy7; BioLegend, London, UK), CD8a (clone OX8; FITC; eBioscience), $CD25^+$ (clone OX39; PE; eBioscience, San Diego, California, USA), CD161 (clone 3.2.3; APC; BioLegend) and CD11b/c (clone OX-42; APC; BioLegend). Background fluorescence level of isotype control (IgG1K; clone P3.6.2.8.1; PE; eBioscience) was used as negative reference. Stained cells were fixed by addition of 1% PFA (final dilution) and analyzed with a FACS Canto II flow cytometer (BD Biosciences). Data were analyzed using Kaluza software version 1.2 (Beckman-Coulter, Brea, California, USA).

Statistical analysis

Statistical tests were performed using IBM SPSS Statistics 25 (IBM Corporation, Armonk, New York, USA). Parametric data (expressed as mean values ± SEM) were analyzed by a paired *t*-test or one-way analysis of variance (ANOVA; for comparisons between ≥ two groups), followed by post hoc correction according to Bonferroni or Dunnett in case of multiple comparisons, where appropriate. Nonparametric data were transformed to natural logarithms before statistical testing (ANP; albuminuria; renal histology scores; GFR; ERPF). Univariate linear associations were assessed by calculation of Pearson's coefficient of correlation. Multiple linear regression analysis was applied to determine the variables affecting *Rcan1*, GSI and albuminuria. Two-tailed *P* values less than 0.05 were considered statistically significant.

TABLE 1. Main characteristics of diabetic TGR(mREN2)27 rats

				Vehicle	Valsartan	Sacubitril/valsartan
		0 weeks	9 weeks	12 weeks		
n		25	25	10	7	8
General	MAP (mmHg)		137 ± 3	133 ± 9	87 ± 2^{v}	90 ± 3^{v}
	Body weight (g)	450 ± 6	434 ± 6°	445 ± 14	481 ± 14	486 ± 7*
	Δ treatment			22 ± 8	46 ± 3*	37 ± 5
	Food intake (mg/day)	26 ± 0.4	35 ± 1.1$^{\#}$	34 ± 1.4	36 ± 1.2	32 ± 1.5
	Water intake (ml/day)	44 ± 1	158 ± 7$^{\#}$	136 ± 12	152 ± 17	137 ± 10
	Insulin (U/day)			4.1 ± 0.2	3.6 ± 0.3	2.8 ± 0.2$^{\ddagger\$}$
Tissue	Tibia length (TL; cm)			4.27 ± 0.03	4.34 ± 0.06	4.25 ± 0.01
	Heart weight / TL (g/cm)			0.41 ± 0.02	0.37 ± 0.01	0.36 ± 0.01*
	Kidney weight / TL (g/cm)			0.54 ± 0.02	0.55 ± 0.01	0.57 ± 0.01
Blood	Glucose (mmol/l)			48 ± 3	51 ± 6	38 ± 2
	Serum urea (mmol/l)			9.4 ± 0.5	9.3 ± 0.9	9.2 ± 0.6
	Serum creatinine (μmol/l)			29.5 ± 3.8	28.5 ± 4.4	22.2 ± 2.7
Urine	Volume (ml/day)	25 ± 1	150 ± 9$^{\#}$	128 ± 11	150 ± 18	133 ± 11
	Sodium excretion (mmol/day)	3.4 ± 0.1	4.8 ± 0.2$^{\#}$	4.1 ± 0.2	4.4 ± 0.2	4.8 ± 0.4
	Urea excretion (μmol/day)	14.2 ± 2.1	27.1 ± 4.7$^{\#}$	23.6 ± 2.6	25.3 ± 5.7	26.5 ± 5.0
	Creatinine excretion (μmol/day)	143 ± 2.7	137 ± 3.4°	131 ± 5.3	136 ± 4.3	165 ± 7.6$^{\dagger\$}$
Renal parameters	Albuminuria (mg/day; IQR)	3.5 (2.4–6.7)	8.9 (4.7–12.8)$^{\#}$	5.9 (2.3–8.4)	1.5 (1.2–2.4)*	1.2 (1.0–1.4)†
	Creatinine clearance (ml/min)			3.1 ± 0.6	3.4 ± 0.9	5.9 ± 0.8*

Data on metabolism and 24-h urines were collected before the induction of diabetes (0 weeks), at 9 weeks of diabetes (9 weeks) and at 12 weeks of diabetes (12 weeks), after 3 weeks of vehicle, valsartan or sacubitril/valsartan. Parametric data, represented as means ± SEM, were analyzed using a paired *t*-test (0 vs. 9 weeks) or one-way ANOVA and post hoc Bonferroni. Nonparametric data, represented as median (IQR), were analyzed using a Wilcoxon signed rank or Kruskal–Wallis test and post hoc Dunn. ($^{\circ}P \leq 0.05$, $^{\#}P \leq 0.0001$ vs. 0 weeks; $^{*}P \leq 0.05$, $^{\dagger}P \leq 0.01$, $^{\ddagger}P \leq 0.001$, $^{v}P \leq 0.0001$ vs. vehicle; $^{\$}P \leq 0.05$ vs. valsartan). MAP, mean arterial pressure.

RESULTS

Sacubitril/valsartan offers blood pressure-independent renoprotection

Upon induction of diabetes, rats developed polydipsia and polyuria accompanied by an arrest in physiological weight gain (Table 1). Prior to treatment (in week 9 of the diabetic phase), mean arterial pressure (MAP) was 137 ± 3 mmHg. Sacubitril/valsartan lowered MAP by −50 ± 4 mmHg, thus yielding a similar antihypertensive effect as valsartan (−43 ± 4 mmHg; $P = 0.3$; Fig. 1a). Although both valsartan and sacubitril/valsartan lowered albuminuria, the effect of dual blockade vs. control ($P < 0.01$) was stronger than that of single blockade ($P < 0.05$; Fig. 1b). Accordingly, only after dual therapy did kidneys demonstrate less globally sclerotic glomeruli and less severe, chronic ischemic damage (Fig. 1c and d). Tubulointerstitial damage was still sporadic at this point in time, and did not differ between treatment groups (Fig. 1e). At the end of treatment, only sacubitril/valsartan-treated rats had lower heart weight (indexed to tibia length; Table 1).

Sacubitril/valsartan preserves effective renal plasma flow and glomerular filtration rate

Sacubitril/valsartan preserved effective renal plasma flow (ERPF; 10.7 ± 1.0 vs. 6.5 ± 0.9 ml/min; $P < 0.05$ vs. control; Fig. 2a), and a similar trend was observed for GFR (2.1 ± 0.4 vs. 1.4 ± 0.2 ml/min; $P = 0.1$ vs. control; Fig. 2b). In accordance, sacubitril/valsartan displayed higher creatinine clearances when compared with control ($P < 0.05$; Table 1), and a similar trend was found vs. valsartan ($P = 0.06$). Neither treatment affected filtration fraction (Fig. 2c). As expected, valsartan lowered urinary atrial natriuretic peptide (ANP) levels vs. control ($P < 0.01$; Fig. 2d), presumably by decreasing atrial stretch [16]. In contrast, sacubitril/valsartan-treated animals maintained high ANP levels, consistent with increased half-life of NEP substrates under NEP inhibition.

Sacubitril/valsartan reduces renal macrophage gene expression, but not immune cell infiltration

Sacubitril/valsartan caused no changes in cortical *Cd3ε* gene expression, a general T-lymphocyte marker (Fig. 3a). Yet, dual therapy did decrease gene expression of the macrophage marker *Cd68* in renal cortex ($P < 0.05$; Fig. 3b), without affecting expression of interleukin-6 (*Il-6*; Fig. 3c). However, with immunohistochemistry, we observed no differences in the number of $ED1^{+}$ or $CD3^{+}$ cells present in glomeruli or tubulointerstitium (Table S2, http://links.lww.com/HJH/B200), contradicting an effect of treatment on the cortical infiltration of macrophages or T lymphocytes, respectively. Furthermore, we found no changes in the concentrations of IL-6, tumor necrosis factor alpha (TNFα), and interleukin 1 beta (IL-1β) in whole kidney homogenate (Fig. 3d–f). To analyze an effect of treatment on specific immune cell subpopulations, we performed FACS of a single cell suspension derived from one whole kidney. Positive staining for $CD45^{+}$, a pan leukocyte marker, identified leukocytes infiltrating the kidney. T lymphocytes and monocytes/macrophages represented most of these cells (Figure S1A, http://links.lww.com/HJH/B198). The proportions of the $CD3^{+}$ T-cell subpopulations characterized by $CD4^{+}$ (T-helper cells), $CD8^{+}$ (cytotoxic T cells), $CD161^{+}$ (natural killer T cells) and $CD4^{+}/CD25^{+}$ bright (regulatory T cells) were similar in all three groups. The same was true for monocyte/macrophage ($CD11b/c^{+}CD3^{-}CD4^{+}$), natural killer (NK)

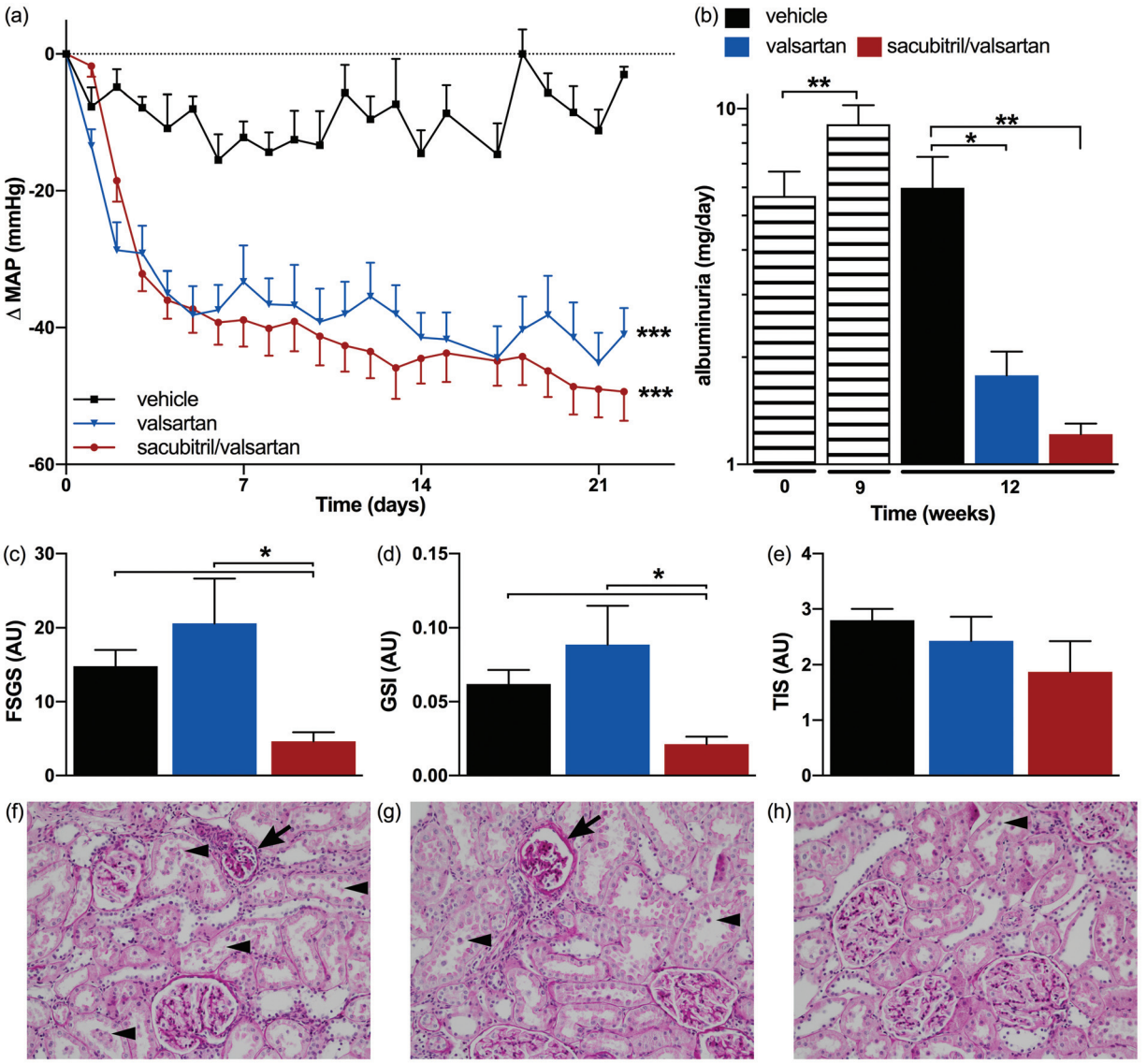

FIGURE 1 Mean arterial pressure, albuminuria, and renal structure. Diabetic TGR(mREN2)27 rats were treated for 21 days with vehicle ($n=10$), valsartan ($n=7$), or sacubitril/valsartan ($n=8$). (a) Change from baseline mean arterial pressure (ΔMAP). (b) 24-h albuminuria prior to diabetes ($t=0$), after 9 weeks of diabetes ($t=9$) and after 3 weeks of vehicle, valsartan or sacubitril/valsartan ($t=12$). (c) Focal segmental glomerulosclerosis (FSGS), (d) glomerulosclerosis index (GSI), and (e) tubulointerstitial score (TIS). Representative, PAS-stained renal histological images of (f) vehicle-treated, (g) valsartan-treated, and (h) sacubitril/valsartan-treated rats. Arrows indicate collapsed glomerular capillaries of ischemic glomeruli; arrowheads indicate intraluminal debris of tubular epithelial cells. Data, represented as means ± SEM, were analyzed using repeated measures or one-way ANOVA and post hoc Bonferroni (after logarithmic transformation for renal parameters; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$ vs. vehicle or otherwise indicated).

cell ($CD161^{+}$ bright) and B-lymphocyte ($CD45RA^{+}$) populations. As expected, B cells were virtually absent in kidney tissue, whereas they were abundant in spleen (Figure S1B, http://links.lww.com/HJH/B198).

Sacubitril/valsartan protects podocyte integrity

Sacubitril/valsartan-treated rats tended to have the lowest nonfasting blood glucose levels ($P=0.09$ vs. control; Fig. 4a), while receiving the least amount of insulin ($P<0.001$ vs. control, $P<0.05$ vs. valsartan; Table 1). Additionally, whereas valsartan reduced glomerular transient receptor potential canonical 6 (TRPC6) channels by 25% ($P=$NS), the reduction in combination with sacubitril amounted to 45% ($P=0.06$ vs. control; $P=0.06$ for linear trend; Fig. 4b) and was accompanied by upregulation of cortical nephrin expression ($P<0.05$ vs. control; Fig. 4c). Nuclear factor of activated T cell (NFATc)-dependent regulator of calcineurin 1 (*Rcan1)* expression correlated positively with TRPC6 abundance ($r=0.56$; $P<0.01$) and was lowered by ~50% after sacubitril/valsartan ($P<0.01$ vs. control and valsartan; Fig. 4d and e). Although no correlation was observed with glucose, *Rcan1* tended to correlate negatively with ANP ($P=0.07$). Multiple linear regression subsequently confirmed that TRPC6 and ANP independently affected *Rcan1* variability, in an opposite manner (Table 2). Blood glucose levels correlated inversely with urinary ANP levels ($r=-0.55$; $P=0.02$; Fig. 4f), GFR ($r=-0.74$; $P=<0.0001$; Fig. 4g) and ERPF ($r=-0.72$; $P=<0.001$; Fig. 4h).

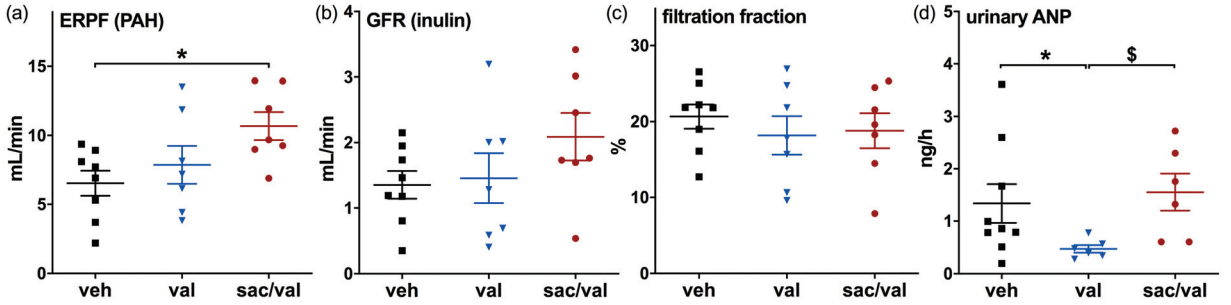

FIGURE 2 Renal function parameters. Renal clearance of (a) para-aminohippuric acid (PAH) (effective renal plasma flow; ERPF) and (b) inulin (glomerular filtration rate; GFR) in diabetic TGR(mREN2)27 after 3 weeks of treatment with vehicle (veh; $n = 10$), valsartan (val; $n = 7$), or sacubitril/valsartan (sac/val; $n = 8$). (c) Filtration fraction. (d) Urinary atrial natriuretic peptide (ANP) excretion per hour. Data, represented as means ± SEM, were analyzed using one-way ANOVA and post hoc Dunnett (after logarithmic transformation of urinary ANP; $^{*}P \leq 0.05$ vs. vehicle; $^{\$}P \leq 0.05$ vs. valsartan).

Determinants of glomerulosclerosis and albuminuria

When analyzed separately, TRPC6, *Rcan1*, and ERPF, but not ΔMAP, GFR, filtration fraction, or blood glucose correlated significantly with GSI (Table S3, http://links.lww.com/HJH/B201). Multiple linear regression of the parameters displaying a significant correlation subsequently revealed that only *Rcan1* determined GSI independently (Table 2). Albuminuria correlated significantly with ΔMAP and *Rcan1*, and a similar trend was observed for GSI ($P = 0.06$; Table S3, http://links.lww.com/HJH/B201). Subsequent multiple linear regression demonstrated that the effects of blood pressure and *Rcan1* on albuminuria were independent (Table 2).

DISCUSSION

Sacubitril/valsartan provides better blood pressure control and improves neurohumoral markers and mortality related to cardiac dysfunction in populations with heart failure or CKD [5,10,20]. Its effects on renal function are less clear, being either similar or superior to that of conventional RAS blockade [7,8,10]. This difference likely depends on the pathogenesis of CKD progression, as particularly in diabetic patients sacubitril/valsartan appears more renoprotective than valsartan [11]. Indeed, in the present study, sacubitril/valsartan reduced chronic renal ischemic damage and globally sclerotic glomeruli better than valsartan in a well known hypertensive rat model, the TGR(mREN2)27 rat, made diabetic with streptozotocin. Furthermore, in addition to these rats displaying the lowest levels of albuminuria, only sacubitril/valsartan preserved ERPF and GFR and reduced cardiac weight (indexed to tibia length). As these beneficial effects occurred at similar antihypertensive efficacy, it can be concluded that sacubitril/valsartan offers drug-class specific, blood pressure-independent cardioprotection and renoprotection. A logical mediator of these effects would be ANP (Fig. 5) [21]. Natriuretic peptides are elevated in TGR(mREN2)27 rats, because of elevated blood pressure and increased atrial stretch [22].

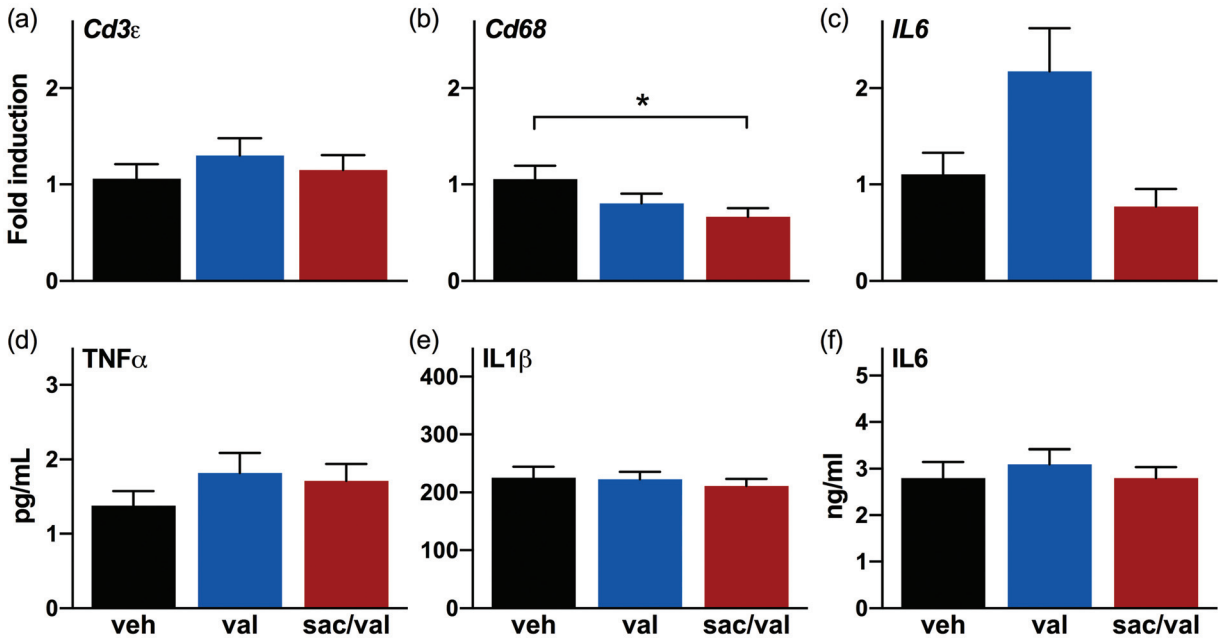

FIGURE 3 Renal inflammatory markers. Gene expression of immune cell markers (a) *Cd3ε* and (b) *Cd68*, and cytokine (c) *IL6* in renal cortex in diabetic TGR(mREN2)27 after 3 weeks of treatment with vehicle ($n = 10$), valsartan ($n = 7$), or sacubitril/valsartan ($n = 8$). Concentration of cytokines (d) TNFα E) IL-1β and (F) IL6 in whole kidney homogenate. Data, represented as means ± SEM (gene expression normalized to vehicle), were analyzed using one-way ANOVA and posthoc Bonferroni ($^{*}P \leq 0.05$).

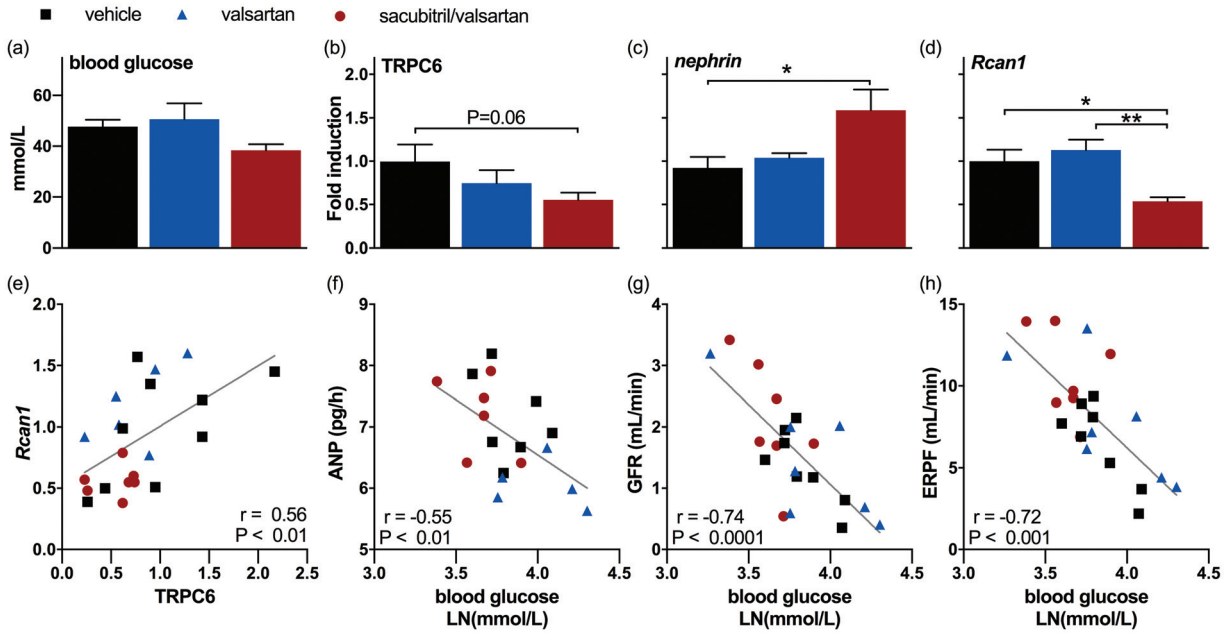

FIGURE 4 Blood glucose and transient receptor potential canonical 6 activity. (a) Blood glucose levels, (b) glomerular transient receptor potential canonical 6 (TRPC6) protein abundance, (c) renal cortex *nephrin*, and (d) regulator of calcineurin 1 (*Rcan1*) mRNA expression of diabetic TGR(mREN2)27 rats treated for 21 days with vehicle ($n=10$; black bars/squares), valsartan ($n=7$; blue bars/triangles), or sacubitril/valsartan ($n=8$; red bars/circles). Relationship between (e) *Rcan1* and TRPC6, (f) atrial natriuretic peptide (ANP) and blood glucose, (g) glomerular filtration rate (GFR) and blood glucose, (h) effective renal plasma flow (ERPF) and blood glucose. Data, represented as means ± SEM, were analyzed using a one-way ANOVA and posthoc Bonferroni ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$). *r* denotes Pearson's correlation coefficient.

Therefore, blood pressure lowering should reduce ANP. This is indeed what was observed after valsartan. Yet, in combination with sacubitril, urinary ANP levels remained elevated, reflecting the consequence of additionally blocking NEP.

ANP, by activating guanylyl cyclase-A (GC-A), regulates natriuresis, diuresis, and renal vascular tone [23]. Podocytic GC-A appears to additionally protect podocyte integrity [24,25]. Podocyte loss during diabetic kidney disease is initiated by a TRPC6 channel-mediated calcium influx, followed by activation of calcineurin-NFATc signal transduction [26,27]. Ang II and hyperglycemia jointly augment the number of TRPC6 channels, thereby exacerbating podocyte damage and glomerulosclerosis [19,28–30]. We previously demonstrated that glomerular TRPC6 expression was significantly increased in TGR(mREN2)27 rats, whereas treatment with the ARB candesartan in a nonhypotensive dose significantly reduced this effect [19]. The present study showed suppression of this pathway by sacubitril/valsartan, reflected by a reduction of TRPC6, a parallel downregulation of *Rcan1* (a proxy for TRPC6 and calcineurin activity [31,32]) and an increase in nephrin gene expression. In contrast, with valsartan we observed at most a downward trend for TRPC6 without any change in *Rcan1* or nephrin. Regression analysis revealed that TRPC6 and ANP independently determined *Rcan1*, in an opposite manner. This is in agreement with the observation that expression of TRPC6 dose-dependently stimulates the NFATc-dependent promoter of *Rcan1* [21], whereas ANP may both reduce the number of TRPC6 channels through attenuation of hyperglycemia [33–35], and, via its second messenger cGMP, inhibit TRPC6 channel activation [21,24]. Indeed, we previously demonstrated that inhibiting cGMP degradation by treatment with sildenafil prevented TRPC6 upregulation and proteinuria in rats with adriamycin-induced nephropathy and mice with hyperglycemia-induced renal injury [36]. ANP-dependent inhibition of TRPC6 activity in particular explains why valsartan alone

TABLE 2. Multiple linear regression to assess variables affecting regulator of calcineurin 1 (*Rcan1*) gene expression, glomerulosclerosis index (GSI) and albuminuria

Dependent variable	Predictor	Coefficient	*P* value	Adjusted R^2
Rcan1	TRPC6	0.516	0.021	0.56
	ANP	−0.307	0.025	
GSI	*Rcan1*	1.301	0.004	0.34
	TRPC6	0.397	0.337	
	ERPF	−0.066	0.193	
Albuminuria	ΔMAP	1.053	0.005	0.46
	Rcan1	0.587	0.049	

GSI, albuminuria and ANP were transformed to natural logarithms before statistical testing. *r* denotes Pearson's correlation coefficient. ΔMAP, change from baseline mean arterial pressure (expressed as area under the curve); ANP, atrial natriuretic peptide; ERPF, effective renal plasma flow; TRPC6, transient receptor potential canonical 6.

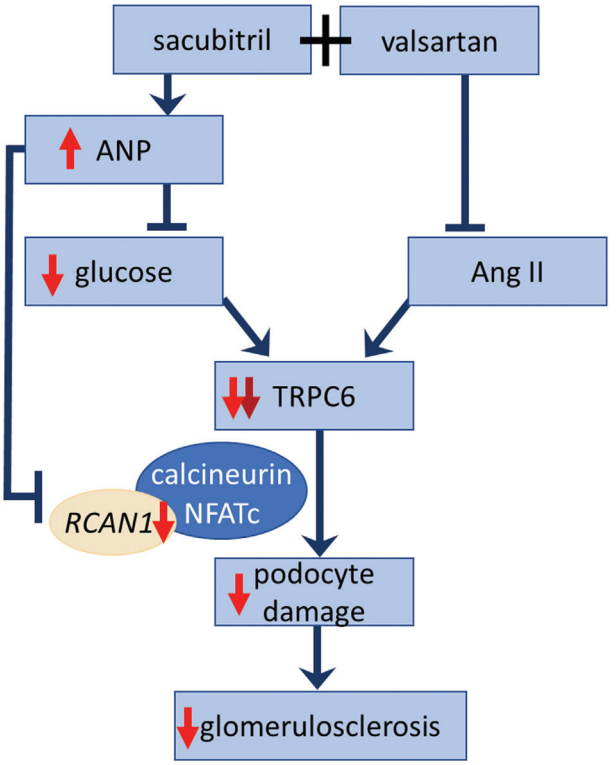

FIGURE 5 Effects of dual angiotensin–neprilysin inhibition vs. angiotensin receptor blockade. Red arrows indicate the observed effects of sacubitril/valsartan. In addition to angiotensin (Ang) II type 1 receptor blockade, sacubitril/valsartan prevents the breakdown of atrial natriuretic peptide (ANP). Raised ANP levels appear to attenuate hyperglycemia and enhance suppression of transient receptor potential canonical 6 (TRPC6) activity, indicated by lowering of regulator of calcineurin 1 (*Rcan*1), a nuclear factor of activated T cell (NFATc)-response gene. This may prevent podocyte damage, reflected by an increase in nephrin expression, and explain why sacubitril/valsartan preserves glomerular function and structure better than valsartan alone.

did not affect *Rcan1*. The fall in ANP during valsartan would be expected to counteract the *Rcan1*-suppressing effect of TRPC6-lowering. Taken together, these data suggest that sacubitril/valsartan provides superior renoprotection to valsartan by better preserving podocyte integrity.

Consistent with the inverse correlation between urinary ANP levels and glycemic control in the current study, sacubitril/valsartan lowered HbA1c values in patients with heart failure and diabetes [37], and increased insulin sensitivity in obese patients with hypertension [38]. Yet, ANP can cause a potentially harmful rise in filtration fraction because of an isolated increase in GFR, at least in healthy individuals. This is unlikely to be observed in patients with CKD [39,40], acute kidney injury [41], hypertension [42,43] and diabetes [44], because of the preexisting afferent vasoconstriction in such subjects, which ANP will counteract, thereby now also increasing ERPF [45,46]. Indeed, no change in filtration fraction was observed in diabetic TGR(mREN2)27 rats after sacubitril/valsartan. Furthermore, glomerular hyperfiltration is unlikely to have occurred, as the GFR after sacubitril/valsartan (2.1 ± 0.4 ml/min) was comparable to that in healthy control rats (2.0 ± 0.2 ml/min), reported by us and others [47–52]. Yet, admittedly, we did not determine baseline ERPF and GFR values before the start of treatment, and thus we cannot rule out that sacubitril/valsartan, instead of preserving, increased these parameters. If so, this might also relate to the lower blood glucose levels in sacubitril/valsartan-treated rats. Here it should be noted that at the elevated glucose levels in the present study, the typically positive correlation between glycaemia and GFR is inverted [53].

In damaged proximal tubules, lysosomal capacity to degrade reabsorbed protein is likely to be exceeded [54]. This will stimulate chemokine production and attract inflammatory cells [14]. Although in the renal cortex, we did observe a sacubitril/valsartan-induced reduction in the macrophage marker $CD68^+$ at the gene expression level, there were no changes at the protein level of IL-6, TNFα, and IL-1β. Moreover, the renal infiltration of macrophages and T-lymphocyte subsets, determined by immunohistochemistry and FACS, was similar in all groups. This renders an actual change in immune cell infiltration, and a sacubitril/valsartan-induced reduction in inflammatory kidney damage, unlikely in our model.

Collectively, these findings indicate that sacubitril/valsartan may be most beneficial in a diabetic context. As CKD was caused by diabetic kidney disease in only a subset of the participants in the UK-HARP III trial, (subgroup) analysis may have been underpowered to distinguish a difference between irbesartan and sacubitril/valsartan on measured GFR [10]. Furthermore, an initial rapid decline in GFR after starting both irbesartan and sacubitril/valsartan distorted the natural development of renal function [10]. Analyses of earlier trials demonstrating superior renoprotection with sacubitril/valsartan were not complicated by this factor, as in these studies, RAS inhibitors previously used by patients were not discontinued for a prolonged period of time, but rather replaced with the study drugs at randomization [5,20]. At present, therapeutics targeting the RAS are the most validated clinical strategy for slowing the progression of diabetic kidney damage, including tubulointerstitial changes and fibrosis. To fully establish the potential of sacubitril/valsartan herein, future studies should specifically target patients with CKD caused by diabetes. Other nephropathies mediated by podocyte loss and glomerulosclerosis, such as obesity-related kidney damage, may represent alternative targets.

In conclusion, when compared with valsartan, dual treatment with sacubitril/valsartan ameliorated chronic renal ischemia and glomerulosclerosis in TGR(mREN2)27 rats with hypertension, diabetes, and kidney damage. These beneficial effects surpassed its antihypertensive properties, as the blood pressure-lowering effects of sacubitril/valsartan and valsartan were similar. In contrast to valsartan, sacubitril/valsartan could prevent the breakdown of ANP, and thus retain high renal/urinary ANP levels. This not only attenuated hyperglycemia, but also suppressed the TRPC6-NFATc-*Rcan1* pathway, thereby potentially improving podocyte integrity and preserving glomerular function and structure. As sacubitril/valsartan seems to particularly prevent disease progression related to podocyte loss and glomerulosclerosis, its renoprotective effect should now be investigated in patients with such pathogenesis, that is, patients with diabetic nephropathy.

ACKNOWLEDGEMENTS

We gratefully acknowledge J.A.H. Dijk, K. den Ouden, and W. Verschoor for their technical assistance.

Authors' contributions: E.U., L.C.W.R., E.J.H., R.Z., T.N., J.A.J., and A.H.J.D. conceived and designed the research. E.U., D.C.H., L.C.W.R., R.v.V., I.M.B.G., R.d.V. acquired the data. M.C.C.v.G. scored renal histology. E.U. performed statistical analysis and drafted the manuscript. E.U., D.C.H., L.C.W.R., M.C.C.v.G, J.v.d.V., R.Z., T.N., E.J.H., J.A.J., and A.H.J.D. made critical revision of the manuscript for key intellectual content. E.J.H., T.N., and A.H.J.D. handled funding and supervision.

A.H.J.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflicts of interest

This work was financially supported by a Kolff Senior Postdoc Career Stimulation Grant for T.N. from the Dutch Kidney Foundation (13OKS023). For the remaining authors, none were declared.

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