Received: 7 September 2021 | Revised: 9 December 2021 | Accepted: 1 January 2022

DOI: 10.1111/dom.14639

ORIGINAL ARTICLE

WILEY

Combined sodium glucose co-transporter-2 inhibitor and angiotensin-converting enzyme inhibition upregulates the renin-angiotensin system in chronic kidney disease with type 2 diabetes: Results of a randomized, double-blind, placebo-controlled exploratory trial

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Funding information
Austrian National Bank Anniversary Fund, Grant/Award Number: 16976; Boehringer Ingelheim, Grant/Award Number: Investigator-Initiiated Study

Abstract

Aim: Sodium glucose co-transporter-2 inhibitors (SGLT-2i) improve cardiorenal outcomes in patients with chronic kidney disease (CKD), with and without type 2 diabetes. The molecular mechanisms underlying these pleiotropic effects remain unclear, yet it is speculated that SGLT-2i elicit a neurohormonal modulation resulting in renin-angiotensin system (RAS) activation. We hypothesized that combined SGLT-2 and angiotensin-converting enzyme inhibition (ACEi) favours RAS regulation towards the beneficial angiotensin-(1-7)-driven axis.

Materials and Methods: This randomized controlled prospective study investigated the effect of 12 weeks treatment with the SGLT-2i empagliflozin on top of ACEi on the molecular RAS dynamics in 24 diabetic and 24 non-diabetic patients with CKD. Systemic RAS peptides were quantified by mass spectrometry.

Results: In patients with type 2 diabetes, combined SGLT-2i and ACEi significantly upregulated plasma renin activity [pre-treatment median and interquartile range 298.0 (43.0-672.0) pmol/L versus post-treatment 577.0 (95.0-1543.0) pmol/L; $p = .037$] and angiotensin I levels [pre-treatment 289.0 (42.0-668.0) pmol/L versus post-treatment 573.0 (93.0-1522.0) pmol/L; $p = .037$], together with a significant increase of angiotensin-(1-7) levels [pre-treatment 14.0 (2.1-19.0) pmol/L versus post-treatment 32.0 (5.7-99.0) pmol/L; $p = .012$]. Empagliflozin treatment resulted in a 1.5 to 2-fold increase in main RAS peptides in patients with diabetes compared with placebo. No significant effect of empagliflozin on top of ACEi on RAS peptides was found in patients with CKD without diabetes.

Conclusion: A distinct RAS modulation by SGLT-2i occurs in diabetic kidney disease reflected by enhancement of the beneficial angiotensin-(1-7) providing a molecular background for this renoprotective therapeutic approach.

KEYWORDS
ACE inhibition, angiotensins, chronic kidney disease, empagliflozin, renin-angiotensin system activation, SGLT-2 inhibition, type 2 diabetes mellitus

1 | INTRODUCTION

Sodium-glucose co-transporter-2 inhibitors (SGLT-2i) exert a glucose-lowering effect by blocking glucose reabsorption in the proximal tubular system.[1] Mounting evidence indicates additional renoprotective properties including an attenuation of kidney function decline and albuminuria, the hallmark of diabetic kidney disease (DKD).[2-4] SGLT-2i have also been shown to reduce cardiovascular and all-cause mortality profoundly in patients both with and without diabetes.[5-8] The detailed underlying mechanisms of these pronounced clinical effects, however, remain unresolved.

The renin-angiotensin system (RAS) is essential in the regulation of blood pressure as well as sodium and fluid homeostasis.[9] Dysregulation of the RAS is critically involved in the development and progression of DKD.[10] Specific modulation of RAS components could delay DKD progression.[11] Key components of the 'classical RAS' include angiotensin-converting enzyme (ACE) and angiotensin (Ang) II, the latter being a highly potent vasoconstrictor with profibrotic and proinflammatory properties. ACE inhibitors (ACEi) decrease Ang II levels in the blood, thereby lowering blood pressure and preventing end-organ damage. In the last decade, the 'alternative' RAS comprising the enzymes ACE2 and neprilysin and their product Ang-(1-7) was identified as a beneficial antagonist of the 'classical' RAS. ACEi increase Ang-(1-7) levels in addition to their inhibitory effects on Ang II.[12] Both Ang II and Ang-(1-7) are further metabolized to smaller downstream metabolites, such as Ang III, Ang IV or Ang-(1-5), respectively. The clinical effects of these peptides remain ambiguous, despite reports on stimulation of atrial natriuretic peptide production through Ang-(1-5) and vasoconstrictive properties of Ang III.[13,14]

SGLT-2i have been hypothesized to result in RAS modulation by affecting the tubuloglomerular feedback[15] and it has been suggested that the renoprotective mechanism(s) of SGLT-2i may be driven by altered intrarenal haemodynamics similar to those of ACEi.[16] We have previously shown that addition of an ACEi to the SGLT-2 blockade results in a distinct increase of Ang-(1-7) in an experimental setting.[17] We thus hypothesized that the selective inhibition of SGLT-2 in addition to existing RAS blockade might exert similar synergistic effects in humans by modulating the RAS metabolism towards upregulation of the alternative RAS axis and its main effector, Ang-(1-7). Therefore, we studied the influence of empagliflozin on top of standard ACEi treatment on the molecular dynamics of the RAS at the systemic level in patients with albuminuric kidney disease, with and without diabetes.

2 | MATERIALS AND METHODS

2.1 | Study population

The present analysis was a prospective, single-centre, randomized, double-blind clinical study according to good clinical practice (EUDRACT no. 2016-002935-14; NCT03078101, http://clinicaltrials.gov). It was approved by the local Ethics Committee of the Medical University of Vienna (no. 1936/2016) and all participants provided written informed consent before participation. The CONSORT flow chart for participants is shown in Figure 1. Overall, 48 patients with chronic kidney disease (CKD) stages 3-4 were included between April 2017 and March 2019; 24 patients had type 2 diabetes and 24 were non-diabetic.

Inclusion criteria for patients with type 2 diabetes at screening were: (a) male or female subjects diagnosed with CKD stages 3-4, defined by an estimated glomerular filtration rate (eGFR; calculated using the MDRD formula) of 15-59 ml/min/1.73m^2 at the time of pre-screening; (b) urinary albumin excretion rates of >30 mg/24 h [or a urinary albumin/creatinine ratio (UACR) >30 mg/g]; (c) fasting plasma glucose levels >126 mg/dl (7 mmol/L) or glycated haemoglobin (HbA1c) levels >6.5% (definition of type 2 diabetes mellitus according to the diagnostic criteria of the American Diabetes Association); and (d) age ≥18 years. Exclusion criteria were: (a) severely impaired renal function (eGFR <15 ml/min/1.73m^2); (b) hyperkalaemia >5.1 mmol/L; (c) total urinary protein excretion ≥3.5 g/day; (d) hypotension (systolic blood pressure <120 mmHg on ambulatory measurement); and (e) pregnant patients or patients planning pregnancy.

Inclusion criteria for patients without type 2 diabetes were the same as for patients with type 2 diabetes above except for diabetes mellitus representing an exclusion criterion.

2.2 | Study procedures

All participants were subjected to a 2-week run-in period for conversion of the current RAS-blocking medication and dose titration to either enalapril 10 mg twice daily or ramipril 10 mg once daily, after which patients underwent blood collection including the first RAS analysis and urinary analysis. Subsequently, patients were randomized to either a 12-week course of empagliflozin 10 mg daily or placebo. All patients attended biweekly clinic visits including laboratory follow-

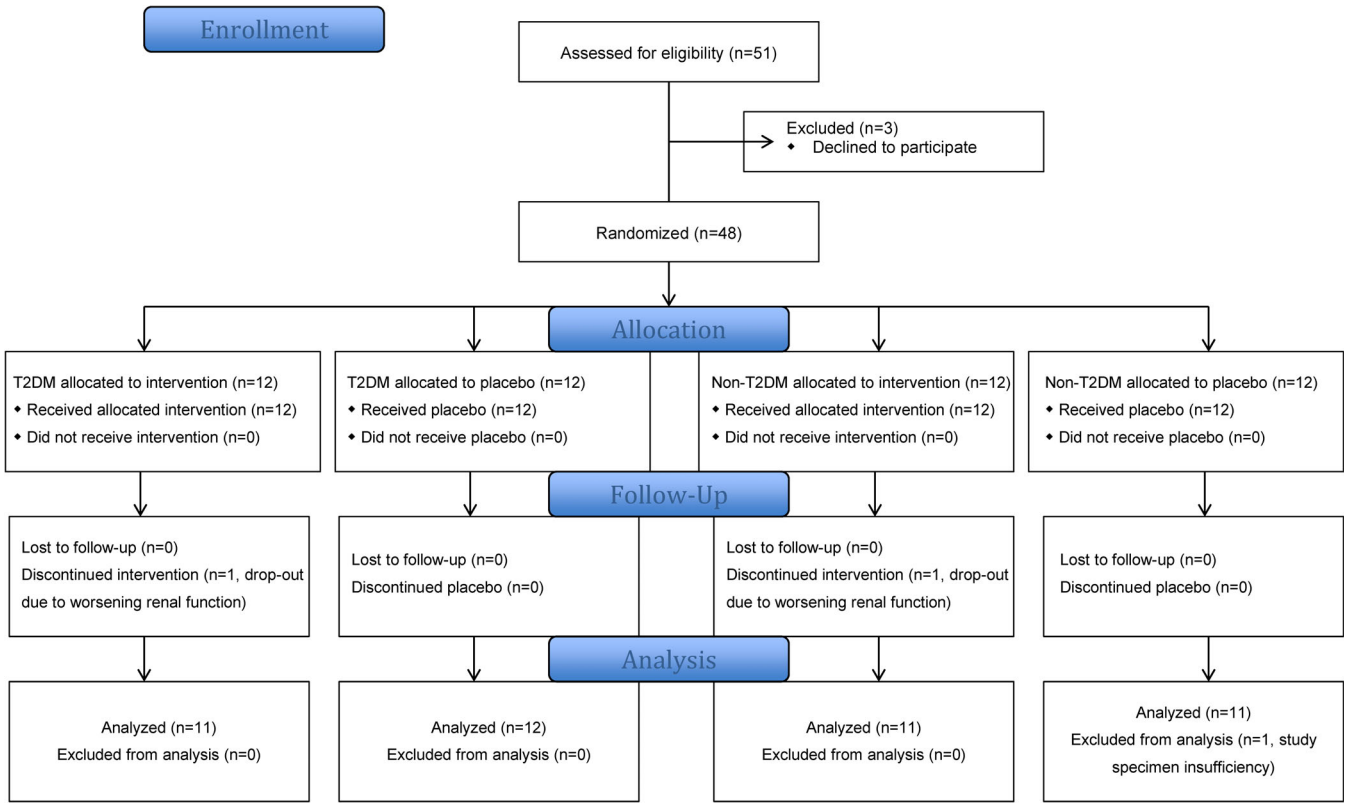

FIGURE 1 CONSORT flow chart of study participants. T2DM, type 2 diabetes

up of electrolytes, plasma and urinary glucose, kidney function (serum creatinine, eGFR, UACR) and blood pressure. At 12 weeks, final RAS analysis, final blood and urinary analysis were carried out.

2.3 | Sample collection and quantification of renin-angiotensin system metabolites

Blood samples were collected using Li-heparin tubes, plasma was obtained by centrifugation at 2000 *g* for 15 min (4°C) and aliquots were stored at −80°C until analysis.

Equilibrium levels of six different RAS Ang peptide metabolites [Ang I, Ang II, Ang III, Ang IV, Ang-(1-7) and Ang-(1-5)] as well as the steroid aldosterone in the samples were quantified in plasma samples by liquid chromatography-tandem mass spectrometry (LC–MS/MS) using previously validated and described methods.[18] Briefly, plasma conditioning for equilibrium analysis was performed at 37°C followed by sample stabilization through blocking Ang metabolism (Attoquant Diagnostics, Vienna, Austria). The biochemical background of the equilibrium approach has recently been described,[19] while previous results have shown similar qualitative outcomes when comparing the quantification of circulating (stabilized immediately at blood drawing) and equilibrium Ang peptide levels.[20,21] For quantification of Ang levels, stabilized equilibrated samples were spiked with stable isotope labelled internal standards for each Ang metabolite and with a deuterated internal standard for aldosterone (aldosterone D4). The samples then underwent C18-based solid-phase extraction and were subjected to LC-MS/MS analysis using a reversed-phase analytical column operating in line with a Xevo TQ-S triple quadruple mass spectrometer (Waters, Milford, MA, USA). Internal standards were used to correct for peptide and steroid recovery of the sample preparation procedure for each analyte in each individual sample. Analyte concentrations are reported in pmol/L and were calculated considering the corresponding response factors determined in appropriate calibration curves in original sample matrix, given that integrated signals exceeded a signal-to-noise ratio of 10. The coefficient of variation is <15% for all analytes over the entire quantification range [range between lower limit of quantification (LLOQ) and upper LOQ]. The quantification ranges for the analytes were 3-3949 pmol/L (Ang I), 2-4894 pmol/L (Ang II), 2-5499 pmol/L (Ang III), 1-6607 pmol/L (Ang IV), 3-5695 pmol/L (Ang 1-7), 1,5-7702 pmol/L (Ang 1-5) and 20-14 204 pmol/L (aldosterone), respectively. ACE-2 was measured by LC-MS/MS as described in.[22]

2.4 | Statistical analyses

The primary endpoint was the change in serum Ang-(1-7) levels from baseline to after 12 weeks of combined empagliflozin and ACEi treatment. The sample size was chosen to evaluate within-group differences in Ang levels. Assuming an SD of 9.6%, a paired *t*-test with a two-sided significance level of .05 and a sample size of 10 subjects would provide 85% power to detect a 15% increase from baseline to 12 weeks. Assuming a 20% drop-out rate, we planned to include

TABLE 1 Demographic and clinical characteristics of the study participants before and after empagliflozin treatment

	Patients with T2DM				Patients without T2DM			
	Empagliflozin group ($n = 11$)		Placebo group ($n = 12$)		Empagliflozin group ($n = 11$)	Placebo group ($n = 11$)		
	Baseline	End of treatment	Baseline	End of treatment	Baseline	End of treatment	Baseline	End of treatment
Male, *n* (%)	10 (91.6)	NA	7 (58.3)	NA	8 (72.7)	NA	6 (54.5)	NA
Age, years	71 ± 6	NA	69 ± 12	NA	53 ± 16	NA	63 ± 13	NA
Weight, kg	92 ± 15	NA	80 ± 13	NA	77 ± 12	NA	79 ± 14	NA
BMI, kg/m^2	31 ± 3.9	NA	28 ± 5.5	NA	26.3 ± 2.7	NA	27.7 ± 3.0	NA
Underlying kidney disease								
Vascular					2 ± 18	NA	3 ± 27	NA
Autoimmune/GN					3 ± 27	NA	2 ± 18	NA
IgA nephropathy					3 ± 27	NA	2 ± 18	NA
Unclear					2 ± 18	NA	2 ± 18	NA
Malignancy/urological					1 ± 9	NA	2 ± 18	NA
History of comorbidities, *n* (%)								
Hypertension	10 (90.9)	NA	11 (91.7)	NA	10 (90.9)	NA	9 (81.8)	NA
Coronary artery disease	3 (27.3)	NA	2 (16.7)	NA	1 (9.1)	NA	2 (18.2)	NA
Peripheral artery disease	1 (9.1)	NA	3 (25.0)	NA	NA	NA	1 (9.1)	NA
Hyperlipidaemia	4 (36.4)	NA	4 (33.3)	NA	3 (27.3)	NA	2 (18.2)	NA
Atrial fibrillation	1 (9.1)	NA	1 (8.3)	NA	2 (18.2)	NA	NA	NA
COPD	2 (18.2)	NA	4 (33.3)	NA	1 (9.1)	NA	3 (27.3)	NA
Concomitant medication, *n* (%)								
Diuretic	5 (45.5)	NA	7 (58.3)	NA	4 (36.4)	NA	3 (27.3)	NA
Beta-blocker	10 (90.9)	NA	6 (50.0)	NA	5 (45.5)	NA	6 (54.5)	NA
Calcium channel blocker	7 (63.6)	NA	5 (41.7)	NA	5 (45.5)	NA	2 (18.2)	NA
Statin	9 (81.8)	NA	8 (66.7)	NA	3 (27.3)	NA	5 (45.5)	NA
Platelet inhibitor	7 (63.3)	NA	8 (66.7)	NA	5 (45.5)	NA	7 (63.6)	NA
Metformin	4 (36.4)	NA	1 (8.3)	NA	NA	NA	NA	NA
Insulin	6 (54.5)	NA	9 (75.0)	NA	NA	NA	NA	NA
Other antidiabetic	9 (81.8)	NA	8 (66.7)	NA	NA	NA	NA	NA
HbA1c, %	6.9 ± 1.3	6.8 ± 1	7 ± 1.1	6.9 ± 1.1	5.4 ± 0.3	5.4 ± 0.3	5.6 ± 0.4	5.6 ± 0.4
Blood glucose, mg/dl	173 ± 114	152 ± 40	137 ± 45	165 ± 72	97 [94-113]	99 ± 10	93 ± 14	92 ± 16
Urinary glucose, mg/dl	0 [0-50]	1000 [500-1000]	0 [0-0]	0 [0-50]	0 [0-0]	500 [100-500]	0 [0-0]	0 [0-0]
Blood pressure, mmHg								
Systolic	146 ± 27	140 ± 18	141 ± 19	130 ± 12	135 ± 7.9	136 ± 19	134 ± 23	127 ± 17
Diastolic	77(12	76 ± 5	76 ± 10	75 ± 9.9	83 ± 11	88 ± 9.4	82 ± 6.2	78 ± 7.8
Estimated GFR, ml/min/1.72 m^2	33 ± 7.5	32 ± 11	37 ± 10	37 ± 12	37 ± 12	35 ± 12	29 ± 8.8	29 ± 6.7
Serum creatinine, mg/dl	1.9 ± 0.5	2.1 ± 0.5	1.8 ± 0.5	1.7 ± 0.3	1.9 ± 0.4	2.1 ± 0.5	2.2 ± 0.7	2.1 ± 0.5
Blood urea nitrogen, mg/dl	28 [22-39]	33 [23-41]	27 [22-49]	32 [25-36]	28 [22-49]	27 [24-41]	30 [24-44]	32 ± 9.3
UACR	1315 [190-1762]	461 [98-1104]	624 [49-1481]	217 [39-2297]	868 [336-1305]	805 [189-1540]	118 [54-333]	205 [47-551]
UPCR	1778 [318-2262]	605 [236-1284]	841 [135-1943]	500 [122-2708]	1079 [680-1866]†	1016 [260-1920]	324 [172-599]†	295 [166-928]

(Continues)

TABLE 1 (Continued)

	Patients with T2DM				Patients without T2DM			
	Empagliflozin group ($n = 11$)		Placebo group ($n = 12$)		Empagliflozin group ($n = 11$)	Placebo group ($n = 11$)		
	Baseline	End of treatment	Baseline	End of treatment	Baseline	End of treatment	Baseline	End of treatment
Aldosterone, pmol/L	96 [78-182]	103 [61-186]	78 [45-114]	107 [58-173]	86 [22-323]	93 [22-363]	183 [90-322]	188 [65-321]
PRA, pmol/L	298 [43-672]	577 [95-1543]*	179 [165-965]	394 [33-1121]*	335.0 [85-844]	370 [146-2077]	364 [63-1210]	566 [142-888]
Serum albumin, g/L	41 ± 2.7	42 ± 2.8	42 ± 3.1	41 ± 2.9	42 ± 3.4	43 ± 3.3	43 ± 2.6	43 ± 3.2
Total cholesterol, md/dl	154 ± 30	154 ± 45	167 ± 41	184 ± 62	209 ± 59	217 ± 67	192 ± 46	194 ± 39
HDL-cholesterol, mg/dl	43 ± 14	43 ± 13	48 ± 20	55 ± 23	50 ± 20	52 ± 20	51 ± 17	50 ± 14
LDL-cholesterol, mg/dl	77 ± 21	83 ± 35	81 ± 48	103 ± 59	130 ± 51	136 ± 55	112 ± 41	113 ± 34
Triglycerides, mg/dl	175 ± 83	138 ± 71	202 ± 145	166 ± 68	153 ± 113	145 ± 73	147 ± 82	173 ± 111
Plasma biochemistry, mEq/L								
Sodium	141 ± 2.4	141 ± 2.8	141 ± 3.3	141 ± 2.4	140 ± 1.8	140 ± 3.9	140 ± 3.3	140 ± 1.3
Potassium	4.6 ± 0.4	4.7 ± 0.4	4.7 ± 0.5	4.7 ± 0.3	4.9 ± 0.6	4.6 ± 0.5	4.7 ± 0.3	4.6 ± 0.4
Chloride	103 ± 3.4	103 ± 3.2	103 ± 3.4	103 ± 2.8	103 ± 3.6	102 ± 5	103 ± 3.6	103 ± 2.2
Phosphorus	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	1 ± 0.3	1 ± 0.2
C-reactive protein	0.3 [0.1-0.7]	0.2 [0.1-0.4]	0.3 [0.1-0.5]	0.6 [0.1-1.6]	0.2 [0.1-0.5]	0.2 [0.1-1]	0.6 [0.1-1.1]	0.7 [0.2-1]

Note: Data are shown as mean ± SD or median [IQR] unless indicated otherwise. $p < .01$; †$p < .05$; *$p < .05$.
Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; GFR, glomerular filtration rate; GN, glomerulonephritis; HbA1c, haemoglobin A1c, HDL, high-density lipoprotein; LDL, low-density lipoprotein; NA, not applicable; PRA, plasma renin activity; T2DM, type 2 diabetes; UACR, urinary albumin-to-creatinine ratio; UPCR; urinary protein-to-creatinine ratio.

12 patients per group. The SD and effect size were estimated based on previous experiments.[20] Ang changes between the respective empagliflozin and placebo groups were compared as part of the secondary analyses.

Data were not normally distributed, confirmed by graphical assessment as histogram and by performing the Shapiro–Wilk Test. Within-group differences were compared using Wilcoxon test (paired analyses) and Mann–Whitney *U*-test (unpaired analyses). Markers for plasma renin activity (PRA) and ACE activity were calculated as PRA: Ang I + Ang II, and ACE activity: Ang II/Ang I, respectively.[19] GraphPad Prism software (Prism 8) was used for statistical analyses.

3 | RESULTS

3.1 | Characteristics of study participants

Demographic and clinical characteristics of the study cohort are presented in Table 1. Overall, 31 patients (69%) were male and the average age was 64 ± 14 years.

The analysed per-protocol study population consisted of 23 patients with CKD with type 2 diabetes of which 11 subjects received empagliflozin (baseline mean ± SD eGFR 33 ± 8 ml/min/1.73m^2; HbA1c 6.9 ± 1.3 %) and 12 subjects received placebo (baseline eGFR 37 ± 10 ml/min/1.73m^2; HbA1c 7.0 ± 1.1 %). Of the 22 patients with CKD without type 2 diabetes, 11 subjects each received empagliflozin (baseline eGFR 37 ± 12 ml/min/1.73m^2) or placebo (baseline eGFR 29 ± 9 ml/min/1.73m^2). The diabetes mellitus disease duration was on average 14.3 ± 9.5 years. We found no statistical differences between baseline characteristics in each of the patient cohorts with the exception of UACR and urinary protein-to-creatinine ratio in the patients with CKD and without type 2 diabetes [placebo versus empagliflozin groups: median (IQR) 118 (54-333) versus 868 (336-1305) $p = .007$ and 324 (172-599) versus 1079 (680-1866) $p = .013$, respectively].

3.2 | Effects of empagliflozin on kidney function, glucose control and laboratory parameters

Empagliflozin treatment led to an initial eGFR decrease at 2 weeks with a relative recovery thereafter (Figure S1). No clinically relevant changes in serum electrolytes, HbA1c and blood glucose levels were observed within each patient subgroup at the end of treatment compared with baseline. Urinary protein excretion and blood pressure tended to decrease moderately in the whole type 2 diabetes group while remaining stable in patients without diabetes. Overall, proteinuria was lower in patients without diabetes. Urinary glucose excretion increased significantly on semiquantitative analysis in the patient subgroups receiving empagliflozin, corroborating study medication adherence during the study period.

3.3 | Effect of empagliflozin on renin-angiotensin system metabolites in patients with chronic kidney disease and with type 2 diabetes

After 12 weeks of treatment, empagliflozin on top of ACEi induced a distinct alternative RAS activation in patients with type 2 diabetes

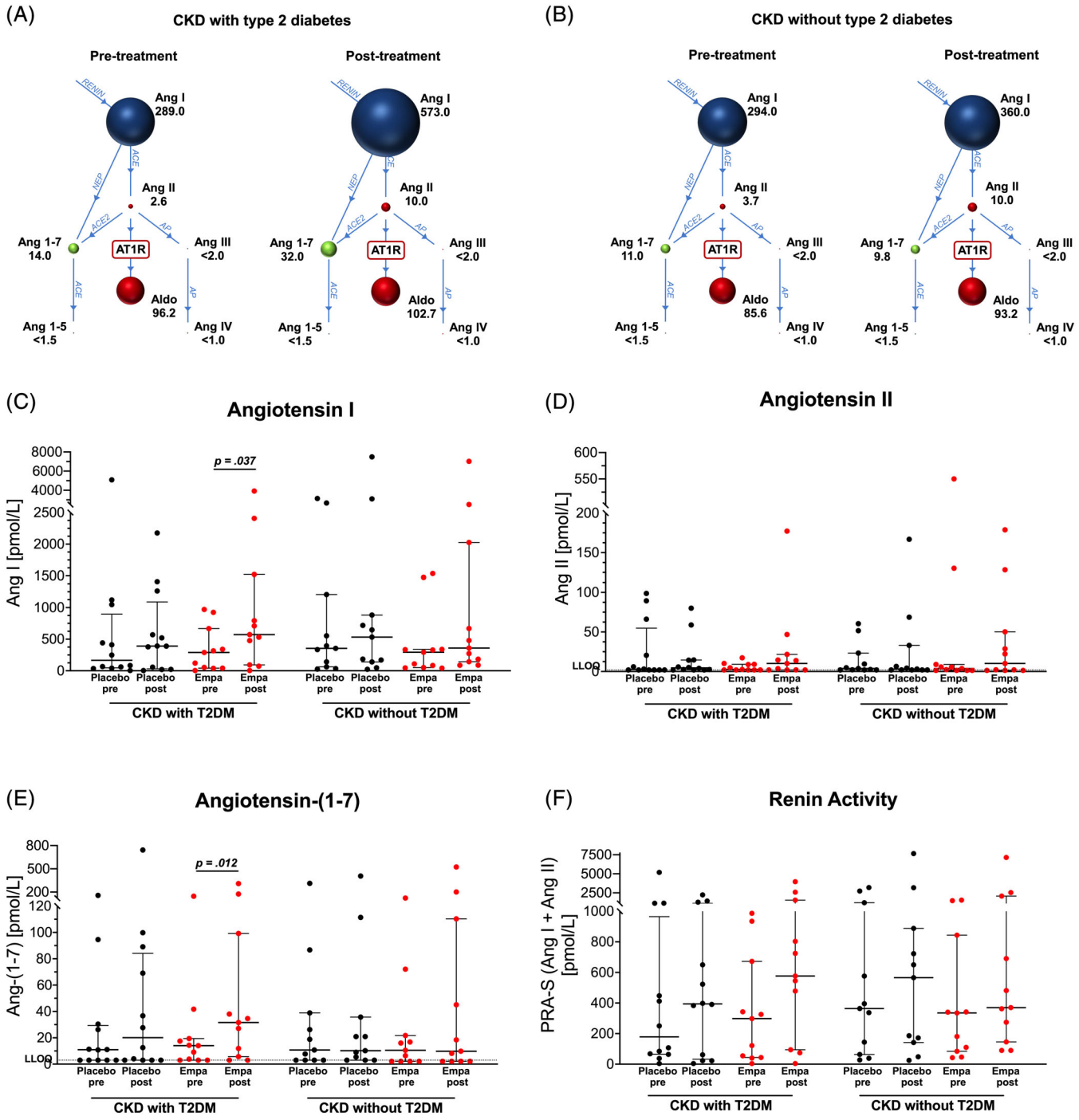

FIGURE 2 Angiotensin levels before and after sodium glucose co-transporter-2 inhibitor treatment on top of angiotensin-converting enzyme inhibition in patients with CKD, with and without T2DM. A, Median angiotensin peptide concentrations in patients with T2DM before and after treatment. B, Median angiotensin peptide concentrations in patients without T2DM before and after treatment. Plasma angiotensin peptide concentrations and renin-angiotensin system enzymatic cascade are depicted as renin-angiotensin system fingerprints. Concentration of indicated angiotensin metabolites are reflected by the size of the corresponding sphere. Blue arrows indicate enzymes that are known to carry out metabolic conversions between connected angiotensin metabolites. Numbers represent median concentrations [pmol/L]. . C, Ang I levels, D, Ang II levels, E, Ang-(1-7) levels, and F, renin activity levels in patients with CKD, with and without T2D, before and after 12 weeks of empagliflozin treatment. CKD, chronic kidney disease; Empa, empagliflozin; T2D, type 2 diabetes; PRA, plasma renin activity (calculated as sum of Ang I and Ang II)

(Figure 2A and Table 2), demonstrated by significantly increased Ang I levels by 2.0-fold ($p = .037$; Figure 2C) and significantly increased Ang-(1-7) levels by 2.3-fold ($p = .012$; Figure 2E) compared with baseline. We observed a statistically non-significant increase in Ang II in empagliflozin-treated subjects [pre-treatment median (IQR) 2.6 (1.4-9.4) pmol/L versus post-treatment 10 (1.4-22.0) pmol/L; $p = .074$; Figure 2D] indicating a global RAS response to empagliflozin treatment as observed in previous studies.[17]

TABLE 2 Angiotensin concentrations in patients with chronic kidney disease, with and without type 2 diabetes, before and after 12 weeks of empagliflozin treatment

	Patients with type 2 diabetes					
	Empagliflozin ($n = 11$)			Placebo ($n = 12$)		
	Pre-treatment	12 weeks treatment	*p*-value	Pre-treatment	12 weeks treatment	*p*-value
Ang I, pmol/L	289.0 [42.0-668.0]	573.0 [93.0-1522.0]	**.037**	166.0 [46.0-896.0]	391.0 [31.0-1090.0]	.898
Ang II, pmol/L	2.6 [1.4-9.1]	10 [1.4-22]	.074	2.3 [1.4-55.0]	4.6 [1.7-15.0]	.547
ACE-S	0.03 [0.01-0.03]	0.02 [0.01-0.03]	.340	0.02 [0.01-0.08]	0.02 [0.01-0.1]	.074
ACE2, ng/ml	1.6 [1.2-2.1]	1.2 [0.9-1.5]	.102	1.5 [0.9-1.7]	1.1 [1.0-1.9]	.835
Ang-(1-7), pmol/L	14.0 [2.1-19.0]	32.0 [5.7-99.0]	**.012**	11.0 [2.1-29.0]	20.0 [2.1-84.0]	.129
Ang-(1-5), pmol/L	<1.5	<1.5	NA	<1.5	<1.5	NA
Ang III, pmol/L	<2.0	<2.0	NA	<2.0	<2.0	NA
Ang IV, pmol/L	<1.0	<1.0	NA	<1.0	<1.0	NA
	Patients without type 2 diabetes					
	Empagliflozin ($n = 11$)			Placebo ($n = 11$)		
	Pre-treatment	12-week treatment	*p*-value	Pre-treatment	12-week treatment	*p*-value
Ang I, pmol/L	294.0 [51.0-339.0]	360.0 [144.0-2026.0]	.123	354.0 [62.0-1206.0]	533.0 [140.0-882.0]	.320
Ang II, pmol/L	3.7 [1.4-8.9]	10.0 [1.4-50.0]	.770	3.8 [1.4-23.0]	3.1 [1.4-33.0]	.301
ACE-S	0.02 [0.01-0.03]	0.02 [0.01-0.03]	.452	0.02 [0.01-0.04]	0.01 [0.01-0.03]	.562
ACE2, ng/ml	1.4 [0.9-2.0]	1.2 [0.9-2.0]	.999	1.0 [0.7-1.3]	1.0 [0.8-1.5]	1.000
Ang-(1-7), pmol/L	11.0 [2.1-22.0]	9.8 [2.1-110.0]	.203	11.0 [2.1-39.0]	10.0 [2.1-36.0]	.250
Ang-(1-5), pmol/L	<1.5	<1.5	NA	<1.5	<1.5	NA
Ang III, pmol/L	<2.0	<2.0	NA	<2.0	<2.0	NA
Ang IV, pmol/L	<1.0	<1.0	NA	<1.0	<1.0	NA

Note: Data are given as median [interquartile range]. ACE-S calculated as Ang II to Ang I ratio.
Abbreviations: ACE, angiotensin-converting enzyme; ACE-S, plasma ACE activity surrogate; Ang, angiotensin; NA, not applicable.

The specific impact of empagliflozin on the systemic RAS was further reflected by a highly increased PRA [pre-treatment 298.0 (43.0-672.0) pmol/L versus post-treatment 577.0 (95.0-1543.0) pmol/L; $p = .037$; Figure 2F). No significant changes in Ang levels compared with baseline were observed in the placebo group (Table 2).

Compared with placebo, all main RAS peptides increased up to 100-fold more in the empagliflozin group, albeit in a non-significant manner, while plasma ACE activity and ACE2 levels remained suppressed (Table 3). Aldosterone remained within the normal range in both groups (Tables 2 and 3). Downstream metabolites Ang-(1-5), Ang III and Ang IV remained below the LLOQ [<1.5 pmol/L for Ang-(1-5), <2 pmol/L for Ang III and <1 pmol/L for Ang IV] in both groups.

Patient-individual Ang level changes throughout the study period are shown in Figure S2.

3.4 | Effect of empagliflozin on renin-angiotensin system metabolites in patients with chronic kidney disease without type 2 diabetes

In patients without diabetes, combined empagliflozin and ACEi treatment resulted in a moderate increase of Ang I levels [294.0 (51.0-339.0) pmol/L versus 360.0 (144.0-2026.0) pmol/L, $p = .123$], whereas Ang-(1-7) levels remained unaffected [11.0 (2.1-22.0) pmol/L versus 9.8 (2.1-110.0) pmol/L, $p = .203$] compared with pre-treatment levels (Figure 2B, Table 2). A statistically non-significant increase in Ang II levels was observed, while plasma ACE activity remained suppressed throughout the treatment period in both groups compared with baseline. Furthermore, plasma aldosterone levels remained stable in both groups throughout the treatment period (Tables 2 and 3).

Placebo-treated subjects showed no significant change in Ang levels compared with baseline (Table 3). Downstream Ang metabolites [Ang-(1-5), Ang III and Ang IV] remained below the LLOQ in both treatment groups. Compared with placebo, all main RAS peptides increased up to four-fold more in the empagliflozin group, however in a non-significant manner (Table 3). Patient-individual Ang level changes are shown in Figure S3.

3.5 | Adverse events

No study drug-related severe adverse events were observed. Throughout the 12-week study phase, three patients were excluded and discontinued the study drug: one male patient from the type 2 diabetes group was excluded because of eGFR

TABLE 3 Changes in angiotensin concentrations in patients with chronic kidney disease, with and without T2DM, after 12 weeks of empagliflozin treatment

	Patients with T2DM			Patients without T2DM		
	Placebo ($n = 12$)	Empagliflozin ($n = 11$)	*p*-value	Placebo ($n = 12$)	Empagliflozin ($n = 11$)	*p*-value
Δ Ang I, pmol/L	−4.8 [−177.0 to 336.0]	411 [0.0-1232.0]	.122	78.5 [−94.0 to 384.0]	319.0 [−66.8 to 1976.0]	.562
Δ Ang II, pmol/L	0.0 [−8.6 to 2.3]	1.1 [0.0-13.1]	.153	0.7 [−1.2 to 8.1]	0.7 [−4.0 to 16.3]	>0.99
Δ Ang-(1-7), pmol/L	5.2 [−5.4 to 65.0]	19.0 [0.0-29.0]	.523	1.3 [0.0 to 10.0]	1.8 [−7.0 to 108.0]	.650
Δ PRA, pmol/L	−3.4 [−176.0 to 320.0]	423 [0.0-1245.0]	.122	79 [−95.0 to 386.0]	328 [−68.0 to 1694.0]	.562
Δ Aldosterone, pmol/L	16.0 [−7.0 to 75.0]	−5.4 [−74.0 to 58.0]	.502	3.1 [−63.0 to 50.0]	7.2 [−0.2 to 99.0]	.357
Δ ACE2, ng/ml	0.1 [−0.5 to 0.4]	−0.4 [−0.7 to 0.1]	.174	0.1 [−0.04 to 0.2]	0.0 [−0.6 to 0.5]	.641
Δ ACE-S	0.0 [−0.01 to 0.04]	0.0 [−0.02 to 0.0]	.555	0.0 [−0.02 to 0.01]	0.0 [−0.01 to 0.02]	.784

Note: Δ Ang, aldosterone, ACE2 and ACE-S levels were calculated individually from each patient as changes from pre-treatment levels after the 12-week treatment period and are given as median [interquartile range]; ACE-S was calculated as Ang II to Ang I ratio; PRA was calculated as the sum of Ang I + Ang II levels.

Abbreviations: ACE, angiotensin-converting enzyme; ACE-S, plasma ACE activity surrogate; Ang, angiotensin; PRA, plasma renin activity; T2DM, type 2 diabetes.

worsening <15 ml/min/1.73m^2 in the context of acute myocardial infarction; one female patient from the non-diabetic group was excluded because of worsening of eGFR <15 ml/min/1.73m^2 and increasing proteinuria >3.5 g/day associated with the ongoing activity of the underlying renal disease (C3 nephritis); and one male patient without diabetes was excluded as the sample was not sufficient for analysis.

Serious adverse events were documented in seven individual patients: four patients were hospitalized because of respiratory tract infections, two required hospitalization because of coronary artery disease during the study phase, both with previously documented coronary disease, and one patient was hospitalized because of a lower leg injury after trauma. All patients recovered without prolonged hospitalization.

No cases of diabetic ketoacidosis and no cases of hypoglycaemia were recorded.

4 | DISCUSSION

The present study is the first, to our knowledge, to investigate the effect of combined SGLT-2 and ACE inhibition on systemic RAS components in patients with CKD with and without type 2 diabetes. We found that empagliflozin on top of therapeutic ACEi treatment induced activation of the alternative RAS axis in patients with CKD and diabetes, corroborating the hypothesis that this drug exerts a modulatory effect on the neurohormonal system.

As SGLT-2i exert their primary effect in the tubular system of the kidney and have been shown to associate with a reduction in blood pressure,[3,23] the influence of neurohormonal regulators by this substance class is probable. SGLT-2i provoke an increase in renin secretion, which then cleaves its substrate angiotensinogen into Ang I. Ang I is further cleaved by ACE, creating Ang II, which exhibits vasoconstrictive, profibrotic and proinflammatory effects, thereby contributing to increased blood pressure and renal damage.[24] The importance of the 'alternative' RAS with Ang-(1-7) as a key opposing effector to Ang II has been well established.[25] The alternative RAS axis is maintained by the enzymes ACE2 and neprilysin, which both lead to the formation of Ang-(1-7) from Ang II or Ang I.[26-28]

To our knowledge, we were the first to show that by combining SGLT-2i and ACEi, a distinct RAS modification ensues in patients with DKD. Suppressed Ang II/Ang I ratios at the baseline visit reflected the intake and effect of ACEi treatment. Subsequent to SGLT-2i treatment, an additional upregulation of PRA, Ang I and, to a very small extent, Ang II, was observed in the diabetic patient group constituting global RAS activation. The overall median Ang II levels in the empagliflozin groups remained at 10 pmol/L in both patients with diabetes and patients without diabetes. These levels reflect the continued ACEi efficacy and are considered physiologically suppressed, which is further indicated by continuously suppressed Ang-(1-5) levels. Ang I levels were increased to a significantly higher extent than Ang II, paralleled by an increased PRA indicating global and proximal RAS activation by combined SGLT-2i and ACEi. We confirmed findings by Ansary et al. who discussed that increases in systemic RAS parameters can be explained as compensatory mechanisms in response to volume reduction by SGLT-2i, while plasma aldosterone levels did not change after SGLT-2i treatment.[29] Furthermore, a RAS modulation toward its alternative axis ensued, reflected by significantly increased Ang-(1-7) concentrations.

Our findings extend recent data of RAS blockade-naïve patients with type 1 diabetes treated with SGLT-2i.[17,30,31] Here, a systemic RAS activation followed by distinct RAS metabolite modulation was observed including an increase of Ang-(1-5) reflecting an upregulated conversion of Ang-(1-7) into Ang-(1-5) and, hence, activation of the alternative RAS. Importantly, our patient cohort did not exhibit

increased Ang-(1-5) concentrations as the metabolization of Ang-(1-7) to Ang-(1-5) occurs via ACE, which continued to be inhibited in this study.

Although RAS-blocking agents such as ACEi efficiently lower blood pressure and proteinuria,[32-34] CKD progression cannot be reversed or halted completely. This might partly be caused by the normalization of plasma Ang II after long-term ACEi therapy despite initial suppression, a phenomenon termed ACEi escape, constituting a significant therapeutic limitation. In this regard, a continued therapeutic suppression of Ang II and/or a pharmacological stimulation of the alternative RAS axis are of importance to ensure adequate patient care. Here, Ang II increased negligibly throughout the 3-month study period, while a significant increase of Ang-(1-7) production was observed. This was probably driven by increased availability of the neprilysin substrate Ang I because of renin upregulation and ACE inhibition together with ACEi-mediated blockade of Ang-(1-7) to Ang-(1-5) conversion. As ACE2 remained stable in all patient groups, a primary generation of Ang-(1-7) via neprilysin seems probable, in accordance with previous findings from kidney tissue analyses.[35] This, however, remains speculative as systemic neprilysin was not directly measured because of low assay reliability. In summary, a long-term beneficial modulation of the RAS appears feasible.

Several large-scale trials have recently demonstrated beneficial effects of SGLT-2i in patients without diabetes.[4,6,8] In our analysis, SGLT-2i effects on the RAS including Ang-(1-7) regulation were less pronounced in patients with CKD without diabetes. The reasons for this finding are not clear yet. While ACEi and SGLT-2i adherence were reflected by suppressed Ang II/Ang I ratios and glucosuria, no additional RAS activation was observed with empagliflozin in this patient group. It is known that an intrarenal RAS activation reflected by increased tissue Ang II occurs in patients with type 2 diabetes[36]; thus RAS stimulation via SGLT-2i and subsequent modulation towards the alternative RAS via ACEi might confer stronger effects on Ang regulation in these patients.

An interesting observation in the cohort of patients with diabetes was the effect of empagliflozin on proteinuria. While we intentionally aimed to include patients with proteinuria [one inclusion criterion was an albumin excretion rate of >30 mg/24 h (UACR >30 mg/g)] we only excluded patients with nephrotic-range proteinuria (exclusion criterion total urinary protein excretion ≥3.5 g/day). The study was double-blinded and randomized; therefore, the difference in proteinuria at baseline appears coincidental and possibly because of the small sample size. While proteinuria was lower in patients with diabetes at the start of the study, it decreased in all patients with diabetes during the study whereas no significant changes were detected in the group of patients without diabetes. To determine a potential influence of RAS modulation on proteinuria in our populations, we performed correlation analyses on the albumin/creatinine ratio and Ang metabolites. However, we did not find any significant associations between changes in UACR and RAS components in either patient cohort (data not shown). Considering the small sample size, pronounced conclusions must be avoided. This evokes speculation that proteinuria in patients with CKD and without diabetes (a) might not be influenced by SGLT-2i, and (b) does not respond in a timely manner to ACE inhibition.

No study drug-related severe adverse events were observed. The included patients exhibited significant cardiovascular comorbidities at inclusion as well as significant impairment of kidney function. Of note, cardiovascular and renal risk reductions have previously been shown to be highest in patients with lower eGFR.[37] In our study, patients with an eGFR as low as 15 ml/min/1.73m^2 were included and none exhibited ketoacidosis or worsening impairment of kidney function because of the study drug, thus constituting a safe therapeutic option also for patients with advanced CKD. No cases of hypoglycaemia occurred both in the diabetic and the non-diabetic group.

Some limitations warrant discussion: because of its exploratory design, the study was relatively small and the sample size calculation was carried out for the primary endpoint only; a larger sample size might have enabled the unveiling of additional subtle differences, including effects on smaller RAS peptides. In addition, Ang values are known to have a large biological variation, particularly in kidney disease populations[20,38] and this was detected and confirmed by the current results, reflecting the highly dynamic nature of the systemic RAS. However, we aimed at increasing the study's quality by showing patient-individual Ang levels, double-blinding and randomization. Further, we focused on RAS modulation with SGLT-2 inhibition of top of ACE inhibition, thus not analysing subjects without any RAS-modulating treatment. However, strengths of the study include its double-blind, parallel-group randomized design including both patients with and without type 2 diabetes and the MS-based molecular approach to the RAS quantification.

In conclusion, our study represents the first molecular analysis of the systemic RAS in patients with CKD, both with and without type 2 diabetes, receiving SGLT-2i on top of ACEi treatment. We detected a distinct modulation of RAS metabolites in patients with diabetes with a clear shift towards beneficial, potentially disease-modifying Ang. This provides a molecular background to this renoprotective therapeutic approach and might help design future multimodal therapy strategies.

ACKNOWLEDGMENTS

This work was supported by funds of the Oesterreichische Nationalbank (Austrian Central Bank, Anniversary Fund, project number: 16976) and by Boehringer Ingelheim.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

CK designed this study, performed data analyses, contributed to discussion and wrote and edited the manuscript. MA was involved in patient recruitment and care and wrote, reviewed and edited the manuscript. OD performed mass spectrometry analyses and reviewed and edited the manuscript. CCK and JJK contributed to discussion and reviewed and edited the manuscript. VR and MMM were involved in patient sample processing and helped with patient recruitment. ES and MH were

involved in patient recruitment and reviewed the manuscript. MP contributed to data analysis and discussion and reviewed and edited the manuscript. MDS contributed to discussion and reviewed and edited the manuscript. CK is the guarantor of this work and, as such, had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analyses.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/dom.14639.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

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How to cite this article: Antlanger M, Domenig O, Kaltenecker CC, et al. Combined sodium glucose co-transporter-2 inhibitor and angiotensin-converting enzyme inhibition upregulates the renin-angiotensin system in chronic kidney disease with type 2 diabetes: Results of a randomized, double-blind, placebo-controlled exploratory trial. *Diabetes Obes Metab*. 2022;24(5):816-826. doi:10.1111/dom.14639