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DISSERTATION

Effects of salt, telmisartan, linagliptin, and empagliflozin on the regulation of SARS CoV2 host factors in the kidney and heart of 5/6 nephrectomized rats

Auswirkungen von Salz, Telmisartan, Linagliptin und Empagliflozin auf die Regulierung von SARS CoV2-Wirtsfaktoren in Niere und Herz von 5/6 nephrektomierten Ratten

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ACE2	Angiotensin-converting enzyme 2;
ACR	Albumin-to-creatinine ratio;
Agt	Angiotensinogen
Agtr2	Angiotensin Receptor Type 2
ARB	Angiotensin II receptor blockers;
BNP45	Brain Natriuretic Peptide-45;
BW	Body weight;
CKD	Chronic kidney disease;
DPP-4	Dipeptidyl peptidase-4;
EDTA	Ethylenediaminetetraacetic acid;
ELISA	Enzyme-Linked Immunosorbent Assay;
HSD	High salt-diet;
ND	Normal diet;
PBS-T	Phosphate-buffered saline/Tween 20;
PBO	Placebo;
RAAS	Renin-angiotensin-aldosterone system;
SGLT2	Sodium-glucose Cotransporter-2;
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2;
SHR	Spontaneously hypertensive rats;
STNx	Subtotal nephrectomy
TMPRSS2	Transmembrane protease, serine-subtype-2.

Abstract

Introduction: The outbreak of the COVID-19 infection triggered many concerns regarding the intake of SGLT2-blockers empagliflozin, the RAAS-blocker telmisartan and the DPP4-inhibitor linagliptin among chronic kidney disease (CKD) patients while the safety of the listed medicine has been awaiting testification. At the same time, it is also unknown whether these three medicines increase the risk of SARS-CoV-2 infection. This research, hence, is intended to shed lights on the above issues. Previous clinical evidence has, by and large, proven that SARS-CoV-2 can infect humans through the angiotensin-converting enzyme 2 (ACE2) and transmembrane protease, serine-subtype 2 (TMPRSS2). Clinical and pre-clinical studies show that RAAS-blocking agents are safe in dealing with SARS-CoV-2 infection, but it remains unclear whether DPP-4 inhibitors or SGLT2-blockers may exacerbate the infection through adding the host viral entry enzymes ACE2 and TMPRSS2 in the presence of the virus.

Methods: We adopted 5/6 nephrectomized rats under high-salt-diet condition to model CKD patients. Those rats were then under the administration of telmisartan, linagliptin, and empagliflozin. By doing so, we examined the effects of the listed medicines on the renal and cardiac expression of ACE2, TMPRSS2, and key RAAS enzymes (REN, AGTR2, AGT) in a non-diabetic experimental 5/6 nephrectomy (5/6 Nx) model. To determine the gene expression of Ace2, Tmprss2, Ren, Agtr2, and Agt, we used qRT-PCR. In addition, the protein expression of ACE2 and TMPRSS2 was determined using immunohistochemistry in the following experimental groups: Sham + normal diet (ND) + placebo (PBO); 5/6Nx + ND + PBO; 5/6Nx + high salt-diet (HSD) + PBO; 5/6Nx + HSD + telmisartan (TELM); 5/6Nx + HSD + linagliptin (LINA); and 5/6N + HSD + empagliflozin (EMPA).

Results: Based on our observation, HSD decreased renal and cardiac ACE2 protein expression. ACE2 expression was not altered at the mRNA level in the kidney despite

the influence of disease and treatment. LINA lowered cardiac ACE2 mRNA levels. The mRNA levels of TMPRSS2 in kidneys were unaffected compared with the 5/6Nx + HSD + PBO group. Notably, TELM, LINA and EMPA did not alter renal TMPRSS2 mRNA levels compared with PBO-treated group but the cardiac TMPRSS2 mRNA levels were not detectable. Besides, the renal TMPRSS2 protein expression was not altered, and the elevated TMPRSS2 protein expression levels were lowered in heart under the administration of the listed three types of medicines. The renal ACE2 protein levels with LINA treatment witnessed a significant increase and did not induce other alterations in the TELM and EMPA treatments compared with PBO-treated group.

Conclusion: It is concluded that no upregulation of ACE2 and TMPRSS2 that facilitate SARS-CoV-2 virus entering into host cells was identified when the high-salt diet, SGLT2-blockers empagliflozin and the RAAS-blocker telmisartan are used. The DPP4-inhibitor linagliptin may potentially enhance the risk of renal SARS-CoV-2 infection. Also, an ongoing clinical trial is required to confirm the preliminary findings from a preclinical experimental model of non-diabetic kidney failure.

Zusammenfassung

Einleitung: Der Ausbruch der COVID-19-Infektion löste viele Bedenken hinsichtlich der Einnahme des SGLT2-Blockers Empagliflozin, des RAAS-Blockers Telmisartan und des DPP4-Hemmers Linagliptin bei Patienten mit chronischer Nierenerkrankung (CKD) aus, während die Sicherheit der aufgeführten Arzneimittel noch nicht geprüft wurde. Gleichzeitig ist es auch nicht bekannt, ob diese drei Arzneimittel das Risiko einer SARS-CoV-2-Infektion erhöhen. Die Untersuchung soll die oben genannten Fragen klarstellen. Frühere klinische Nachweise haben gezeigt, dass SARS-CoV-2 den Menschen über das Angiotensin-konvertierende Enzym 2 (ACE2) und die Transmembranprotease, Serin-Subtyp 2 (TMPRSS2) infizieren kann. Klinische und präklinische Studien zeigen, dass RAAS-Blocker sicher bei der Behandlung von SARS-CoV-2-Infektionen sind, aber es ist noch nicht klar, ob DPP-4-Hemmer oder SGLT2-Blocker die Infektion verstärken können, durch Zugabe der viralen Wirtseingangsenzyme ACE2 und TMPRSS2 in Gegenwart des Virus.

Methoden: Wir haben 5/6 nephrektomierte Ratten mit einer salzreichen Diät als Modell für CKD-Patienten verwendet. Diese Ratten wurden dann mit Telmisartan, Linagliptin und Empagliflozin behandelt. Auf diese Weise untersuchten wir die Auswirkungen der aufgeführten Medikamente auf die renale und kardiale Expression von ACE2, TMPRSS2 und wichtigen RAAS-Enzymen (REN, AGTR2, AGT) in einem nicht-diabetischen experimentellen 5/6-Nephrektomie-Modell (5/6 Nx). Um die Genexpression von Ace2, Tmprss2, Ren, Agtr2 und Agt festzustellen, verwendeten wir qRT-PCR. Darüber hinaus wurde die Proteinexpression von ACE2 und TMPRSS2 in den folgenden Versuchsgruppen mittels Immunhistochemie festgestellt: Sham + normale Diät (ND) + Placebo (PBO); 5/6Nx + ND + PBO; 5/6Nx + salzreiche Diät (HSD) + PBO; 5/6Nx + HSD + Telmisartan (TELM); 5/6Nx + HSD + Linagliptin (LINA); und 5/6N + HSD + Empagliflozin (EMPA).

Ergebnisse: Auf Grundlage unserer Beobachtungen verringerte HSD die renale und kardiale ACE2-Proteinexpression. Die ACE2-Expression auf mRNA-Ebene wurde in

der Niere trotz des Einflusses von Krankheit und Behandlung nicht verändert. LINA senkte die mRNA-Levels von kardialem ACE2. Die mRNA-Levels von TMPRSS2 in den Nieren blieben im Vergleich zur 5/6Nx + HSD + PBO-Gruppe unbeeinflusst. Bemerkenswerterweise veränderten TELM, LINA und EMPA die renalen TMPRSS2-mRNA-Levels im Vergleich zur mit PBO behandelten Gruppe nicht, jedoch waren die kardialen TMPRSS2-mRNA-Levels nicht nachweisbar. Darüber hinaus wurde die renale TMPRSS2-Proteinexpression nicht verändert, und die erhöhten TMPRSS2-Proteinexpressionen wurden im Herzen unter der Verabreichung der genannten drei Arten von Medikamenten gesenkt. Die renalen ACE2-Proteinlevels zeigten mit LINA-Behandlung einen signifikanten Anstieg und induzierten im Vergleich zur mit PBO behandelten Gruppe keine anderen Veränderungen in den TELM- und EMPA-Behandlungen.

Fazit: Es wird festgestellt, dass keine Hochregulierung von ACE2 und TMPRSS2 identifiziert wurde, die das Eindringen des SARS-CoV-2-Virus in Wirtszellen erleichtern, wenn eine salzreiche Diät, SGLT2-Blocker wie Empagliflozin und der RAAS-Blocker Telmisartan verwendet werden. Der DPP4-Inhibitor Linagliptin könnte potenziell das Risiko einer renalen SARS-CoV-2-Infektion erhöhen. Eine laufende klinische Studie ist ebenfalls erforderlich, um die vorläufigen Ergebnisse aus einem präklinischen experimentellen Modell für nicht-diabetisches Nierenversagen zu bestätigen.

1. Introduction

Covid-19 poses extra requirements on the treatment of cardiac and renal diseases, which are perceived to be risk factors exacerbating the severity as well as the adverse outcomes of infection, resulting in a higher mortality rate among CKD patients. For those suffering from diabetes, it is vital to set strict regulations for glucose control so to prevent diabetic complications during the Covid-19 pandemic. At the same time, this will reduce their susceptibility to the virus as well as alleviating the severity of COVID-19 if they are infected. For those with type 2 diabetes and COVID-19, medicines that block the renin-angiotensin-aldosterone system (RAAS) or dipeptidyl peptidase 4 (DPP4) have been proven safe according to recent researches [1-3]. Besides, sodium-glucose cotransporter 2 (SGLT2) blockers are likely to be an effective adjunct therapy for patients with type 2 diabetes mellitus (T2DM) and SARS-CoV2 infection, in spite of the rising risk of diabetic ketoacidosis along with the extended ketonemia[4].

Specifically, the angiotensin-converting enzyme 2 (ACE2), being a part of the renin-angiotensin-system (RAS), is the key to the management of RAAS and regulates pathological processes, encompassing cardiac dysfunction, diabetes and hypertension[5]. ACE2 deploys its protective function through targeting Ang-II as well as converting it to Ang 1–7, thus regulating several pathological processes like inflammation, fibrosis and vasodilatation. ACE2 is found to be the primary receptor for coronaviruses, involving SARS-CoV and SARS-CoV-2. Once connected to ACE2 via the receptor-binding domain in the viral spike protein, it requires priming by a type of proteases, Transmembrane protease serine 2 (TMPRSS2), which can cleave the coronavirus spike protein and improve the cell membrane fusion for viral entry[6, 7]. ACE2- and TMPRSS2-dependent could be used as an alternative approach for viral entry which is achieved through the direct fusion of the viral envelope together with the cell membrane [8]. The enhanced ACE2 expression was noted in response to

acute lung problems, including fibrosis and inflammation as well as heart failure [9-12] resulting in an elevated AngII level and easier viral entry. By contrast, AngII gives rise to the internalization of ACE2 along with the following degradation into lysosomes through an AT1R-dependent mechanism[13]. In addition, The MERS-CoV infection pathway involves the binding of the virus and human DPP4/CD26[14], and a recent study predicted the structure of SARS-CoV-2 spike glycoprotein and the glycan shield pattern suggests that DPP4/CD26 may be a SARS-CoV-2 receptor[15], which needs further investigation. The prolonged existence of ACE2 or DPP4 or TMPRSS2 in infected patients may contribute to the exacerbation of their disease.

The effects of RAAS blocking medications on cardiac and renal ACE2 mRNA and protein expression in experimental preclinical models have produced contradictory results. Cardiac ACE2 mRNA expression was enhanced in normotensive rats after being medicated with ACE inhibitor (ACEi) lisinopril or angiotensin receptor blocker (ARB) losartan[16]. In contrast, there is no observable growth on cardiac ACE2 mRNA levels after coronary artery ligation and treatment with ARB valsartan, ACEi ramipril alone or in combination compared to control[17]. In kidneys, the administration of ARB telmisartan led to a significant increase in renal ACE2 mRNA expression in comparison to vehicle-treated mice[18]. There is a lack of verification from previous studies that renal mRNA expression of ACE2 and TMPRSS2 after telmisartan or ramipril treatment keeps stable [19]. ACE2 protein in kidney membranes was shown to be decreased by captopril and telmisartan, according to a recent study, without having a significant effect on the amount of protein present in total renal lysates. Captopril, in particular, remarkably decreased ACE2 protein in kidney membranes while raising ACE2 levels in cytosolic[20]. Notably, a previous study discovered that mice with comorbid diabetes (old age, high fat diet, and streptozotocin-induced diabetes) have encouraged renal Ace2 mRNA expression; however, this effect is not exacerbated by telmisartan treatment. As a result, it is reasonable to conclude that the improved ACE2 level is caused by the

comorbidity rather than being the impact of RAAS blockade [19].

Dietary consumption of salt is a widely acknowledged risk factor contributing to cardiovascular as well as renal derangement in hypertension and is linked to RAAS imbalance. Spontaneously hypertensive rats (SHR) fed by high-salt diet presented marginally reduced cardiac Ace2 mRNA and protein expression[21]. At the same time, renal expression was significantly down-regulated in uni-nephrectomized and high-salt-diet-fed rats [22]. However, the effectiveness of RAAS blocking medicines in a salt-induced experimental model have yet to be determined.

Considering that certain groups of patients with chronic kidney disease, who take in excessive amounts salt from diets and normally prescribed RAAS blocking medications and DPP4-inhibitors and SGLT2 blockers, are faced with the threat of severe COVID-19 outcomes, we examined the expression profiles of ACE2 and TMPRSS2 as well as other genes included in the RAAS in the kidney and the heart in a rat model that resembles this phenotype (weakened kidney function together with exceeding intake of salt – the majority of patients, sadly, have a much higher salt consumption than currently recommended, which is contributing to hypertension). In this study, we utilized the rat 5/6 nephrectomy model, one of the most well-established experimental non-diabetic CKD models featured by elevated hypertension, inflammation, and fibrosis.

2. Objective

As demonstrated above, the rising occurrence of ACE2 or DPP4 or TMPRSS2 in infected patients may contribute to the severity of their COVID as they are host factors which are vital to virus entry. These host factors can be modulated by both the underlying comorbidities (hypertension, kidney disease) of the patient and the treatment of comorbidities (RAS-blockers, SLGT2-inhibitors, DPP4-inhibitors). As it is virtually impossible to untangle both effects in a human study, because it would be unethical to withhold treatment to patients, we chose an experimental approach. Our objective was to compare the effects on the mentioned above host factors exerted by kidney disease and hypertension independent from those exerted by widely used treatments like RAS-, DPP4-, or SGLT2-inhibition. Thus, we decided to use a well-established animal model for those comorbidities (the 5/6 nephrectomized rat aggravated by high-salt diet) with and without those treatment options in order to answer the following questions:

1.Is there a different expression of the crucial COVID host factors ACE2, TMPRSS2, and key RAAS enzymes (REN, AGTR2, AGT) due to 5/6 Nx and high-salt induced kidney disease and hypertension?

2.Is there a different expression of the crucial COVID host factors ACE2, TMPRSS2, and key RAAS enzymes (REN, AGTR2, AGT) due to RAS-, DPP4-, or SGLT2-inhibition?

3.Is there an organ-specific effect (f.e. heart versus kidney) on the host factor expression due to disease or treatment?

3. Materials and methods

3.1 Animals

The animal experiment was approved by the laboratory animal ethics committee (20170904092822, Jinan University, Guangzhou, China). The experiment was conducted in accordance with the University Guidelines for the Use of Laboratory Animals, and successfully carried out at the Laboratory Animal Center of Jinan University, Guangzhou, China. The qRT-PCR and immunohistochemistry were carried out at the institute of pharmacy at Freie Universität Berlin. 91 male Wistar rats were randomly assigned to one of the following groups:

Group 1: Sham + ND + PBO (n=14);

Group 2: 5/6 Nx + ND + PBO (n=12);

Group 3: 5/6 Nx + HSD+ PBO (n=23);

Group 4: 5/6 Nx + HSD + telmisartan (5 mg/kg/day; n=15);

Group 5: 5/6 Nx + HSD + linagliptin (3 mg/kg/day; n=14);

Group 6: 5/6 Nx + HSD + empagliflozin (1.2 mg/kg/day; n=13).

The normal diet was standardized with AIN93M[23] and the high-salt diet was adjusted to contain 2% sodium chloride on this basis. The two feeds were produced according to the codes LAD 3001M and LAD0011HF2 (Trophic Animal Feed High-tech Co., Ltd, China). The doses of telmisartan and linagliptin have been used in previous studies[24, 25]. From week 3 until the sacrifice (week 11), drug treatment was administered through gavage. The rats were sacrificed at week 11 and anesthesia was administered intraperitoneally using pentobarbital sodium (36-39 mg/kg body weight). Urine and perfused kidney and heart samples were collected and frozen for further analysis (Figure 1).

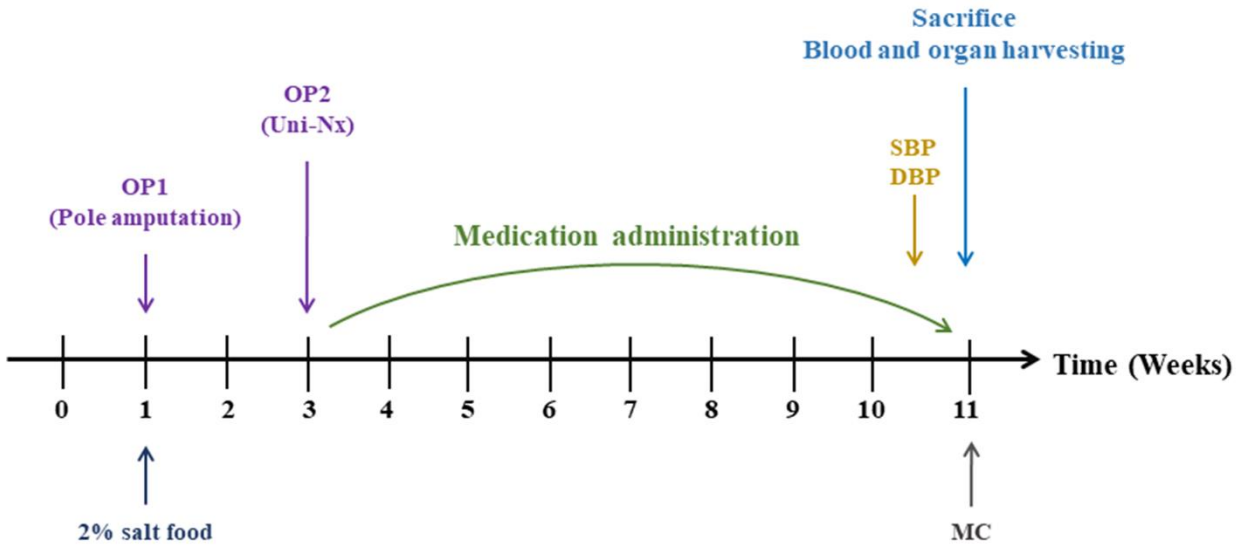

Figure 1. Time course of the animal study.

SBP=systolic blood pressure measurement; DBP=diastolic blood pressure measurement; MC=metabolic cages; OP1=amputation of the poles of the left kidney; OP2=uninephrectomy on the right side; Uni-Nx=unilaterally nephrectomized. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

3.2 5/6 nephrectomized rats model

The procedure 5/6Nx was carried out to simulate renal insufficiency. Surgical procedures 5/6Nx included amputation of the poles of one kidney and uninephrectomy (Uni-nx) of the other kidney. The first operation was performed on the rats in groups 2, 3, 4, 5 and 6 during the first week of the animal study. The second operation was carried out two weeks later, during the third week of the animal study. (Figure 1).

The following was the procedure for the first operation, which was an amputation of the poles of the left kidney: To begin the operation, the animals were sedated with pentobarbital sodium (36-39 mg/kg body weight) intraperitoneally before the procedure started. Once the animals had been anesthetized, they were removed from the cage and the left flank of the rat was shaved with a skin preparation knife and

moistened with a disinfectant before being placed back in the cage. The procedure was carried out while the rat was in a prone position. The kidney was exposed through a flank incision, and the renal pedicle was temporarily clamped while about two-thirds of the cortex was removed. The hemostatic sponge (Guangzhou Kuai Kang Medical Instruments Co., Ltd., Guangzhou, CHN) comprises biologic absorptive collagen and treats hemostasis. Afterward, once the bleeding from the cut surfaces had stopped, the kidney was reinserted into its proper position within the abdominal cavity and the wound closed.

The second operation, Uni-Nx of the right kidney, was performed at week 3. The anesthesia was administered in the same method for the first operation. The right flank of the rat was then shaved and moistened with a disinfectant, and the procedure was repeated. A skin incision was made in the right flank area, and the muscles were incised. In this procedure, the right kidney's afferent and efferent blood vessels and the urethra were ligated, and then the right kidney was removed entirely. After that, the muscles and skin were sutured back.

At the same time, sham operations were performed on the rats in Group 1. The same procedures as for amputation of the poles of the left kidney and Uni-Nx of the right kidney were used during sham surgery, but no resections were performed. The kidney was only gently manipulated on each occasion before the incision was closed and recovery began.

Following the operations, each rat was monitored until it awoke. To warm the animals, the cages were illuminated from a distance with red light. The animals were checked several times a day (every 2 hours) on the day of the operation, and then the muscles and skin were sutured. An intramuscular injection of penicillin G 20,000U was administered following each operation to prevent infection.

3.3 Blood pressure measurement

The animals’ blood pressure (BP) and pulse were measured using non-invasive

tail-cuff plethysmography of the tail artery at week 11 of the study's duration (Figure 1). Initially, the animal was restrained in a restrainer, which was a tubular construction with only the tail of the rat protruding from it. Then, similar to how blood pressure is measured on the human arm, a blood pressure cuff and an electronic transducer were attached to the animal’s tail. The results were recorded. We waited until the animals were comfortable and relaxed with the restrainer and had become accustomed to it. At least three measurements were taken at intervals of 30 seconds to obtain reliable means of heart rate and blood pressure. To get the animals used to this procedure, they were trained before the actual measurement was performed. The tail-cuff plethysmography blood pressure systems from IITC Life Science were used to record and evaluate the blood pressure diagrams and pulses.

3.4 High salt diet and medication administration

Except for groups 1 and 2, the feeding of the high salt diet (2%) began after operation one and was continued throughout the study until the animals were sacrificed (Figure 1).

Telmisartan (5 mg/kg/day; qd), linagliptin (3 mg/kg/day; qd), 5/6 Nx + HSD + empagliflozin (1.2 mg/kg/day; bid) were gavage-administered to rats in groups 3, 4, 5 and 6 from weeks 3 to weeks 11.

3.5 Urine collection

The animals were placed in metabolic cages for 24 hours at week 11 of the study to collect urine. The values obtained during these experiments were extrapolated to obtain values for 24 hours. The animals had unrestricted access to chow and water. The samples were frozen and stored at -80°C for subsequent analysis, including urinary creatinine, urinary albumin and protein measurements.

3.6 Animal sacrifice

The rats were sacrificed at week 11 (Figure 1). 1% pentobarbital sodium (36-39 mg/kg body weight) was administered intraperitoneally to anesthetize the rats. Once the rats had been anesthetized, they were removed from the room and their thoracic and abdominal cavities were opened. The blood samples were drawn from the abdominal aorta into heparinized tubes and centrifuged for 20 minutes at 4500 revolutions per minute (rpm). The plasma samples were removed from the abdominal aorta using a micropipette and transferred into new microcentrifuge tubes. Afterward, the plasma samples were frozen and stored at -80°C in preparation for further analysis. The renal veins were severed after the blood samples were eliminated, and a large amount of saline solution was injected through the left ventricle until the kidney became pale. The kidneys were then removed from the body and weighed. To conduct histological analysis, the kidneys were cut lengthwise and any half of the kidney was processed further. The other half of the kidney was stored at -80°C for later analysis.

3.7 Biochemical assays

Plasma creatinine, urea, glucose and insulin levels, urinary creatinine and total protein were determined using an automatic biochemical analysis system (Roche Cobas 6800, Roche Ltd, Switzerland). Plasma BNP45 and urinary albumin levels were quantified using the Rat BNP 45 ELISA kit (Abcam, Cat#ab108816) and the Rat Albumin ELISA kit (Abcam, Cat#ab235642). Glomerular filtration rate to body weight ratio (GFR) and albumin to creatinine ratio (ACR) were calculated. At a 1 mg/day dose, empagliflozin does not affect urinary sodium and potassium excretion[27].

3.8 RNA isolation and quantitative real-time PCR (qRT-PCR)

Snap frozen kidney and heart tissues were homogenized with Precellys lysis with Precellys Steel 2.8 mm beads (PeqLab Biotechnology, Erlangen, Germany). Total

RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany). 500 µl of the provided ready-to-use lysis buffer were added to 10 mg of each -80°C-frozen powdered tissue sample, and the powdered tissues were lysed using ultrasound sonication three times at five cycles for 10 seconds. The lysate was then centrifuged for 3 minutes at full speed. The supernatant was carefully removed by pipetting and transferred into a new microcentrifuge tube. 350 µl of 70% ethanol were added to the cleared lysate, supernatant, and mixed by pipetting. The samples, including any precipitates that may have been formed, were then transferred to RNeasy spin columns placed in 2 ml collection tubes. The lids were closed gently and the assemblies of spin columns and collection tubes were centrifuged for 15 seconds at 10000 rpm and the flow-through was discarded. Then, 700 µl of the first washing buffer was added to each RNeasy spin column and the lids were closed and the assemblies were centrifuged for 15 seconds at 10000 rpm and the flow-through was discarded. The last washing step was repeated twice using 500 µl of the second washing buffer. These washing steps were carried out to thoroughly wash the spin column membrane and the adsorbed RNA. The collection tubes with the flow-through were discarded, the RNeasy spin columns were then placed in new 2 ml collection tubes, the lids were gently closed and the assemblies were centrifuged for one minute at full speed to eliminate any possible carryover of the washing buffer. Then, the RNeasy spin columns were placed in new 1.5 ml collection tubes and 50 µl of RNase-free water was placed directly on the spin column membrane. The lids were gently closed and the assemblies were centrifuged for one minute at 10000 rpm to elute RNA. Quality control and total RNA yield were quantified using the NanoDrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, United States, DE).

Angiotensin I Converting Enzyme 2 (Ace2; Rn01416293_m1), Transmembrane Protease Serine Subtype 2 (Tmprss2; Rn00590459_m1), Renin (Ren; Rn02586313_m1), Angiotensin Receptor Type 2 (Agtr2; Rn00560677_s1), and Angiotensinogen (Agt; Rn00593114_m1) mRNA levels in the kidney and heart were

measured using an SDS7900HT real-time PCR system (Applied Biosystems by ThermoFisher Scientific). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (Gapdh; Rn01775763_g1) was used, and the experimental details were previously described[28]. All samples were run in duplicates and raw ct values were calculated using the SDS software version 2.4. All values were normalized to the mean expression level of the control group (Sham + ND + PBO) and the fold-change of expression compared to the control was calculated using the comparative Ct method (2-ΔΔct)[29].

3.9 Immunohistochemistry

3.9.1 Tissue processing and embedding

In order to get tissue samples ready for histological examination, they were first fixed for 24 hours in acid-free, phosphate-buffered Roti®-Histofix solutions (PH7) from Carl Roth (Karlsruhe, Germany). These solutions contained 4 % formaldehyde and were free of phosphate. Once the samples had been fixed in formalin, they were dehydrated in ethanol at various concentrations according to the following protocol: 24 hours in 70% ethanol, one hour in 96% ethanol, and three successive one-hour periods in 100% ethanol were required for this experiment. Then, samples were cleared in Roticlear® from Carl Roth (Karlsruhe, Germany).

The samples were then embedded in Richard-Allan Scientific ™ paraffin from ThermoFisher Scientific (Massachusetts, USA) for 4 hours to produce paraffin blocks. Tissues were first immersed in pure molten Richard-Allan Scientific ™ paraffin type 6 from ThermoFisher Scientific (Massachusetts, USA) at 56 °C for two hours before being transferred to a second Richard-Allan Scientific ™ paraffin type 9 bath from ThermoFisher Scientific (Massachusetts, USA) bath for another 2 hours. These procedures were carried out automatically in the Shandon Citadel 1000 automatic tissue processor for an entire night (Thermo Scientific, Germany). The histocassettes were then embedded in the Microm EC-350 modular paraffin embedding center from

ThermoFisher Scientific (Massachusetts, USA), with paraffin wax surrounding the tissues and providing sufficient support for section cutting when cooled and solidified.

3.9.2 Preparation of the tissue sections

The paraffin blocks were cut into 3 µm thick sections with a Jung RM 2025 microtome from Leica Biosystems (Wetzlar, Germany). Tissue sections were then placed in a water bath and transferred on glass slides from Carl Roth (Karlsruhe, Germany). The slides were placed in a warming cabinet for 30 minutes to dry before being stored in a slide box.

3.9.3 Staining

The sections were deparaffinized twice by immersing the slides in xylol (Carl Roth, Karlsruhe, Germany) two times for five minutes. Then the sections were rehydrated by immersing the slides in graded ethanol as follows: 100% ethanol for two minutes, 96% ethanol for two minutes, 80% ethanol for two minutes and 70% ethanol for two minutes. After antigen-retrieval in microwave, sections were blocked with 5% non-fat dry milk in phosphate-buffered saline/Tween 20 (PBS-T) for one hour and incubated respectively with primary antibodies specific to ACE2 (1:100 dilution; ab15348, Abcam, Cambridge, MA) and TMPRSS2 (1:50 dilution; EPR3861, ab92323, Abcam, Cambridge, MA) in 5% non-fat dry milk in PBS-T overnight at 4°C. The sections were then repeatedly washed five times with PBS-T, incubated with matching fluorescent secondary antibody (1:200, ab150075; Abcam) in PBS-T for one hour, and mounted with DAPI containing Fluoroshield mounting medium (ab104139; Abcam) to counterstain the nuclei.

The fluorescent images at 20x magnification were captured using a BZ-9000 compact fluorescence microscope from Keyence (Osaka, Japan) and analyzed using ImageJ software from the National Institutes of Health (Maryland, USA). The total corrected cellular fluorescence (TCCF) = media of integrated density - media of

measurements selected areas * mean fluorescence of background readings was calculated. TCCF was then equalized against the mean TCCF of the neighboring area in the same field of view for each section. Averages of normalized intensity values of around 20 tissue areas were calculated.

3.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad, La Jolla, CA). Normally distributed data were compared using analysis of variance and Bonferroni post hoc test, and data are presented as mean ± SEM. For non-normally distributed data, the Kruskal-Wallis test and Dunn's post hoc test were used. Data were presented as median (25th - 75th percentile). In all cases, differences were considered statistically significant if $P < 0.05$.

4. Results

4.1 Effect of HSD

4.1.1 Clinical and biochemical parameters

The body weights of the animals were recorded at week 11 before sacrifice. After 5/6 nephrectomy, the levels of systolic and diastolic blood pressure, urinary ACR, 24h urinary protein excretion, plasma urea, plasma creatinine and plasma glucose were significantly increased. High-salt-diet remarkably increased the relative heart weight and urinary ACR, and it also noticeably decreased plasma glucose compared to 5/6Nx + ND + PBO rats (Table 1).

Table 1. Effect of HSD on body and organ weight, blood pressure, plasma and urinary parameters

	Sham + ND + PBO (n=12-13)	5/6Nx + ND + PBO (n=12-13)	5/6Nx + HSD + PBO (n=15-23)
Final body weight (g)	475.35±12.04	448.50±17.38	443.35±10.65
Relative left kidney weight (mg/g)	3.20(2.93-3.46)	3.44(2.93-3.46)	3.64(3.31-4.35)
Relative heart weight (mg/g)	2.74±0.06	3.03±0.08	3.74±0.21*
Systolic blood pressure (mm Hg)	124.66(118.33-130.50)*	153.66(118.33-130.50)	153.00(149.00-163.66)
Diastolic blood pressure (mm Hg)	101.56 ±2.43*	122.77±3.68	123.61±2,21
Urinary ACR (mg/mmol)	1.51(1.27-2.27)*	6.89(5.51-15.82)	38.49(11.10-282.90)*
24h urinary protein excretion (mg/24h)	4.81(4.23-5.79)*	7.17(6.35-10.47)	11.63(7.48-36.08)
Plasma urea (mmol/l)	4.78(4.36-5.67)*	11.21(8.61-13.24)	6.90(5.91-12.57)
Plasma creatinine (μmol/l)	46.92 ± 0.76*	72.38 ± 2.27	75.67 ± 2.00
Plasma glucose (mmol)	6.48±0.34*	8.58±0.59	6.64±0.33*
Plasma BNP45 (ng/ml)	2.23±0.49	1.92±0.30	2.13±0.32

Normally distributed data were given as mean ± SEM. Non-normally distributed data were presented as median (25th–75th percentile).

*p <0.05 vs. 5/6Nx+ND+PBO. This table is adopted from our published paper (Xiong Y, et al./2022)[26].

4.1.2 Effect of HSD on mRNA expression of genes associated with SARS-CoV-2 host factors and RAAS

qRT-PCR was used to examine the impacts of high-salt diet on the gene expression levels of the two critical SARS-CoV-2 host factors Ace2 and Tmprss2, as well as genes incorporated in the RAAS, such as Ren, Agtr2, and Agt, in the kidney and heart. Ace2 expression was unaffected in all experimental groups in both the kidney and heart. Compared to Sham + ND + PBO rats, 5/6Nx remarkably decreased the renal Tmprss2 and Ren gene expression, and high-salt diet only decreased cardiac Agt mRNA levels. Cardiac Tmprss2 and Ren mRNA expression was lower than the detection limit.

Table 2. Effect of HSD on mRNA expression of SARS-CoV-2 host factors and genes involved in RAAS

	Sham + ND + PBO (n=6)	5/6Nx + ND + PBO (n=6)	5/6Nx + HSD + PBO (n=6)
Renal mRNA expression			
Ace2	1.02±0.09	1.19±0.30	1.01±0.33
Tmprss2	1.03±0.12*	0.50±0.09	0.86±0.09
Ren	1.06±0.18*	0.06±0.02	0.06±0.04
Agtr2	1.03±0.13	0.78±0.21	1.08±0.19
Agt	1.10±0.24	1.13±0.28	1.02±0.24
Cardiac mRNA expression			
Ace2	1.04±0.19	0.94±0.06	1.17±0.08
Tmprss2	–	–	–
Ren	–	–	–
Agtr2	1.01±0.06	1.06±0.07	1.13±0.09
Agt	1.03(0.80-1.24)	1.66(1.33-2.29)	0.94(0.86-1.05)*

Normally distributed data were given as mean ± SEM. Non-normally distributed data were presented as median (25th–75th percentile). *p <0.05 vs. 5/6Nx+ND+PBO. This table is adopted from our published paper (Xiong Y, et al./2022)[26].

4.1.3 Effect of HSD on renal and cardiac protein expression of SARS-CoV-2 host factors

The next stage involved using polyclonal ACE2 and TMPRSS2 antibodies to examine the protein expression of ACE2 and TMPRSS2 in the kidney and heart, as has been previously described[19]. Compared to Sham + ND + PBO rats, 5/6Nx only decreased cardiac TMPRSS2 protein expression. The levels of renal and cardiac ACE2 protein expression in PBO-treated and high-salt-diet-fed (5/6Nx + HSD + PBO) group were notably weakened (Table 3, Figure 12). High-salt diet led only to a numerical, non-significant decrease in renal and cardiac TMPRSS2 protein levels in the high-salt-diet-fed 5/6Nx rats (Table 3).

Table 3. Effect of HSD on renal and cardiac protein expression of SARS-CoV-2 host factors

	Sham + ND + PBO (n=12-13)	5/6Nx + ND + PBO (n=12-13)	5/6Nx + HSD + PBO (n=15-23)
Renal protein expression			
ACE2	29.89 (20.82-36.55)	27.10 (19.39-30.73)	13.96 (12.36-19.14)*
TMPRSS2	13.44 (11.64-17.31)	13.88 (11.89-15.73)	12.77 (8.53-14.21)
Cardiac protein expression			
ACE2	27.77 (25.58-32.96)	33.11 (30.74-36.34)	29.12 (23.97-32.42)*
TMPRSS2	11.90 (10.63-15.00)*	29.57 (25.12-33.16)	25.23 (16.18-30.46)

Normally distributed data were given as mean ± SEM. Non-normally distributed data were presented as median (25th–75th percentile). *p <0.05 vs. 5/6Nx+ND+PBO. This table is adopted from our published paper (Xiong Y, et al./2022)[26].

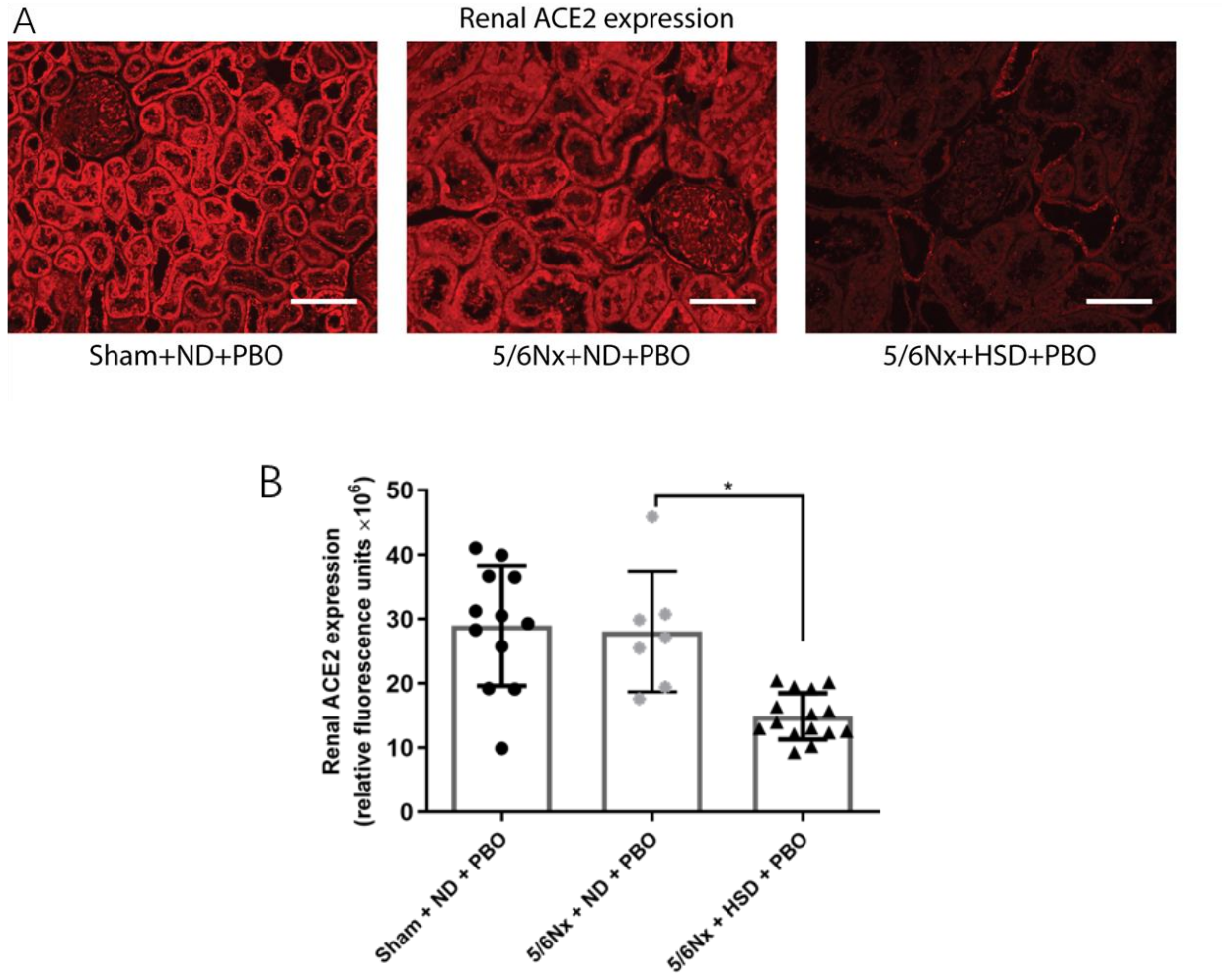

Figure 2. Effect of HSD on renal ACE2 protein expression in different groups

A. Photomicrographs of immunofluorescence-stained kidneys. The red color indicates ACE2. B. Renal protein expression of ACE2. Magnification ×20 (scale bars = 100 μm). **p* <0.05 vs. 5/6Nx+ND+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26]

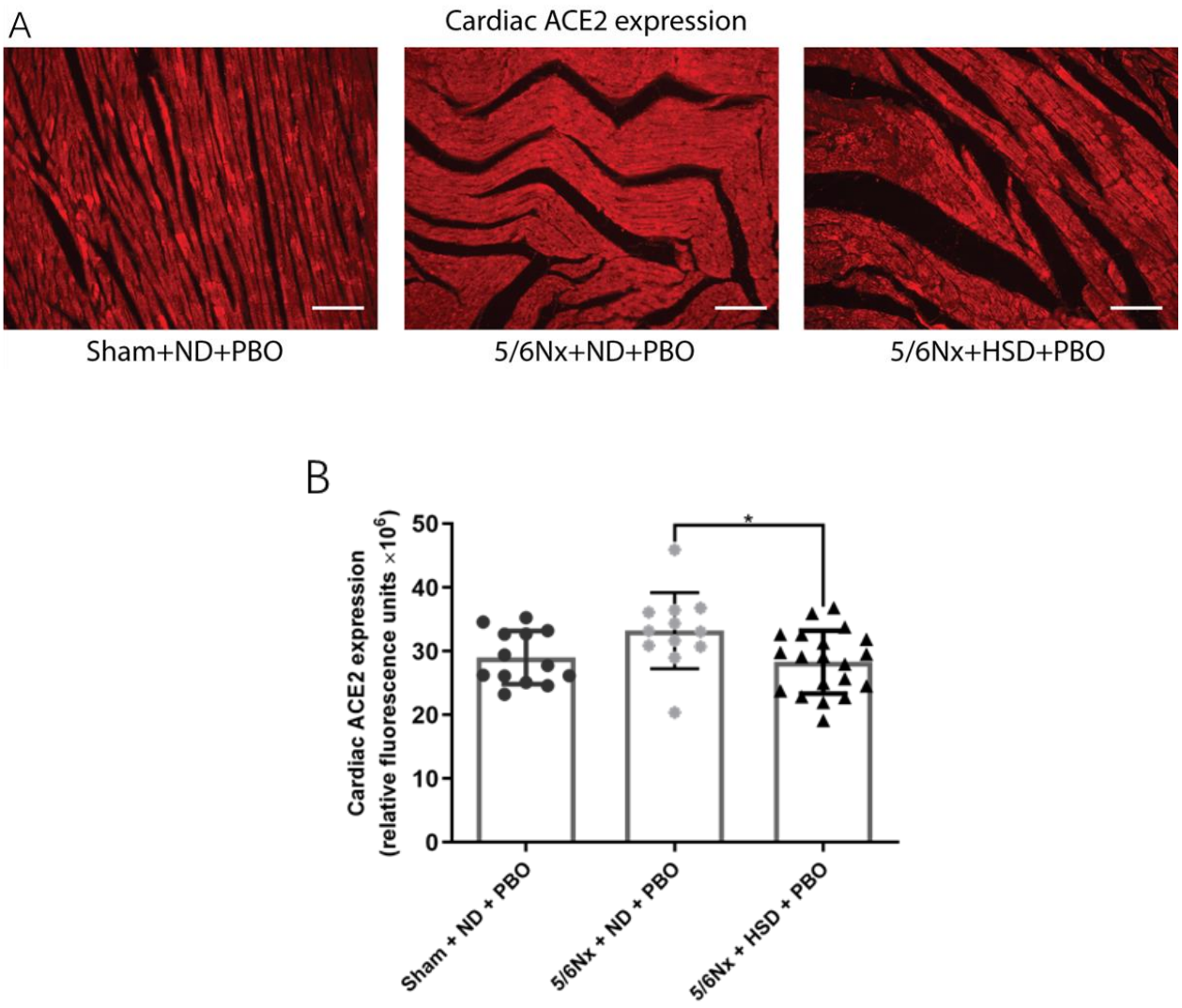

Figure 3. Effect of HSD on cardiac ACE2 protein expression in different groups

A. Photomicrographs of immunofluorescence-stained kidneys. The red color indicates ACE2. B. Renal protein expression of ACE2. Magnification ×20 (scale bars = 100 μm). **p* <0.05 vs. 5/6Nx+ND+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26]

4.2 Effect of treatments with TELM, LINA, EMPA

4.2.1 Effect of treatments on body and organ weight

The body weights of the animals were recorded at week 11 before sacrifice. In high-salt-diet-fed 5/6 Nx rats, treatment with telmisartan (5/6 Nx + HSD + TELM) and linagliptin (5/6 Nx + HSD + LINA) showed significant ($p<0.05$) lighter body weights in comparison to treated with PBO. Relative left kidney weight and Relative heart weight were unaffected among all the treatment groups. (Table 4).

Table 4. Effect of treatments on body and organ weight

	Sham + ND + PBO(n=12-13)	5/6Nx + HSD + PBO (n=15-23)	5/6Nx + HSD + TELM (n=11-15)	5/6Nx + HSD + LINA (n=13-15)	5/6Nx + HSD + EMPA (n=10-11)
Body weight (g)	475.35±12.04	443.35±10.65	394.65±12.51#	382.39±12.44#	420.03±11.68
Relative left kidney weight (mg/g)	3.20(2.93-3.46) #	3.64(3.31-4.35)	3.56(3.15-4.26)	3.72(3.41-4.13)	3.94(3.49-4.46)
Relative heart weight (mg/g)	2.74±0.06#	3.74±0.21	3.97±0.33	3.77±0.23	3.58±0.13

Normally distributed data were given as mean ± SEM. Non-normally distributed data were presented as median (25th–75th percentile). #*p* <0.05 vs. 5/6Nx+HSD+PBO. This table is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.2 Effect of treatments on systolic and diastolic blood pressures

5/6Nx and HSD caused a considerable ($p<0.05$) surge in systolic and diastolic blood pressures in 5/6 Nx + HSD + PBO rats compared to Sham + ND + PBO. In high-salt-diet-fed 5/6 Nx rats, treatment with telmisartan (5/6 Nx + HSD + TELM) and empagliflozin (5/6 Nx + HSD + EMPA) noticeably lowered the systolic and diastolic blood pressures, treatment with linagliptin (5/6 Nx + HSD + LINA) only reduced the systolic blood pressures. It did not affect significantly diastolic blood pressures versus 5/6 Nx + HSD + PBO rats (Figure 4).

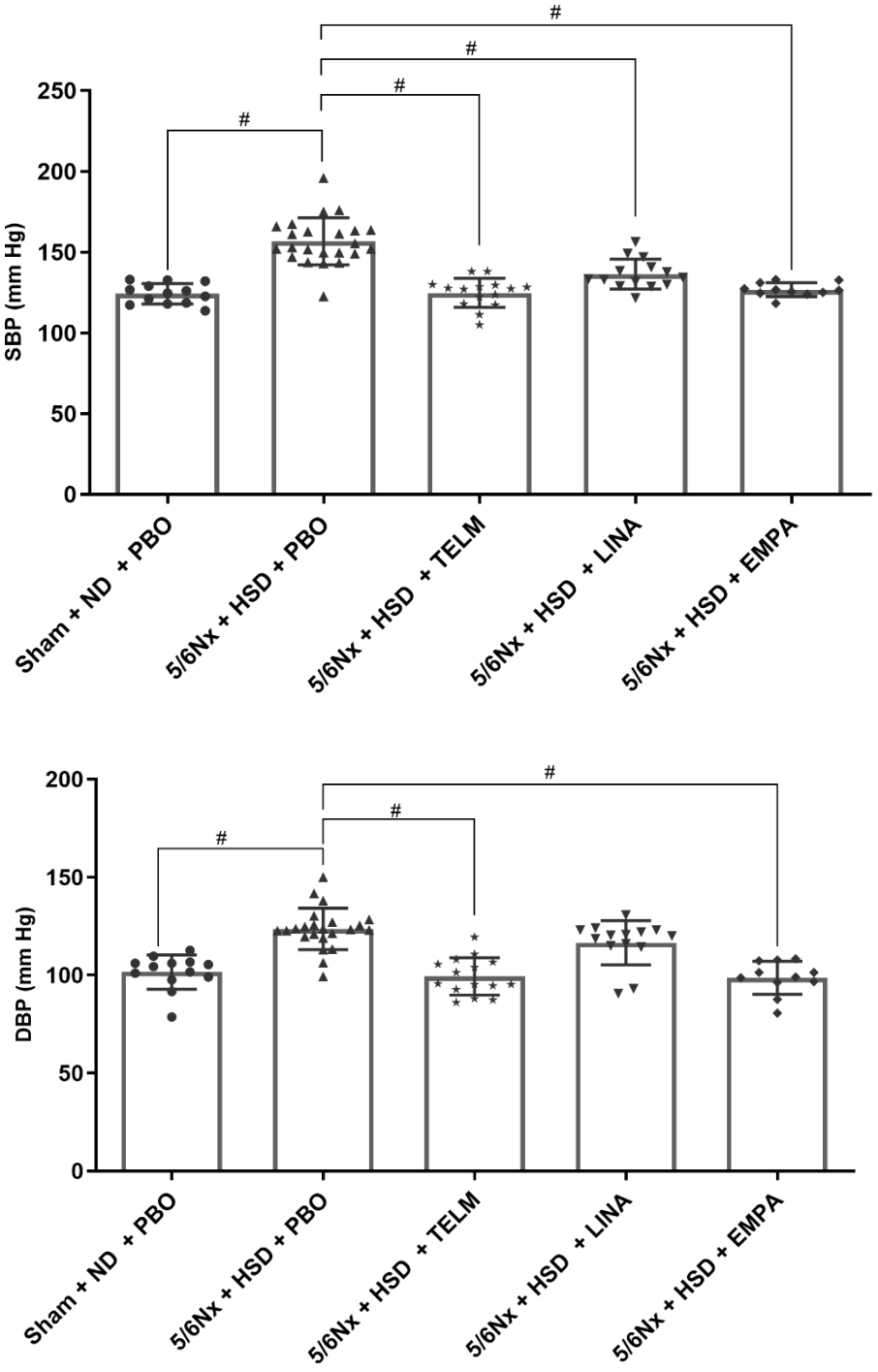

Figure 4. Effect of treatments on the systolic and diastolic blood pressures

Values are given as mean ± SEM. $^{\#}P<0.05$ vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.3 Effect of treatments on plasma creatinine

High-salt-diet-fed placebo-treated 5/6 Nx rats (5/6 Nx + HSD + PBO) had noteworthy ($p<0.05$) increases in the level of plasma creatinine compared to normal-diet fed placebo-treated sham control (Sham + HSD + PBO) rats. Treatment with telmisartan (5/6 Nx + HSD + TELM) remarkably increased plasma creatinine level, Treatment with linagliptin (5/6 Nx + HSD + LINA) and empagliflozin (5/6 Nx + HSD + EMPA) did not result in a significant effect on plasma creatinine compared to (5/6 Nx + HSD + PBO) rats (Figure 5).

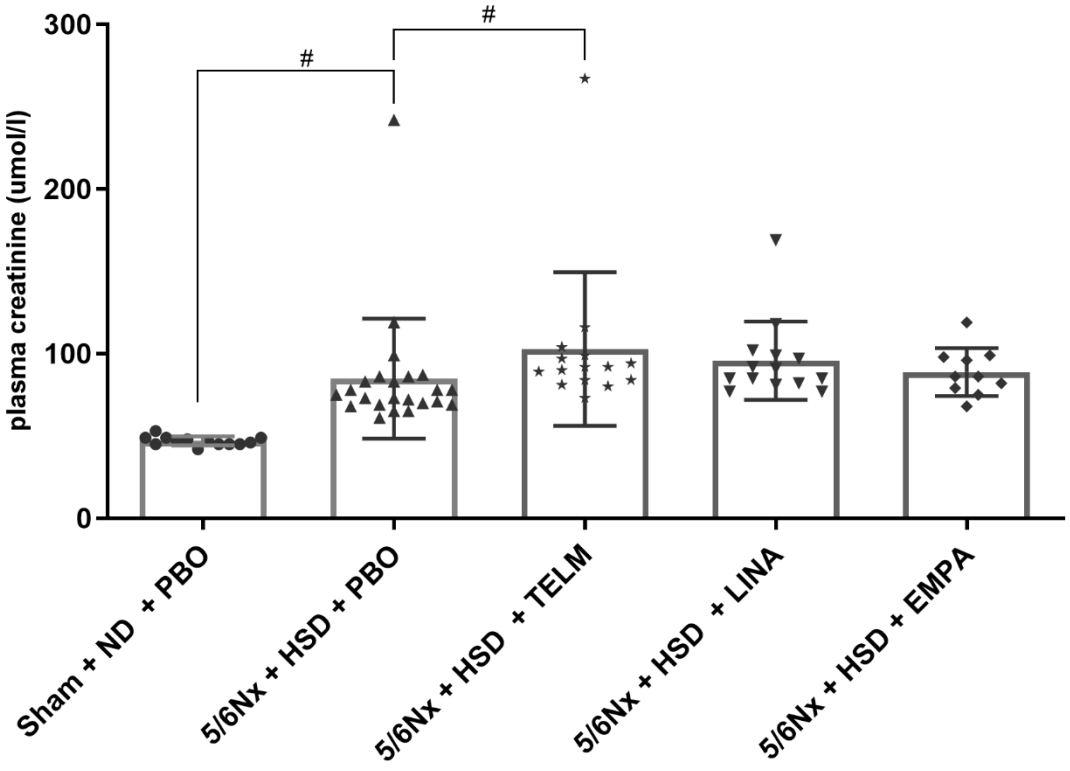

Figure 5. Effect of treatments on plasma creatinine

Values are given as mean ± SEM. $^{\#}P<0.05$ vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.4 Effect of treatments on plasma urea

5/6Nx and high-salt diet fed led to a significant increase of plasma urea compared to PBO-treated sham and normal-diet-fed rats. TELM treatment caused a significant rise in plasma urea in 5/6Nx and high-salt-diet fed rats. LINA and EMPA treatment merely resulted in a numerical, non-significant growth of plasma urea in the high-salt diet fed 5/6Nx rats (Figure 6).

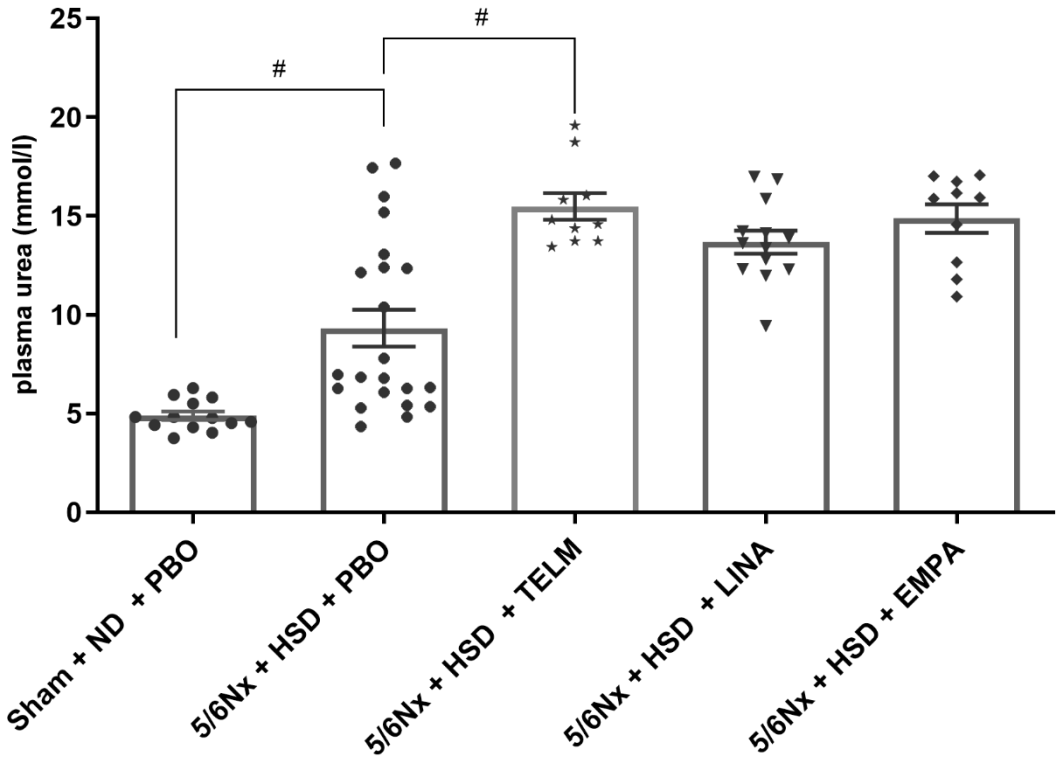

Figure 6. Effect of treatments on plasma urea

Values are given as mean ± SEM. $^{\#}P<0.05$ vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.5 Effect of treatments on plasma glucose

5/6Nx and high-salt diet non-significantly (p<0.05) up-regulated plasma glucose in comparison normal-diet fed placebo-treated sham control (Sham + HSD + PBO) rats. TELM, LINA and EMPA treatment did not impose notable impact on plasma glucose in 5/6 Nx and high salt diet fed rats (Figure 7).

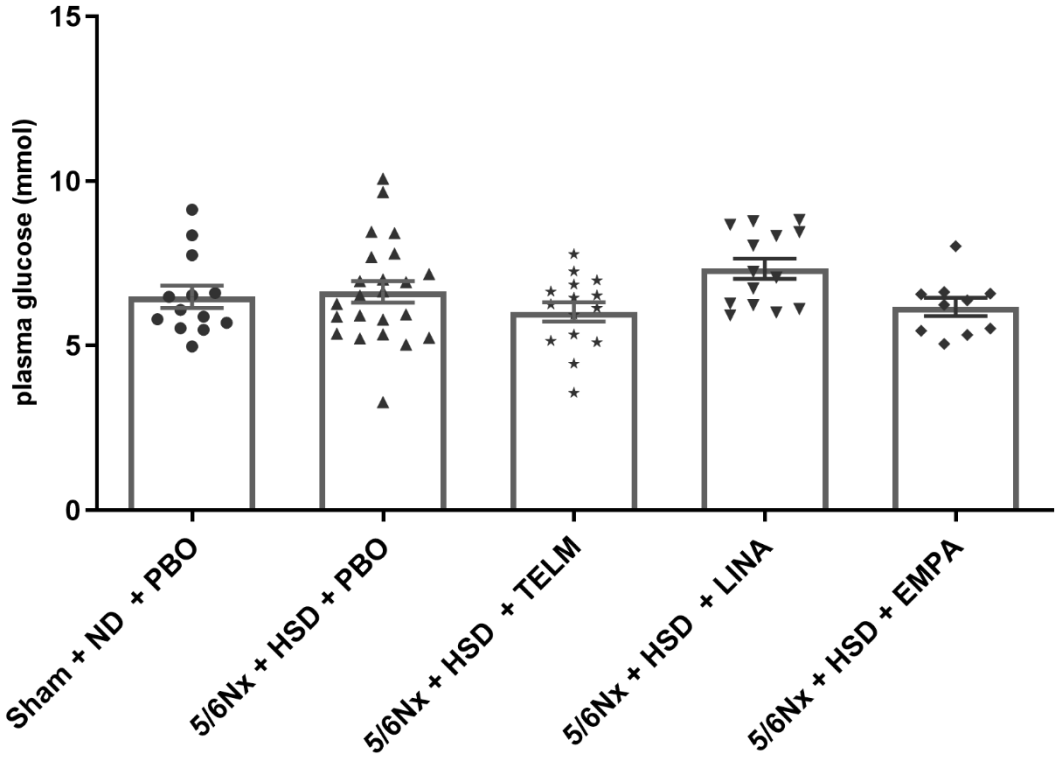

Figure 7. Effect of treatments on plasma glucose

Values are given as mean ± SEM. $^{\#}P$<0.05 vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26]

4.2.6 Effect of treatments on plasma insulin

TELM treatment significantly (p<0.05) down-regulated plasma insulin in 5/6 Nx and high-salt diet fed rats in contrast with to normal-diet fed and PBO-treated sham rats. LINA and EMPA treatment did not let to a significant alteration of plasma insulin levels. (Figure 8). ,

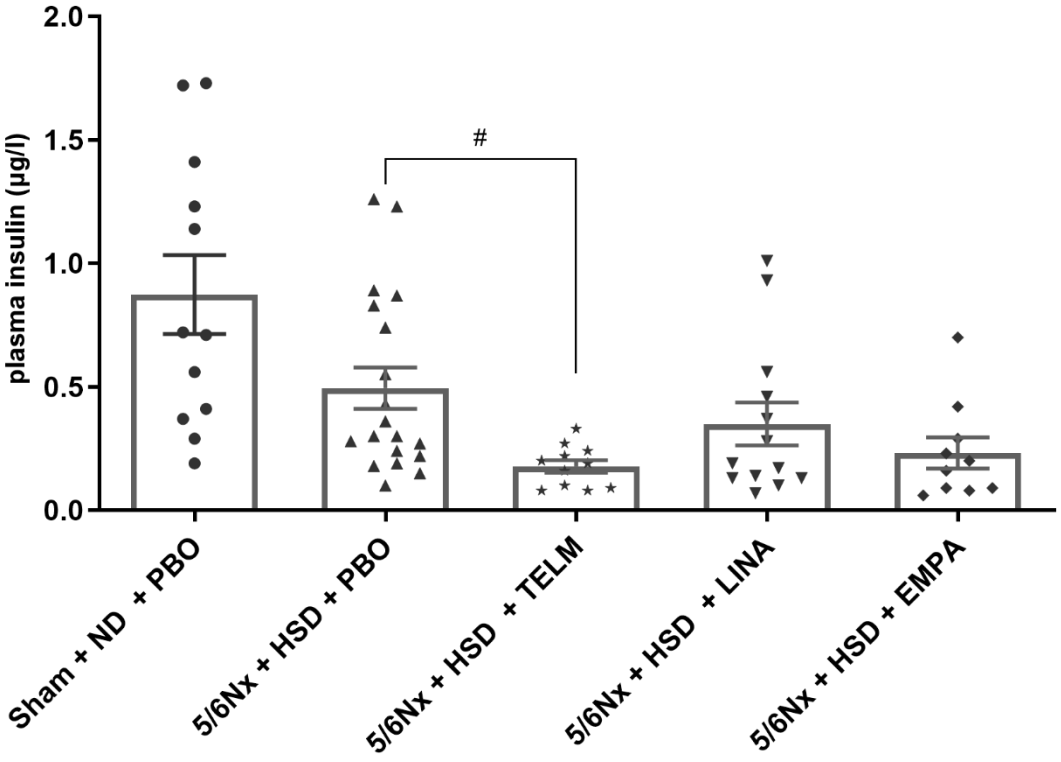

Figure 8. Effect of treatments on plasma insulin

Values are given as mean ± SEM. $^{\#}P$<0.05 vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26]

4.2.7 Effect of treatments on plasma BNP45

The differences in plasma BNP45 were not significant among the groups as shown in Figure 9.

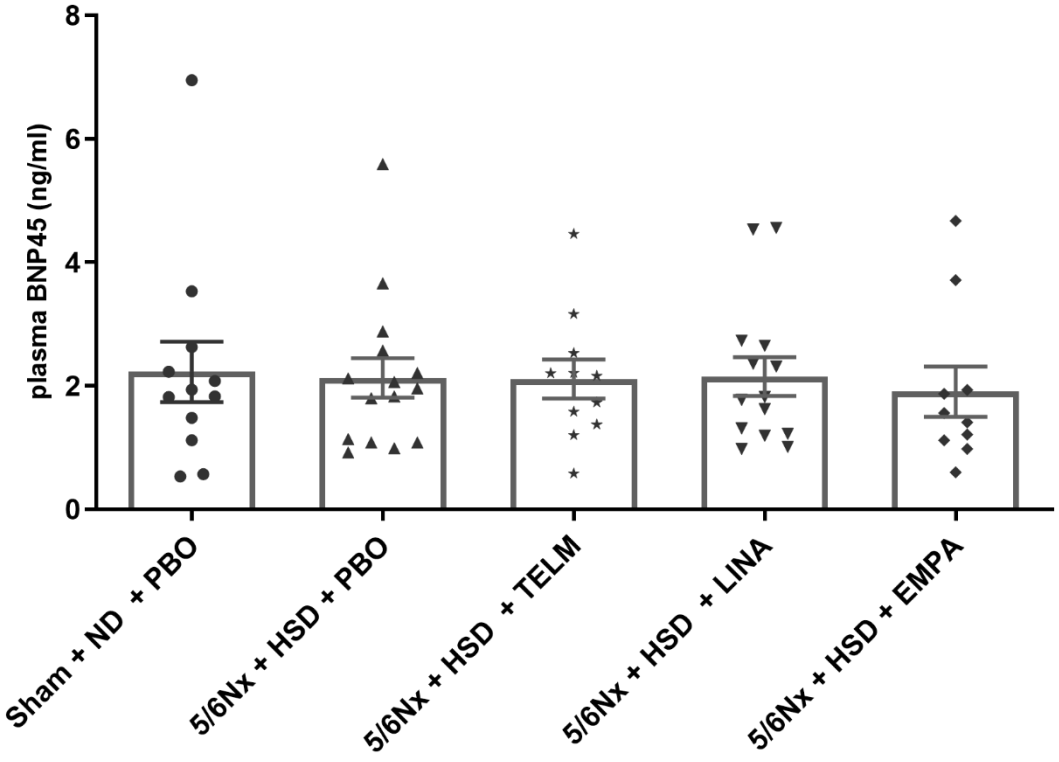

Figure 9. Effect of treatments on plasma BNP45

Values are given as mean ± SEM. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.8 Effect of treatments on GFR

TELM and LINA treatment significantly (p<0.05) down-regulated GFR in 5/6Nx and high-salt-diet-fed rats contrasted to PBO-treated and 5/6Nx high-salt diet fed rats, as shown in Figure 10.

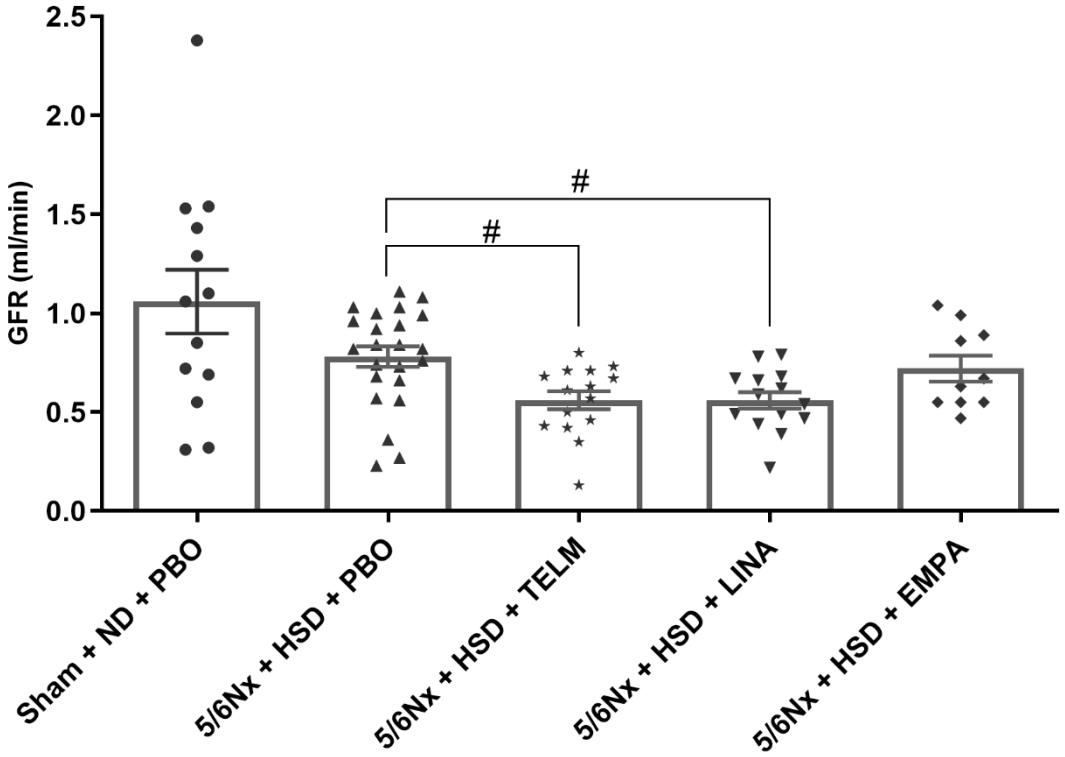

Figure 10. Effect of treatments on GFR

GFR (ml/min) = [urinary creatinine * urinary flow (ml/min)]/serum creatinine. Values are given as mean ± SEM. $^{\#}P$<0.05 vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.9 Effect of treatments on urinary creatinine

TELM and LINA treatment significantly (p<0.05) down-regulated urinary creatinine in 5/6 Nx and high-salt diet fed rats contrasted with PBO-treated and 5/6Nx high-salt diet fed rats (Figure 11).

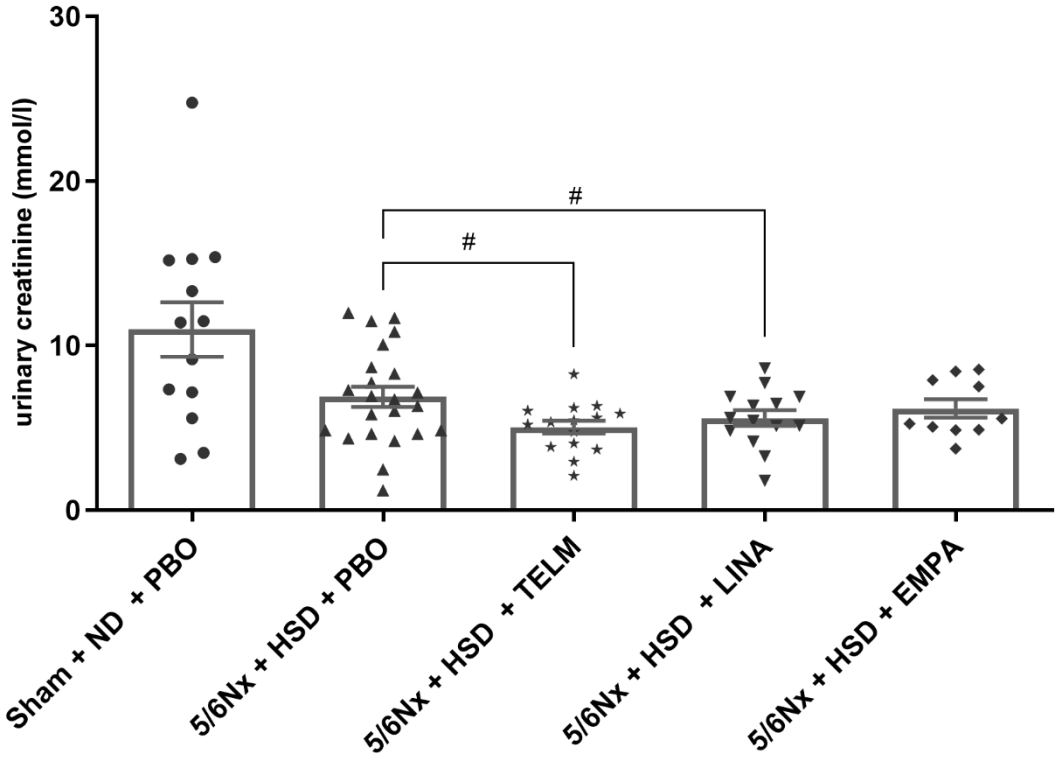

Figure 11. Effect of treatments on urinary creatinine

Values are given as mean ± SEM. $^{\#}P$<0.05 vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.10 Effect of treatments on urinary ACR

5/6 Nx and high-salt diet fed rats displayed significantly higher urinary ACR than Sham control rats. TELM, LINA and EMPA treatment did not result in alterations in urinary ACR compared to the high-salt diet fed and PBO-treated 5/6Nx rats (Figure 12).

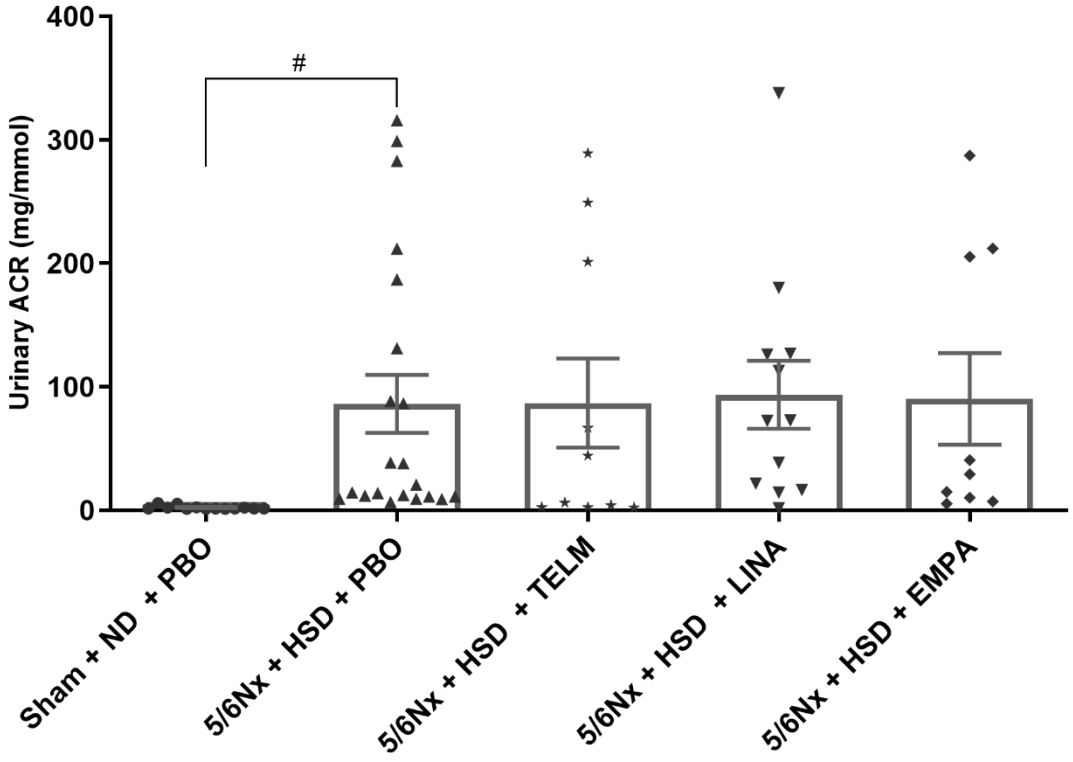

Figure 12. Effect of treatments on urinary ACR

Urinary ACR (mg/mmol) = urinary albuminuria / urinary creatinine.

Values are given as mean ± SEM. $^{\#}P<0.05$ vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.11 Effect of treatments on 24h urinary protein excretion

5/6Nx and high-salt diet fed caused a significant increase of 24h urinary protein excretion. TELM, LINA and EMPA treatment led only to a numerical, non-significant increase of urinary protein excretion in the high-salt-diet-fed 5/6Nx rats (Figure 13).

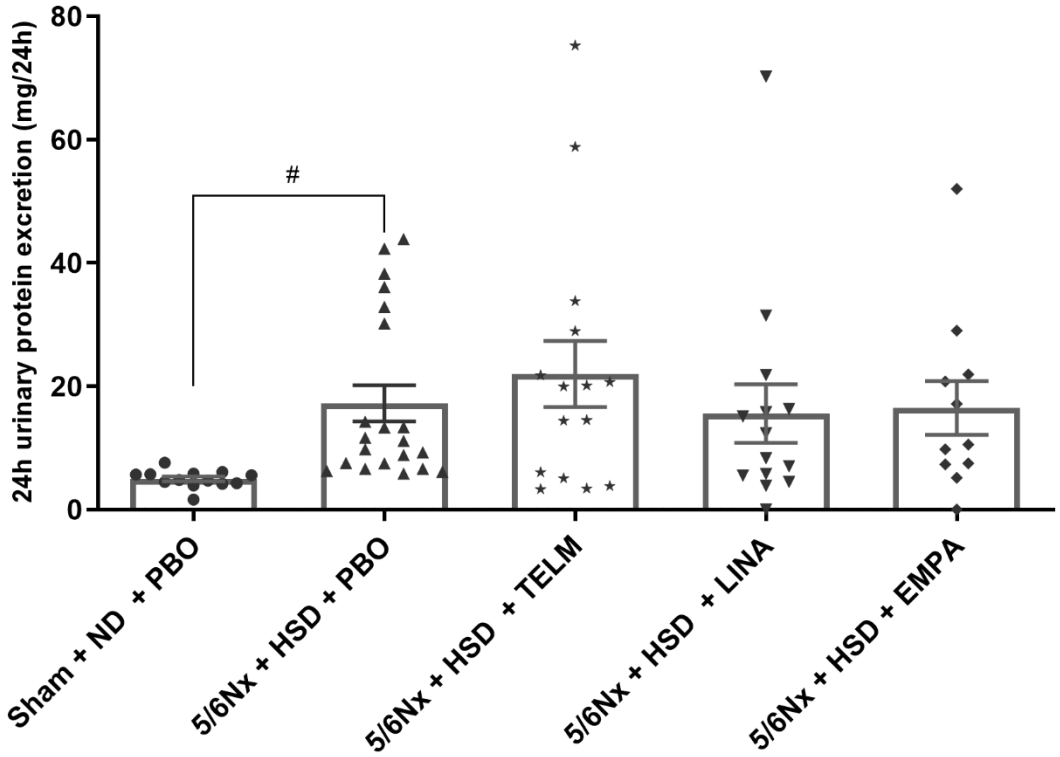

Figure 13. Effect of treatments on 24h urinary protein excretion

Values are given as mean ± SEM. $^{\#}P<0.05$ vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.12 Effect of treatments on mRNA expression of genes associated with SARS-CoV-2 host factors and RAAS

qRT-PCR was used to examine the impacts of telmisartan, linagliptin, and empagliflozin on the gene expression levels of the two critical SARS-CoV-2 host factors Ace2 and Tmprss2, as well as genes incorporated in the RAAS, such as Ren, Agtr2, and Agt, in the kidney, Ace2 and Tmprss2 mRNA expression was unaffected in

all experimental groups. Compared to PBO-treated and high-salt-diet-fed 5/6Nx rats, LINA remarkably lowered the cardiac Ren gene expression, but cardiac Tmprss2 and Ren expression was lower than the detection limit.Cardiac Tmprss2 and Ren mRNA levels were lower than the detection limits.(Table 5).

Table 5. Effect of treatments on mRNA expression of SARS-CoV-2 host factors and genes involved in RAAS

	Sham + ND + PBO (n=6)	5/6Nx + HSD + PBO (n=6)	5/6Nx + HSD + TELM (n=6)	5/6Nx + HSD + LINA (n=6)	5/6Nx + HSD + EMPA (n=6)
Renal mRNA expression					
Ace2	**1.02±0.09**	**1.01±0.33**	**1.14±0.14**	**1.47±0.47**	**1.33±0.33**
Tmprss2	**1.03±0.12**	**0.87±0.21**	**2.12±0.41**	**0.97±0.08**	**4.32±2.05**
Ren	**0.97(0.72-1.34)**[#]	**0.03(0.01-0.14)**	**1.46(0.43-2.31)**[#]	**0.09(0.06-0.22)**	**1.06(0.57-5.27)**[#]
Agtr2	**1.03±0.13**	**1.08±0.19**	**1.02±0.13**	**0.84±0.16**	**3.09±1.41**
Agt	**0.87(0.79-1.38)**	**1.22(0.44-1.50)**	**1.57(1.09-1.96)**	**1.64(1.19-1.95)**	**2.51(1.08-9.26)**
Cardiac mRNA expression					
Ace2	**1.04±0.19**	**1.17±0.08**	**1.08±0.08**	**0.77±0.06**[#]	**1.03±0.08**
Tmprss2	**–**	**–**	**–**	**–**	**–**
Ren	**–**	**–**	**–**	**–**	**–**
Agtr2	**1.01±0.06**	**1.13±0.10**	**0.98±0.06**	**0.91±0.09**	**0.97±0.5**
Agt	**1.04±0.12**	**0.95±0.04**	**1.19±0.20**	**0.77±0.09**	**0.92±0.07**

Normally distributed data were given as mean ± SEM. Non-normally distributed data were presented as median (25th–75th percentile). [#]p <0.05 vs. 5/6Nx+HSD+PBO. This table is adopted from our published paper (Xiong Y, et al./2022)[26].

4.2.13 Effect of treatments on proteins expression associated with SARS-CoV-2 host factors

The next stage involved using polyclonal ACE2 and TMPRSS2 antibodies to examine the protein expression of ACE2 and TMPRSS2 in the kidney and heart, as has been previously described[19]. In 5/6Nx rats, the levels of renal ACE2 protein expression in PBO-treated and high-salt-diet-fed (5/6Nx + HSD + PBO) were notably weakened compare with the PBO-treated and normal-diet-fed sham control group. LINA remarkably improved the renal ACE2 protein levels in 5/6Nx and high-salt diet

fed rats compare with the PBO-treated and high-salt-diet-fed 5/6Nx rats. TELM and EMPA treatment led only to a numerical, non-significant decrease in renal ACE2 protein levels in the high-salt-diet-fed 5/6Nx rats (Table 6, Figure 14). In the corresponding heart tissues, there existed no significant difference in ACE2 protein levels among all experimental groups (Table 6).

The levels of the TMPRSS2 protein in the kidneys were not considerably affected by the respective treatments (Table 6). On contrary, the levels of cardiac TMPRSS2 expression were increased dramatically in PBO-treated and high-salt-diet-fed (5/6Nx + HSD + PBO) group in contrast to Sham + ND + PBO control group. Notably, when compared to PBO-treated and high-salt-diet-fed (5/6Nx + HSD + PBO) rats, telmisartan, linagliptin, and empagliflozin were found to be effective in down-regulated the elevated cardiac TMPRSS2 level. (Table 6, Figure 15).

Table 6. Effect of treatments on renal and cardiac protein expression of SARS-CoV-2 host factors

	Sham + ND + PBO (n=12-13)	5/6Nx + HSD + PBO (n=15-23)	5/6Nx + HSD + TELM (n=11-15)	5/6Nx + HSD + LINA (n=13-15)	5/6Nx + HSD + EMPA (n=6-9)
Renal protein expression					
Ace2	**29.89 (20.82-36.55)#**	**13.96 (12.36-19.14)**	**14.14 (9.35-19.34)**	**33.25 (18.55-39.25)#**	**17.07 (12.59-19.96)**
Tmprss2	**13.44 (11.64-17.31)**	**12.77 (8.53-14.21)**	**12.35 (10.79-18.26)**	**12.21 (9.72-13.90)**	**11.60 (10.62-13.29)**
Cardiac protein expression					
Ace2	**28.99±1.16**	**28.30±1.10**	**27.70±0.99**	**25.71±1.17**	**31.45±2.02**
Tmprss2	**11.90 (10.63-15.00)#**	**25.21 (16.15-29.53)**	**10.41 (8.60-18.57)#**	**7.85 (6.03-13.39)#**	**12.00 (8.59-16.27)#**

Normally distributed data were given as mean ± SEM. Non-normally distributed data were presented as median (25th–75th percentile). #p <0.05 vs. 5/6Nx+HSD+PBO. This table is adopted from our published paper (Xiong Y, et al./2022)[26].

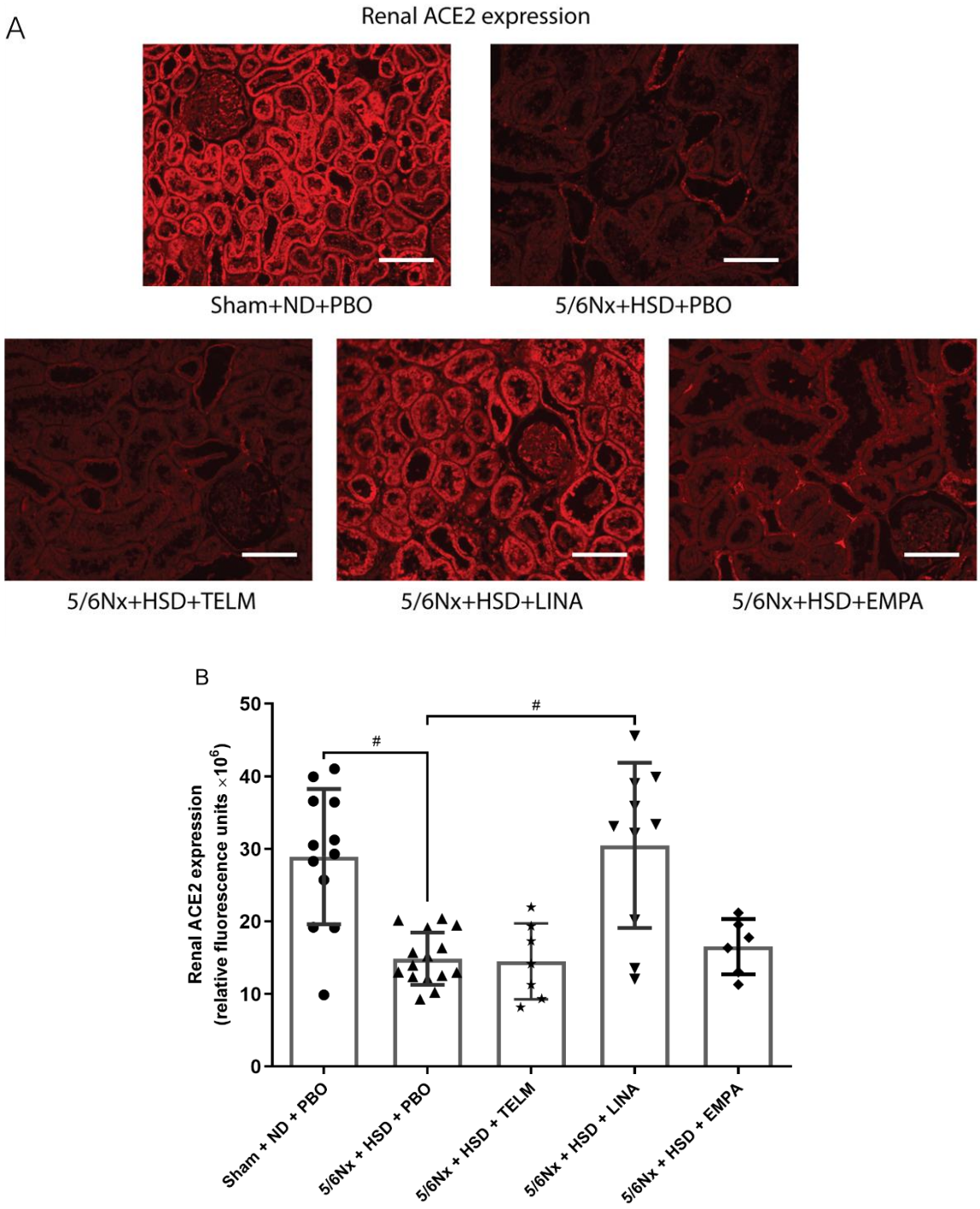

Figure 14. Effect of treatments on renal ACE2 protein expression in different groups

A. Photomicrographs of immunofluorescence-stained kidneys. The red color indicates ACE2. B. Renal protein expression of ACE2. Magnification ×20 (scale bars = 100 μm). $^{\#}p$ <0.05 vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26]

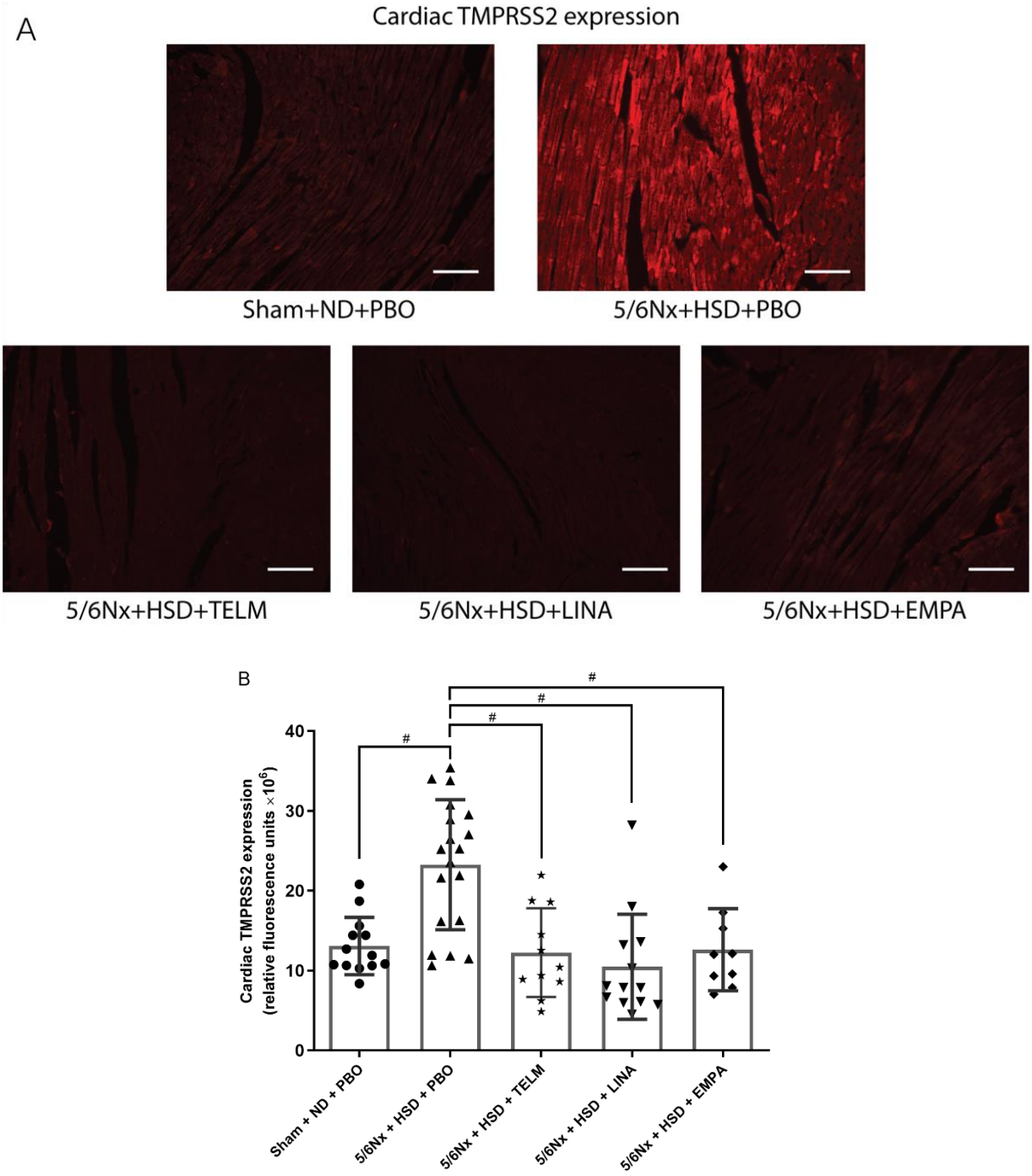

Figure 15. Effect of treatments on cardiac TMPRSS2 protein expression in different groups

A. Photomicrographs of immunofluorescence-stained hearts. The red color indicates TMPRSS2. B. Cardiac protein expression of TMPRSS2. Magnification ×20 (scale bars = 100 μm). $^{\#}p$ <0.05 vs. 5/6Nx+HSD+PBO. This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

4.3 Cellular localization of renal and cardiac ACE2 and TMPRSS2 expression

Our research has thoroughly investigated the distribution of ACE2 and TMPRSS2 in both kidney and heart. According to our observation of ACE2 in kidney, it occurs in epithelial cells of the proximal tubule and distal tubule. On the other hand, there is only a weak glomerular visceral ACE2 staining as shown in Figure 14A. By contrast, we can also notice that the parietal and visceral epithelial cells were reasonably positive as seen in Figure 14A. These findings are in consistency with previous studies [30, 31]. Apart from those discoveries, we found that ACE2 was also present in arterial endothelial cells (Figure 16B). In particular, it was detected primarily in tubules. On contrary, the value of ACE2 in glomeruli can hardly be called significant. This phenomenon is in agreement with findings from other studies conducted in rat kidneys, which discovered that ACE2 mRNA expression is substantially higher in tubules than in glomeruli [32, 33]. At the same time, our observation of the change in heart revealed more details. ACE2 was more abundant in myocytes than in arteries [34] (Figure 16C). Besides ACE2, we also gave weight to TMPRSS2 in our research. In the kidney, TMPRSS2 demonstrated disparate distribution in four distinct areas, more abundant in the distal convoluted tubule than in the proximal tubule [35](Figure 16D), glomeruli and arteries (Figure 16E). When we examined TMPRSS2 in heart tissue, it displayed a novel expression primarily in myocytes (Figure 16F).

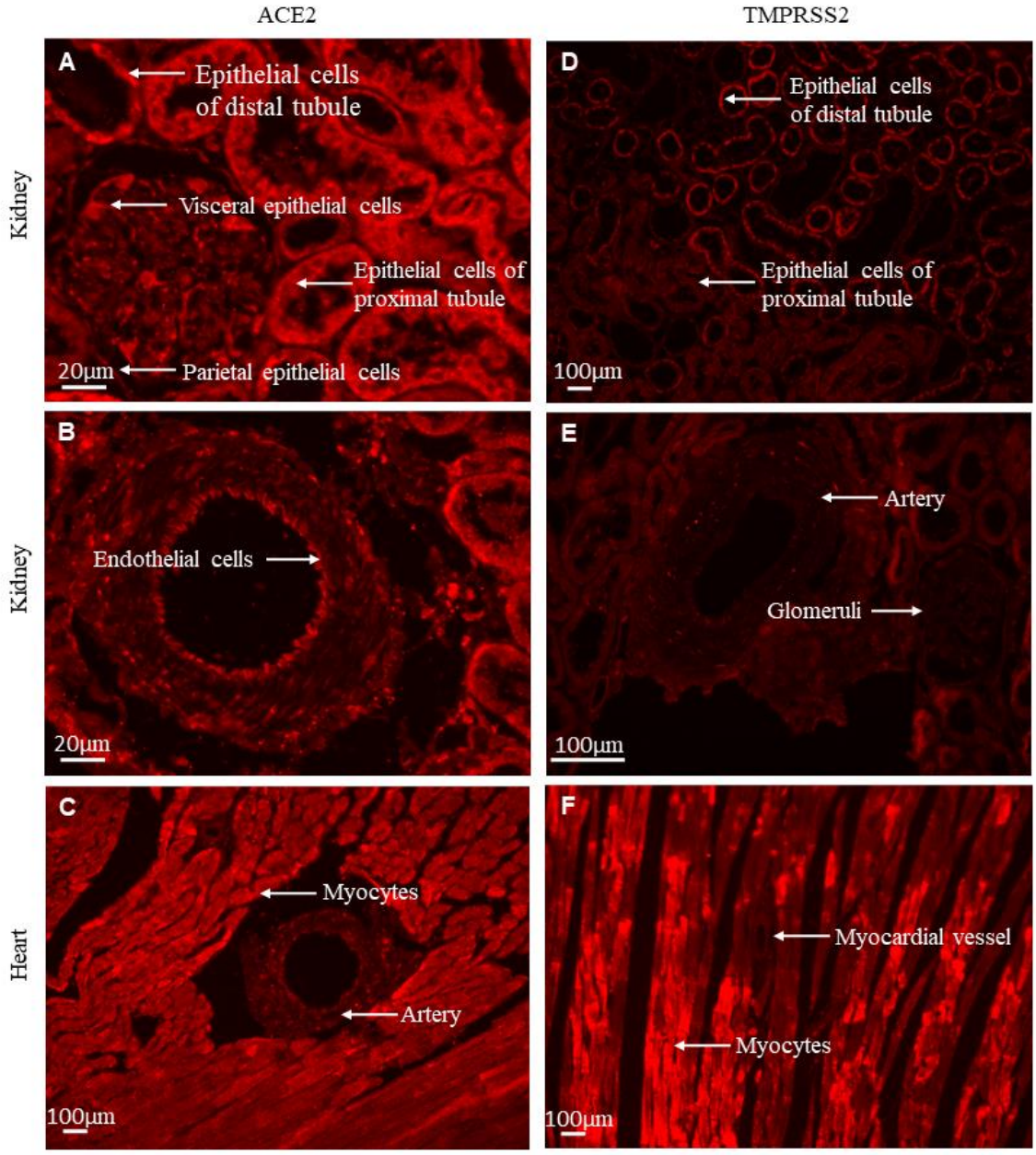

Figure 16. Cellular localization of renal and cardiac ACE2 and TMPRSS2 expression

Photomicrographs of immunofluorescence-stained kidneys and hearts. The red color indicates ACE2 and TMPRSS2. Magnification × 20. Upper panels show immunofluorescence staining of ACE2 in the glomerulus, proximal and distal tubules (A), renal arterial endothelium (B), cardiac myocytes and artery (C). Lower panels show immunofluorescence staining of TMPRSS2 in proximal and distal tubules (D), renal artery and glomerulus (E), cardiac myocytes and myocardial vessel (F). This figure is adopted from our published paper (Xiong Y, et al./2022)[26].

5. Discussion

5.1 Short summary of results

In summary, under disease or treatment, ACE2 expression was not altered at the mRNA level in the kidney and heart. Compared with sham group, the mRNA levels of TMPRSS2 in kidneys were unaffected. Compared with model group, TELM and EMPA improved renal TMPRSS2 mRNA levels but no notable alteration was detected in comparison to the sham control rats. Besides, the TMPRSS2 mRNA levels in the heart were under detectable level, and the elevated TMPRSS2 protein expression levels were normalized in heart under the administration of the listed three types of medicines. The renal ACE2 protein levels with LINA treatment were found to increase significantly compared with model group but normalized when compared with sham group. Regarding SARS CoV-2 infection, there is no significant clue indicating facilitated virus entrance into host cells through ACE2 and TMPRSS2 given the induced effects on renal and cardiac mRNA and protein expression of the listed two factors.

Apart from that, our experimental model demonstrated that no impact was posed on the expression of the ACE2 protein in either the kidney or the heart by the 5/6 nephrectomy. In addition, only the expression of Ren was altered by a more than 10-fold lowered level in 5/6 Nx rats given with placebo. Specifically, it was telmisartan but not linagliptin that restored the Ren mRNA expression levels in this study. By contrast, empagliflozin normalized the Ren mRNA expression levels in 5/6 Nx rats.

5.2 Interpretation of results

The ARB telmisartan, the DPP-4 inhibitor linagliptin, and the SGLT2 blocker empagliflozin were all administered at doses that resulted in positive pharmacodynamic action on systolic and diastolic blood pressures for all drugs when administered under high-salt-diet conditions in the well-established experimental non-

diabetic rat 5/6 nephrectomy model. In our findings, the induced effects on renal and cardiac mRNA and protein expression of the two critical host factors for SARS CoV-2 viral host cell entrance (ACE2 and TMPRSS2) show no clue of easier SARS CoV-2 virus infection via the aforementioned host receptors. In contrast, the ACE2 protein levels were elevated in this diabetic model, regardless of the treatment regimen [19]. Also, our experimental model demonstrated that 5/6 nephrectomy did not place impacts on the expression of the ACE2 protein in either the kidney or the heart. It is interesting to note that high salt conditions resulted in considerably decreased ACE2 levels in the kidney, which were restored by linagliptin treatment, whereas the cardiac levels remained unchanged. Linagliptin administration remarkably enhanced renal ACE2 level; however, this expression level was proved to be in similarity with the control groups sham and 5/6 Nx normal-diet-fed rats. Recently, a study investigated that linagliptin treatment dramatically enhanced the ACE2 activity in Ang II induced rats[27]. Meanwhile, another study discovered a highly significant positive association between ACE2 protein abundance and ACE2 activity in renal cortex tissue extracts from two diabetic models and their respective controls [36], which indicates that linagliptin could upregulate the level of ACE2 protein expression, though this has yet to be adequately illustrated. Furthermore, our investigation also demonstrated no noticeable impacts on the TMPRSS2 level (mRNA and protein) in the kidney, similar to the result of the earlier experimental diabetes model[19]. On contrary, the cardiac TMPRSS2 protein expression levels were dramatically raised after 5/6 Nx and all medication interventions resulted in normalized cardiac TMPRSS2 showing positive influence on decreased viral entry targets.

The disparity in the changes of ACE2 and TMPRSS2 mRNA and protein expression observed in previous in mice and human investigations[19, 36-38] revealing that ACE2 and TMPRSS2 expression is regulated at the post-transcriptional level. Recent studies showed that post-transcriptional regulation of ACE2 could be formed via translational repression[39] or proteolytic shedding occurring in the

extracellular region by ADAM17[40]. Single-cell RNA sequencing analysis indicated that ACE2 is by and large expressed on the brush border apical membrane of the proximal tubules, whereas TMPRSS2 is mostly expressed in the distal tubules[41, 42]. In the heart, a previous study revealed that cardiomyocytes and cardiovascular progenitor cells respectively contain 0.8% and 0.4% TMPRSS2-expressing cells[43]. It might account for why cardiac TMPRSS2 mRNA levels were undetectable in our investigation.

Similar to a recent result that the proximal tubular renin mRNA expression was significantly suppressed in uninephrectomized rats[44], our study demonstrated that only the expression of Ren was altered by a more than 10-fold lowered level in 5/6 Nx rats given with placebo. In this study, telmisartan but not linagliptin restored the Ren mRNA expression levels, which is consistent with a previous demonstration that the Ren expression in the kidneys of telmisartan-treated 5/6 Nx rats was approximately 16-fold higher than in linagliptin-treated rats[45]. In addition, empagliflozin normalized the Ren mRNA expression levels in 5/6 Nx rats. In a previous sub‐study of a double‐blind, randomized, placebo‐controlled, multicenter study (EMPA‐RESPONSE‐AHF) empagliflozin medication significantly increased plasma renin in comparison to placebo treated patients[46]. In sham operated rats, renin mRNA and protein was exclusively detected in the juxtaglomerular apparatus and not tubular epithelium. In contrast, the altered distribution of renin mRNA expression was observed in the nephrectomized kidney, resulting in de novo renin expression in renal tubular epithelial cells but the minimal or absent expression in the juxtaglomerular apparatus[47]. Areas distant from the infarct scar in perindopril-treated subtotal nephrectomy (STNx) rats showed a similar pattern of renin gene transcription compared to that of control rats, which is in consistency with our findings observed in telmisartan and empagliflozin treated animals.

5.3 Embedding the results into the current state of research

Effective though it is, the application of telmisartan, linagliptin, and empagliflozin in treating CKD patients has always been a concern because of the safety issue it triggers. Given the rising worries about the well-being of CKD patients, this research contributed to exploring the potential of these medicines and thus expanding their range of usage, for example, safe administration in patients with type 2 diabetes mellitus and SARS-CoV-2 infection.

Besides that, this research eliminated the uncertainty faced by CKD patients amid Covid-19 pandemic and reduced their worries about the medication increasing infection rate. In this way, it could stimulate further preclinical studies to better understand, define and characterize the safety of the clinical application of the listed medicines. This conclusion is in line with a recent comprehensive meta-analysis which revealed that RAAS-blocking drugs are not accompanied by higher risks of severe outcomes for COVID-19 patients, and they may further decrease all-cause mortality among COVID-19 patients[1].

In addition, DPP4 functions as a co-receptor in SARS-CoV-2 infection, and elevated levels of sDPP4 are found in obese and type 2 diabetic patients, making the disease more complicated if these patients are infected with COVID-19[2]. As a result of their anti-inflammatory effects at the vascular level, DPP-4 inhibitors are currently being studied as a potential therapeutic approach for preventing cardiovascular complications in COVID-19. Several clinical trials involving SGLT2 inhibitors (DARE-19 (NCT04350593)), RAAS-blocking drugs (BRACE-CORONA (NCT04364893)), linagliptin trials NCT04371978 & NCT04341935), and gliptins (SIDIACO (NCT04365517) are currently being conducted in COVID-19 patients.

5.4 Strengths and weaknesses of the study

There are several strengths of this study, for instance, the adaptation of a reliable amount of individual data based on 5/6 nephrectomized rats as CKD model, which

makes our findings comparatively more convincing.

However, it is undeniable that our study has limitations. First of all, it should be clarified that our rat CKD model data are applicable to humans. Association and linkage investigations in humans and rats have thus far not been very successful identifying. It is also essential to study additional animal models to determine whether our findings in terms of the regulation of SARS CoV-2 host factors can be found and generalized in other CKD animal models. Additionally, we quantified ACE2 and TMPRSS2 expression solely through computer-assisted image analysis of immunostaining. Futhermore, CKD animal models, especially those with diabetes approximate many patients' conditions are also keys to understanding its pathogenesis and developing rational treatment strategies. Finally, The ACE2 and TMPRSS2 activity is also a critical factor to be considered.

6. Conclusion

Using the qRT-PCR and immunofluorescence microscopy, the observed high-salt diet and reno-protective effects of telmisartan and empagliflozin were not associated with up-regulation of the renal and cardiac expression of ACE2 and TMPRSS2 in terms of mRNA and protein levels under high-salt conditions in comparison with a sham control and normal diet fed 5/6 nephrectomy rats. Linagliptin may potentially enhance the risk of renal but not cardiac SARS-CoV-2 infection.

Finally, it is noted that further confirmation should be acquired in a preclinical, experimental model of non-diabetic kidney failure to fully understand the implications of these findings. The involvement of the aforementioned drugs in ongoing clinical trials amid COVID-19 pandemic will potentially unfold the risks of using those medicines.

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8. Statutory Declaration

“I, Yingquan Xiong, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic “**Effects of salt, telmisartan, linagliptin, and empagliflozin on the regulation of SARS CoV2 host factors in the kidney and heart of 5/6 nephrectomized rats**”, “**Auswirkungen von Salz, Telmisartan, Linagliptin und Empagliflozin auf die Regulierung von SARS CoV2-Wirtsfaktoren in Niere und Herz von 5/6 nephrektomierten Ratten**”, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

9. Declaration of individual contribution to the following publication:

Xiong Y, Delic D, Zeng S, Chen X, Chu C, Hasan AA, Kramer BK, Klein T, Yin L, Hocher B. Regulation of SARS CoV-2 host factors in the kidney and heart in rats with 5/6 nephrectomy-effects of salt, ARB, DPP4 inhibitor and SGLT2 blocker. *BMC Nephrol* 2022, 23(1):117. DOI: 10.1186/s12882-022-02747-1(IF= 2.879)

Contribution in detail:

The aim of this publication was to explore whether DPP-4 inhibitors or SGLT2-blockers may promote COVID-19 by increasing the host viral entry enzymes ACE2 and TMPRSS2 in the presence of the virus. This project creatively aligned the designated research on the medication of CKD patients with the emerging risks caused by infection. It delivered pharmaceutically significant data that were crucial to improving patients' welfare during the pandemic. As the co-first-author with Denis Delic of this article, I took a leading role in many phases of the research.

I conducted animal experiments with the involvement of 88 rats and immunohistochemistry. I was the person in charge of the whole procedure including sample selection, medicine preparation, 5/6 nephrectomy, sacrifice, tissue section cutting and staining. My elaborate work greatly facilitated data collection, after which I completed data analysis using cutting-edge techniques including the one-way ANNOVA and Kruskal-Wallis test.

To present our findings, I created Figure.1, the time course of the animal study. In addition, I analysed all of the biochemical parameters and the protein expression in table 1&3. Figure.2 was also designed by me to illustrate renal ACE2 protein expression and cardiac TMPRSS2 protein expression in various groups. I have added Figure.3 to present cellular localisation of renal and cardiac ACE2 and TMPRSS2 expression.

I wrote sections for the paper that included methods, results, a part of the discussion and the introduction. Also, I processed reviewers' comments and suggestions, based on which I re-analysed all data. After many rounds of proofreading, I

finalised the last draft revision.

Being a co-first-author of the paper and one of the key project members, my contribution extends beyond the items listed, while the overlapping points should serve as a reflection of our team spirit, which encompasses development of concepts, promotion of research design, and lubrication of project progress.

_____

Signature, date and stamp of first supervising university professor / lecturer

_____

Signature of the doctoral candidate

10. Approval of animal ethical and welfare

暨南大学 JINAN UNIVERSITY　Institutional Animal Care and Use Committee（IACUC）

实验动物福利伦理审查表

Affidavit of Approval of Animal Ethical and Welfare

伦理申请编号	2017090409	批准编号 Approval No.	20170904092822

本动物实验方案经过暨南大学实验动物伦理委员会审核，符合动物保护、动物福利和伦理原则，符合国家实验动物福利伦理的相关规定。方案的相关信息如下：

The Animal Experiment Protocol listed below has been reviewed and approved by Laboratory Animal Ethics Committee of Jinan University.

实验名称 Protocol Title	在试验性慢性肾病模型中，探讨SGLT2抑制剂，DPP4抑制剂，sGC激活剂和sGC刺激剂对心脏和肾脏功能的影响			
	Effects of SGLT2 blocker, DPP4 inhibitor, sGC activators and stimulators on cardiac and renal function in an experimental CKD model			
申请人姓名	刘璠娜	邮箱	lfn-au@126.com	
实验负责人	刘璠娜	邮箱	lfn-au@126.com	
院系（部门）	第一临床医学院		申请日期	2017-08-23
拟实验时间	2017-09-01 – 2018-01-14	实验动物使用许可证	SYXK（粤）2017-0174	
审核意见	√符合动物福利伦理要求，可以进行实验。 Agree □调整方案后，可以进行实验。 Agree after modify			
兽医师			日期	2017-8-30

暨南大学实验动物管理与伦理委员会
Laboratory Animal Ethics Committee of Jinan University

中心IACUC主席（Chairman）：宋琳亮

日期(Date)：2017-08-30

地址：中国广东省广州市黄埔大道西601号 邮编：510632
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11. Excerpt from Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2021** Selected Editions: SCIE,SSCI
Selected Categories: **"UROLOGY and NEPHROLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 90 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfaktor
1	Nature Reviews Nephrology	13,479	42.439	0.02314
2	EUROPEAN UROLOGY	42,327	24.267	0.05321
3	KIDNEY INTERNATIONAL	57,360	18.998	0.04285
4	Nature Reviews Urology	6,129	16.430	0.00870
5	JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY	50,050	14.978	0.04855
6	AMERICAN JOURNAL OF KIDNEY DISEASES	30,051	11.072	0.02292
7	Clinical Journal of the American Society of Nephrology	24,549	10.614	0.02658
8	EUROPEAN UROLOGY SUPPLEMENTS	777	10.417	0.00090
9	European Urology Oncology	2,263	8.208	0.00571
10	JOURNAL OF UROLOGY	51,678	7.600	0.02913
11	NEPHROLOGY DIALYSIS TRANSPLANTATION	31,698	7.186	0.02182
12	World Journal of Mens Health	1,264	6.494	0.00144
13	Kidney International Reports	4,141	6.234	0.00991
14	Kidney International Supplements	3,874	6.083	0.00178
15	BJU INTERNATIONAL	23,142	5.969	0.01853
16	European Urology Focus	3,951	5.952	0.00866
17	Clinical Kidney Journal	4,774	5.860	0.00698
18	PROSTATE CANCER AND PROSTATIC DISEASES	3,875	5.455	0.00583
19	Sexual Medicine Reviews	1,592	5.345	0.00286
20	Minerva Urology and Nephrology	1,518	5.214	0.00228

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfaktor
21	AMERICAN JOURNAL OF NEPHROLOGY	6,498	4.605	0.00667
22	SEMINARS IN NEPHROLOGY	3,843	4.472	0.00334
23	JOURNAL OF NEPHROLOGY	4,712	4.393	0.00504
24	CardioRenal Medicine	937	4.360	0.00139
25	JOURNAL OF RENAL NUTRITION	3,382	4.354	0.00290
26	ADVANCES IN CHRONIC KIDNEY DISEASE	3,045	4.305	0.00389
27	Kidney Research and Clinical Practice	1,096	4.172	0.00173
28	AMERICAN JOURNAL OF PHYSIOLOGY-RENAL PHYSIOLOGY	19,034	4.097	0.01247
29	PROSTATE	8,983	4.012	0.00589
30	Journal of Sexual Medicine	14,476	3.937	0.01053
31	WORLD JOURNAL OF UROLOGY	9,909	3.661	0.01387
32	PEDIATRIC NEPHROLOGY	12,252	3.651	0.00939
33	NEPHRON	4,477	3.457	0.00395
34	CURRENT OPINION IN NEPHROLOGY AND HYPERTENSION	4,111	3.416	0.00373
35	BLOOD PURIFICATION	3,496	3.348	0.00316
36	RENAL FAILURE	4,890	3.222	0.00327
37	Therapeutic Advances in Urology	1,115	3.130	0.00150
38	Clinical Genitourinary Cancer	3,694	3.121	0.00681
39	KIDNEY & BLOOD PRESSURE RESEARCH	3,154	3.096	0.00370
40	NEFROLOGIA	1,899	3.084	0.00159
41	Prostate International	529	3.070	0.00082

Selected JCR Year: 2021; Selected Categories: "UROLOGY and NEPHROLOGY"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfaktor
42	ASIAN JOURNAL OF ANDROLOGY	5,329	3.054	0.00379
43	International Braz J Urol	3,022	3.050	0.00294
44	International Neurourology Journal	1,067	3.038	0.00140
44	European Urology Open Science	166	3.000	0.00027
46	Kidney Diseases	746	3.000	0.00115
47	UROLOGIC ONCOLOGY-SEMINARS AND ORIGINAL INVESTIGATIONS	7,309	2.954	0.00884
48	INTERNATIONAL JOURNAL OF UROLOGY	5,434	2.896	0.00450
49	SEMINARS IN DIALYSIS	3,315	2.886	0.00242
50	PERITONEAL DIALYSIS INTERNATIONAL	4,080	2.879	0.00324
51	Current Urology Reports	2,136	2.862	0.00317
52	Urolithiasis	1,600	2.861	0.00213
53	CURRENT OPINION IN UROLOGY	2,310	2.808	0.00290
54	UROLOGIC CLINICS OF NORTH AMERICA	2,320	2.766	0.00177
55	UROLOGY	24,074	2.633	0.01471
56	JOURNAL OF ENDOUROLOGY	8,166	2.619	0.00651
57	Clinical and Experimental Nephrology	3,569	2.617	0.00382
58	BMC Nephrology	9,042	2.585	0.01308
59	Sexual Medicine	1,026	2.523	0.00140
60	Translational Andrology and Urology	3,395	2.479	0.00637
61	INTERNATIONAL JOURNAL OF IMPOTENCE RESEARCH	3,264	2.408	0.00175

Selected JCR Year: 2021; Selected Categories: "UROLOGY and NEPHROLOGY"

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RESEARCH Open Access

Regulation of SARS CoV-2 host factors in the kidney and heart in rats with 5/6 nephrectomy—effects of salt, ARB, DPP4 inhibitor and SGLT2 blocker

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Abstract

Background: Host factors such as angiotensin-converting enzyme 2 (ACE2) and the transmembrane protease, serine-subtype-2 (TMPRSS2) are important factors for SARS-CoV-2 infection. Clinical and pre-clinical studies demonstrated that RAAS-blocking agents can be safely used during a SARS-CoV-2 infection but it is unknown if DPP-4 inhibitors or SGLT2-blockers may promote COVID-19 by increasing the host viral entry enzymes ACE2 and TMPRSS2.

Methods: We investigated telmisartan, linagliptin and empagliflozin induced effects on renal and cardiac expression of ACE2, TMPRSS2 and key enzymes involved in RAAS (REN, AGTR2, AGT) under high-salt conditions in a non-diabetic experimental 5/6 nephrectomy (5/6 Nx) model. In the present study, the gene expression of *Ace2*, *Tmprss2*, *Ren*, *Agtr2* and *Agt* was assessed with qRT-PCR and the protein expression of ACE2 and TMPRSS2 with immunohistochemistry in the following experimental groups: Sham + normal diet (ND) + placebo (PBO); 5/6Nx + ND + PBO; 5/6Nx + high salt-diet (HSD) + PBO; 5/6Nx + HSD + telmisartan; 5/6Nx + HSD + linagliptin; 5/6Nx + HSD + empagliflozin.

Results: In the kidney, the expression of *Ace2* was not altered on mRNA level under disease and treatment conditions. The renal TMPRSS2 levels (mRNA and protein) were not affected, whereas the cardiac level was significantly increased in 5/6Nx rats. Intriguingly, the elevated TMPRSS2 protein expression in the heart was significantly normalized after treatment with telmisartan, linagliptin and empagliflozin.

Conclusions: Our study indicated that there is no upregulation regarding host factors potentially promoting SARS-CoV-2 virus entry into host cells when the SGLT2-blocker empagliflozin, telmisartan and the DPP4-inhibitor blocker linagliptin are used. The results obtained in a preclinical, experimental non-diabetic kidney failure model need confirmation in ongoing interventional clinical trials.

Keywords: SARS CoV-2 host factors, 5/6 nephrectomy, High-salt diet, ARB, DPP4 inhibitor, SGLT2 blocker

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Background

Cardiovascular and renal diseases are considered as risk factors for increased coronavirus disease 2019 (COVID-19) disease severity and worse outcomes, including higher mortality. During the COVID-19 pandemic, tight control of glucose levels and prevention of complications associated with diabetes might be crucial in patients with

diabetes to lower the susceptibility and severe course of COVID-19. Recent studies suggest that drugs interfering with the renin–angiotensin–aldosterone system (RAAS) or dipeptidyl peptidase 4 (DPP4) inhibitors can be used safely in patients with diabetes mellitus and COVID-19 [1–3]. In addition, the use of sodium-glucose cotransporter 2 (SGLT2) blockers seems to be a promising adjunct treatment option in patients with SARS-CoV2 infection and type 2 diabetes mellitus (T2DM) whereas an increased risk of protracted ketonemia and diabetic ketoacidosis was also reported [4].

ACE2 plays a central role in the regulation of RAAS and is involved in cardiac function, the development of hypertension and diabetes mellitus [5]. ACE2 exerts its protective effects by converting pro-inflammatory and pro-hypertensive AngII into anti-inflammatory and anti-hypertensive Ang1-7. ACE2 has been identified as a receptor for coronaviruses, including SARS-CoV-2. Once attached to ACE2 through the binding with the receptor binding domain in the viral spike protein, it is primed by the host TMPRSS2, which can enhance this endocytic way of entry but is not essential [6, 7]. An alternative route of viral entry is the direct fusion of the viral envelope and the cell membrane which is ACE2- and TMPRSS2-independent [8]. Increased ACE2 expression was observed as a response to inflammation, heart failure, lung injury and fibrosis [9–12] which led to increased AngII level and might facilitate the viral entry. In contrast, AngII can induce the internalization and degradation of ACE2 in an AT1R-dependent manner [13]. In addition, MERS-CoV binds to human DPP4/CD26 to infect host cells [14] and a recent study predicts the structure of the SARS-CoV-2 spike glycoprotein and its glycan shield pattern suggests that DPP4/CD26 might be a receptor for SARS-CoV-2 [15] which needs further validation. The increased presence of ACE2 or DPP4 might contribute to increased disease severity of infected patients.

In experimental preclinical models, the effects of RAAS blocking drugs on cardiac and renal ACE2 mRNA and/or protein expression led to controversial results. *Ace2* mRNA expression was increased in the left ventricle of normotensive rats after lisinopril or losartan treatment [16] whereas no increase in *Ace2* mRNA level was observed after coronary artery ligation and treatment with valsartan, ramipril or both compared to control [17]. In kidneys, telmisartan treatment resulted in increased expression of renal *Ace2* mRNA expression [18]. No effects on renal *Ace2* and *Tmprss2* mRNA expression after telmisartan treatment were previously verified in an independent study [19]. In a recent study, it was shown that captopril and telmisartan both decrease kidney ACE2 protein in kidney membranes without significantly affecting protein abundance in total kidney lysates. Captopril significantly reduced ACE2 protein in kidney membranes while cytosolic ACE2 was increased [20]. Importantly, mice with comorbid diabetes (aging, high fat diet and streptozotocin-induced diabetes) are characterized by increased renal *Ace2* mRNA expression but not further affected after telmisartan treatment which led to the conclusion that the increased ACE2 level is a consequence of the comorbidity and not an effect after RAAS blockade [19].

Dietary salt intake is a known risk factor for hypertension and is associated with an imbalance of the RAAS. The high salt diet fed spontaneously hypertensive rats (SHR) showed slightly decreased cardiac *Ace2* mRNA and protein expression [21] and renal expression was attenuated in uni-nephrectomized rats with subsequent high salt diet intake [22] but the effects of RAAS blocking drugs in a salt-induced experimental model have not been investigated yet. Recognizing that people with chronic kidney disease, who are often consuming a high-salt diet and commonly prescribed RAAS blocking drugs and/or DPP4-inhibitor and/or SGLT2 blocker, are at increased risk of severe COVID-19 outcomes, we studied the expression profiles of ACE2 and TMPRSS2 and other genes involved in the RAAS in the kidney and the heart in a rat model that mimics this phenotype (impaired kidney function combined with a high salt intake – most patients consume unfortunately several times more salt than they actually should control blood pressure). Here we used the rat 5/6 nephrectomy model, one of the most well-established experimental non-diabetic CKD model which is characterized by increased hypertension, inflammation and fibrosis.

Methods

Animals

The animal experiment was approved by the laboratory animal ethics committee (20,170,904,092,822, Jinan University, Guangzhou, China) following University Guidelines for Use of Laboratory Animals. A total of 91 male Wistar rats were assigned to the following groups: Sham + ND + PBO ($n=14$); 5/6 Nx ND + PBO ($n=12$); 5/6 Nx + HSD + PBO ($n=23$); 5/6 Nx + HSD + telmisartan (5 mg/kg/day; $n=15$); 5/6 Nx + HSD + linagliptin (3 mg/kg/day; $n=14$); 5/6 Nx + HSD + empagliflozin (1.2 mg/kg/day; $n=13$). The normal diet was standardized using AIN93M [23] and the high salt diet was adjusted to a 2% level of sodium chloride on this basis. The two feeds were produced under the codes LAD 3001 M and LAD0011HF2 (Trophic Animal Feed High-Tech Co., Ltd, China). The doses of telmisartan and linagliptin have been used in previous studies [24, 25]. Drug treatment via gavage was administered from week

3 until sacrifice (week 11). The rats were sacrificed at week 11 and plasma. Pentobarbital sodium (36–39 mg/kg body weight) was used to anesthetize the rats, which was administered intraperitoneally. Urine and perfused kidney and heart samples were collected and frozen until further analysis (Fig. 1). All experimental procedures (surgery, blood pressure measurements, metabolic cages, as well as plasma and urine analyses) were done as describe previously [26].

Blood pressure measurement

Blood pressure was measured by non-invasive tail cuff plethysmography of the tail artery at week 11. The animal was placed in a restrainer, i.e. a tubular construction from which only the tail of the animal protruded. Then a blood pressure cuff and an electronic transducer were fixed to the tail of the animal. We waited until the animals were relaxed and got used to the restrainer. At intervals of 30 s, at least three measurements were taken to obtain reliable means of blood pressure. To get the animals used to this procedure, animals were trained before the actual measurement. The blood pressure diagrams and pulses were recorded and evaluated using the IITC Life Science tail cuff plethysmography blood pressure systems (IITC Life Science Inc., Woodland Hills, CA, USA).

Biochemical evaluations

EDTA was added to blood samples followed by centrifugation (4,500 rpm) for 20 min at 4 ℃, then plasma was collected and stored at -20 ℃ until analysis. Urine samples were centrifuged (12,000 rpm) for 10 min at 4 ℃. The supernatant was frozen in liquid nitrogen until analysis. Levels of plasma creatinine, urea, glucose, and insulin as well as urinary creatinine, and total protein were detected using an automatic biochemistry analyzing system (Roche Cobas 6800, Roche Ltd, Switzerland). Levels of plasma BNP45 and urinary albumin were determined quantitatively using Rat BNP 45 ELISA Kit (Abcam, Cat#ab108816) and Rat Albumin ELISA Kit (Abcam, Cat#ab235642). The glomerular filtration rate-to-body weight ratio (GFR), albumin-to-creatinine ratio (ACR) were calculated. At a dose of 1 mg/day of empagliflozin urinary sodium and potassium excretion are not affected (data not shown).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Snap frozen kidney and heart tissues were homogenized with Precellys lysis with Precellys Steel 2.8 mm beads (PeqLab Biotechnology, Erlangen, Germany) and total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany). Quality control and total RNA yield were quantified using the NanoDrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, United States, DE). Renal and cardiac mRNA levels of Angiotensin I Converting Enzyme 2 (*Ace2*), Transmembrane Protease Serine Subtype 2 (*Tmprss2*), Renin (*Ren*), Angiotensin Receptor Type 2 (*Agtr2*) and Angiotensinogen (*Agt*) were analyzed by qRT- PCR on a SDS7900HT real-time PCR system (Applied Biosystems by ThermoFisher Scientific). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as a housekeeping gene and experimental details were detailed previously

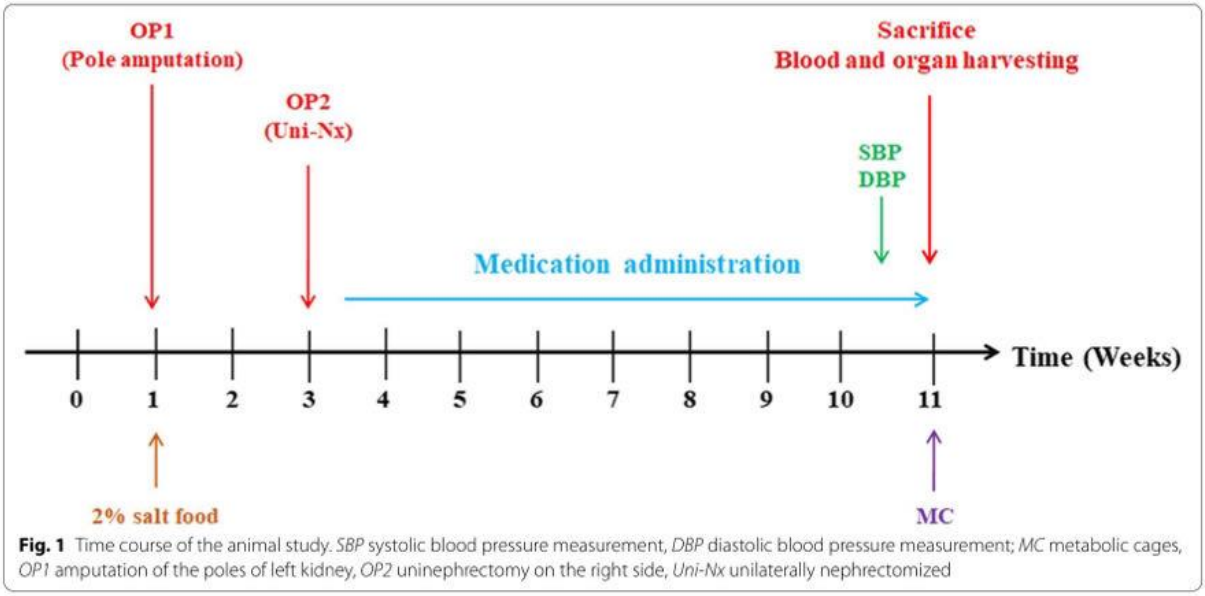

Fig. 1 Time course of the animal study. *SBP* systolic blood pressure measurement, *DBP* diastolic blood pressure measurement; *MC* metabolic cages, *OP1* amputation of the poles of left kidney, *OP2* uninephrectomy on the right side, *Uni-Nx* unilaterally nephrectomized

[27]. All samples were run in duplicates and raw ct values were calculated using the SDS software v.2.4. All values were normalized to the mean expression level of the control group (Sham + ND + PBO) and the fold-change of expression compared to the control was calculated using the comparative Ct method (2-ΔΔct) [28].

Immunohistochemistry

Kidney and cardiac tissue specimens were embedded in paraffin after fixation with 4% paraformaldehyde, cut into 3-µm paraffin sections for immunohistochemical staining. Sections were de-waxed twice using xylene and rehydrated with graded ethanol. After microwave antigen-retrieval, sections were blocked with 5% non-fat dry milk in phosphate-buffered saline/Tween 20 (PBS-T) for one hour and incubated respectively with primary antibodies specific to ACE2 (1:100 dilution; ab15348, Abcam, Cambridge, MA) and TMPRSS2 (1:50 dilution; EPR3861, ab92323, Abcam, Cambridge, MA) in 5% non-fat dry milk in PBS-T overnight at 4 °C. The sections were repeatedly washed 5 times with PBS-T, incubated with matching fluorescent secondary antibody (1:200, ab150075; Abcam) in PBS-T, and mounted with Fluoroshield mounting medium with 4′,6-diamidino-2-phenylindole (ab104139; Abcam). The fluorescent images were captured as described recently and analyzed using a computer-aided image analysis system as described previously [26].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad, La Jolla, CA). The analysis of variance test followed by the Bonferroni post hoc test was applied for comparison of normally distributed data, and the data were presented as mean ± SEM. The Kruskal–Wallis test followed by Dunn's post hoc test was used for non-normally distributed data, and the data were presented as median (25th—75th percentile), In all cases, differences were regarded as statistically significant if $P < 0.05$.

Results

Effects of salt, telmisartan, linagliptin and empagliflozin on clinical and biochemical parameters

At the end of the study high salt diet-fed placebo-treated 5/6 Nx rats (5/6 Nx + HSD + PBO) were characterized by significantly higher relative left kidney and relative heart weights, final systolic and diastolic blood pressures, final plasma creatinine, final urinary ACR level and final 24 h urinary protein excretion compared to normal-diet fed placebo-treated sham control rats (Table 1). In high salt diet-fed 5/6 Nx rats, treatment with telmisartan (5/6 Nx + HSD + TELM) significantly decreased the final body weight, final systolic and diastolic blood pressures versus 5/6 Nx + HSD + PBO rats (Table 1). Linagliptin treatment of high salt diet-fed 5/6 Nx rats (5/6 Nx + HSD + LINA) resulted in significantly decreased final body weight and final systolic blood pressure, whereby empagliflozin treatment led to significantly decreased relative liver weight compared to 5/6 Nx + HSD + PBO rats (Table 1).

Effects of salt, telmisartan, linagliptin and empagliflozin on renal and cardiac mRNA expression of genes associated with SARS-CoV-2 host factors and RAAS

In order to investigate the effects of salt, telmisartan, linagliptin and empagliflozin on the gene expression levels of the two key SARS-CoV-2 host factors *Ace2* and *Tmprss2* and genes involved in the RAAS, such as *Ren*, *Agtr2* and *Agt*, in the kidney and heart qRT-PCR was performed. Overall, the expression of *Ace2* was not affected in both kidney and heart in all experimental groups. Telmisartan and empagliflozin significantly increased the renal *Tmprss2* gene expression compared to 5/6 Nx + HSD + PBO rats whereas the cardiac *Tmprss2* expression was below the detection limit (Table 2). Importantly, telmisartan and empagliflozin increased *Tmprss2* mRNA levels are not significantly altered compared to the control group (Sham + ND + PBO).

Renal *Ren* expression was significantly decreased in 5/6 Nx + ND + PBO, 5/6 Nx + HSD + PBO and 5/6 Nx + HSD + LINA groups compared to the Sham + ND + PBO control group. Telmisartan and empagliflozin significantly normalized the renal expression of *Ren* versus 5/6 Nx + HSD + PBO rats (Table 2), whereas *Agtr2* and *Agt* were not significantly affected in any experimental groups (Table 2).

Effects of salt, telmisartan, linagliptin and empagliflozin on renal and cardiac expression of proteins associated with SARS-CoV-2 host factors

In the next step we examined the ACE2 and TMPRSS2 protein expressions in the kidney and heart using polyclonal ACE2 and TMPRSS2 antibodies as described previously [19]. The renal ACE2 protein expression was significantly decreased in the placebo or telmisartan treated high-salt diet fed 5/6 Nx rats compared to Sham + ND + PBO rats whereby linagliptin significantly increased the ACE2 protein levels in 5/6 Nx + HSD rats (Fig. 2A, B, Table 2) characterized by normalized ACE2 protein levels compared to Sham + ND + PBO or 5/6 Nx + ND + PBO rats (Fig. 2A, B, Table 2). In the corresponding heart tissues, there was no major change in ACE2 protein levels in all experimental groups (Table 2).

In kidneys the TMPRSS2 protein level was not significantly altered by the respective treatments. In contrast, in 5/6 Nx + ND + PBO and 5/6 Nx + HSD + PBO rats the cardiac TMPRSS2 expression was significantly increased

Table 1 Clinical/Biochemical parameters

	Sham + ND + PBO ($n = 12–13$)	5/6Nx + ND + PBO ($n = 12–13$)	5/6Nx + HSD + PBO ($n = 15–23$)	5/6Nx + HSD + TELM ($n = 11–15$)	5/6Nx + HSD + LINA ($n = 13–15$)	5/6Nx + HSD + EMPA ($n = 10–11$)
Final body weight (g)	475.35 ± 12.04	448.50 ± 17.38	443.35 ± 10.65	394.65 ± 12.51[ab]	382.39 ± 12.44[ab]	420.03 ± 11.68[a]
Relative left kidney weight (mg/g)	3.20(2.93–3.46)[b]	3.44(2.93–3.46)	3.64(3.31–4.35)[a]	3.56(3.15–4.26)	3.72(3.41–4.13)[a]	3.94(3.49–4.46)[a]
Relative heart weight (mg/g)	2.74 ± 0.06[b]	3.03 ± 0.08	3.74 ± 0.21[a]	3.97 ± 0.33[a]	3.77 ± 0.23[a]	3.58 ± 0.13[a]
Relative liver weight (mg/g)	24.03(23.27–25.62)	24.25(23.27–25.62)	26.46(22.36–27.54)	21.94(21.24–24.01)	22.88(21.80–24.56)	21.70(20.99–22.58)[ab]
Final systolic blood pressure (mm Hg)	124.66(118.33–130.50)[b]	153.66(118.33–130.50)[a]	153.00(149.00–163.66)[a]	127.33(118.00–129.66)[b]	133.83(129.75–142.25)[b]	126.33(124.66–131.00)
Final diastolic blood pressure (mm Hg)	101.56 ± 2.43[b]	122.77 ± 3.68[a]	123.61 ± 2,21[a]	99.36 ± 2.47[b]	116.52 ± 3.01[a]	98.63 ± 2.56[b]
Final plasma creatinine (µmol/l)	46.92 ± 0.76[b]	72.38 ± 2.27	84.78 ± 7.61[a]	102.80 ± 12.04[a]	95.71 ± 6.38[a]	88.80 ± 4.64[a]
Final plasma urea (mmol/l)	4.89 ± 0.21	11.18 ± 1.08	12.66 ± 3.46	16.94 ± 1.86[a]	15.84 ± 2.22[a]	14.87 ± 0.72
Final plasma glucose (mmol)	6.08(5.62–7.18)	9.03(5.62–7.18)[#]	6.52(5.36–7.69)	6.25(5.14–6.85)	7.16(6.20–8.51)	6.31(5.42–6.59)
Final plasma insulin (µg/l)	0.72(0.38–1.37)	0.53(0.38–1.37)	0.30(0.22–0.83)	0.20(0.10–0.33)[a]	0.19(0.13–0.51)[a]	0.18(0.09–0.32)[a]
Final plasma BNP45 (ng/ml)	2.23 ± 0.49	1.92 ± 0.30	2.13 ± 0.32	2.11 ± 0.32	2.15 ± 0.31	1.91 ± 0.41
GFR/BW (ml/24 h/g)	1.97(1.23–3.15)	1.62(1.23–3.15)	1.99(1.47–2.11)	1.47(1.22–1.72)	1.41(1.31–1.73)	1.74(1.34–2.15)
Final urinary creatinine (mmol/l)	11.41(6.39–15.23)	6.49(6.39–15.23)	6.74(4.64–8.69)	5.34(3.84–6.05)[a]	5.53(4.67–6.89)[a]	5.42(4.89–8.03)
Final urinary ACR (mg/mmol)	1.51(1.27–2.27)[b]	6.89(5.51–15.82)[ab]	38.49(11.10–282.90)[a]	44.10(2.57–249.2)[a]	92.80(20.31–219.70)[a]	34.87(9.45–230.80)[a]
Final 24 h urinary protein excretion (mg/24 h)	4.81(4.23–5.79)[b]	7.17(6.35–10.47)[b]	11.63(7.48–36.08)[a]	19.97(5.04–28.9)[a]	12.38(5.51–21.84)[a]	10.56(7.35–21.92)[a]

GFR/BW (ml/24 h/g) = [urinary creatinine * urinary flow (ml/min)]/[serum creatinine * body weight]

Urinary ACR (mg/mmol) = urinary albuminuria / urinary creatinine

Normally distributed data were given as mean ± SEM. Non-normally distributed data were given as median (25th–75th percentile)

[#a] $p < 0.05$ vs. Sham + ND + PBO, [*b] $p < 0.05$ vs. 5/6Nx + HSD + PBO

compared to Sham + ND + PBO control rats (Fig. 2C, D, Table 2). Notably, telmisartan, linagliptin and empagliflozin normalized the increased cardiac TMPRSS2 level compared to 5/6 Nx + HSD + PBO rats (Fig. 2C, D, Table 2).

We observed that in the kidney ACE2 is present in epithelial cells of the proximal tubule and distal tubule and a weak glomerular visceral ACE2 staining was observed, whereas the parietal and visceral epithelial cells were moderately positive (Fig. 3A) which was described previously [29, 30]. ACE2 is also observed in arterial endothelial cells (Fig. 3B). Moreover, ACE2 was predominantly found in tubules and a lesser extent in glomeruli. This is consistent with other studies also performed in rat kidney that found *Ace2* mRNA expression in tubules to be significantly higher expressed compared with in glomeruli [31,

Table 2 Renal and cardiac mRNA expression of SARS-CoV-2 host factors and genes involved in RAAS

	Sham + ND + PBO ($n=6$)	5/6Nx + ND + PBO ($n=6$)	5/6Nx + HSD + PBO ($n=5–6$)	5/6Nx + HSD + TELM ($n=6$)	5/6Nx + HSD + LINA ($n=6$)	5/6Nx + HSD + EMPA ($n=6$)
mRNA expression (kidney)						
Ace2	1.02 ± 0.09	1.19 ± 0.30	1.01 ± 0.33	1.14 ± 0.14	1.47 ± 0.47	1.33 ± 0.33
Tmprss2	1.03 ± 0.12	0.50 ± 0.09	0.86 ± 0.09	2.11 ± 0.41[b]	0.97 ± 0.08	4.23 ± 2.92[b]
Ren	1.06 ± 0.18	0.06 ± 0.02[ab]	0.06 ± 0.04[a]	1.56 ± 0.56[b]	0.12 ± 0.03[a]	2.52 ± 1.17[b]
Agtr2	1.03 ± 0.13	0.78 ± 0.21	1.08 ± 0.19	1.02 ± 0.13	0.84 ± 0.16	3.09 ± 1.41
Agt	1.10 ± 0.24	1.13 ± 0.28	1.02 ± 0.24	1.63 ± 0.29	1.57 ± 0.17	4.32 ± 1.67
mRNA expression (heart)						
Ace2	1.04 ± 0.19	0.94 ± 0.06	1.17 ± 0.08	1.08 ± 0.08	0.77 ± 0.06	1.03 ± 0.08
Tmprss2	n.d	n.d	n.d	n.d	n.d	n.d
Ren	n.d	n.d	n.d	n.d	n.d	n.d
Agtr2	1.01 ± 0.06	1.06 ± 0.07	1.13 ± 0.09	0.98 ± 0.06	0.91 ± 0.09	0.97 ± 0.5
Agt	1.04 ± 0.12	1.79 ± 0.23	0.95 ± 0.04	1.19 ± 0.20	0.77 ± 0.09	0.92 ± 0.07
	Sham + ND + PBO ($n=12–14$)	5/6Nx + ND + PBO ($n=7–12$)	5/6Nx + HSD + PBO ($n=15–20$)	5/6Nx + HSD + TELM ($n=7–11$)	5/6Nx + HSD + LINA ($n=10–13$)	5/6Nx + HSD + EMPA ($n=6–9$)
protein expression						
ACE2 (kidney)	29.89 (20.82–36.55)	27.10 (19.39–30.73)	13.96 (12.36–19.14)[a]	14.14 (9.35–19.34)[a]	33.25 (18.55–39.25)[b]	17.07 (12.59–19.96)
ACE2 (heart)	27.77 (25.58–32.96)	33.11 (30.74–36.34)	29.12 (23.97–32.42)	27.42 (24.57–31.01)	24.39 (22.32–29.63)	33.21 (26.57–35.92)
TMPRSS2 (kidney)	13.44 (11.64–17.31)	13.88 (11.89–15.73)	12.77 (8.53–14.21)	12.35 (10.79–18.26)	12.21 (9.72–13.90)	11.60 (10.62–13.29)
TMPRSS2 (heart)	11.90 (10.63–15.00)	29.57 (25.12–33.16)[a]	25.23 (16.18–30.46)[a]	10.40 (8.60–18.57)[b]	7.85 (6.03–13.39)[b]	12.00 (8.59–16.27)[b]

Normally distributed data were given as mean ± SEM. Non-normally distributed data were given as median × 10^6 (25th–75th percentile × 10^6). [a]$p<0.05$ vs. Sham + ND + PBO, [b]$p<0.05$ vs. 5/6Nx + HSD + PBO

32]. In the heart, ACE2 was found stronger expressed in myocytes than in arteries [33](Fig. 3C). TMPRSS2, in the kidney, was higher expressed in the distal convoluted tubule, but less expressed in the proximal tubule [34] (Fig. 3D), arteries and glomeruli (Fig. 3E) whereas in the heart, TMPRSS2 is predominantly expressed in myocytes (Fig. 3F).

Discussion

We used the ARB telmisartan, the DPP-4 inhibitor linagliptin and the SGLT2 blocker empagliflozin, in doses where we found positive pharmacodynamic action on systolic and diastolic blood pressures for all drugs under high salt diet conditions in the well-established experimental non-diabetic rat 5/6 nephrectomy. Our study shows that the induced effects on renal and cardiac mRNA and protein expression of the two key host proteins for SARS CoV-2 viral host cell entry (ACE2 and TMPRSS2) do not provide any evidence about facilitating SARS CoV-2 virus infection via the above-mentioned host receptors. The renal and cardiac gene expression level of *Ace2* was not affected either under disease conditions or under treatment conditions. Recently, it was demonstrated that the renal *Ace2* expression was not significantly altered in a STZ/high fat diet induced diabetic mouse model even if the animals were treated with ramipril (ACE inhibitor) or telmisartan [19], whereas the ACE2 protein expression was increased in this diabetic model independent of any treatment regimen. Our experimental model showed no effects on ACE2 protein expression after 5/6 nephrectomy in both the kidney and heart. Interestingly, high salt conditions led to significantly lower ACE2 level in the kidney which was normalized by linagliptin treatment, whereas the cardiac levels were unaffected. Linagliptin treatment significantly increased renal ACE2 level whereas this expression level was similar to the control groups sham and 5/6 Nx normal diet-fed rats. A recent study demonstrated that the administration of linagliptin significantly increased the ACE2 expression, which is consistent with this finding[35]. In addition, our study also revealed no significant effects on TMPRSS2 level (mRNA and protein) in the kidney which is consistent with previous finding in the experimental diabetes model [19]. However, the cardiac TMPRSS2 protein expression was significantly increased in the heart after 5/6 Nx and all drug interventions led to normalized cardiac TMPRSS2 suggesting a beneficial effect with regards to lower viral entry targets.

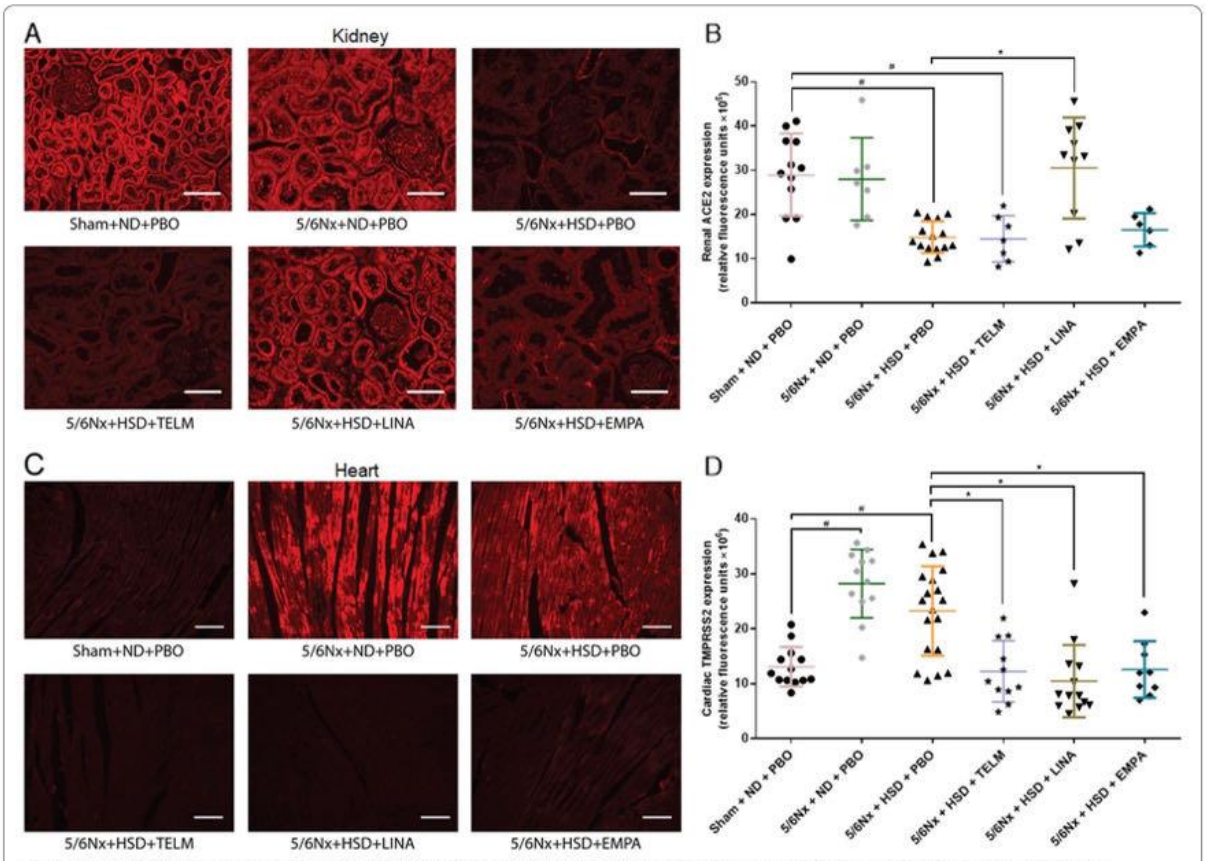

Fig. 2 Renal ACE2 protein expression and cardiac TMPRSS2 protein expression in different groups. **A-B** Effects of high salt diet, telmisartan, linagliptin and empagliflozin on renal protein expression of ACE2 and cardiac protein expression of TMPRSS2. **A** Photomicrographs of immunofluorescence-stained kidneys. The red color indicates ACE2. **B** Renal protein expression of ACE2. **C** Photomicrographs of immunofluorescence-stained hearts. The red color indicates TMPRSS2. **D** Cardiac protein expression of ACE2. Magnification × 20 [scale bars = 100 µm]. $^{\#}p < 0.05$ vs. Sham + ND + PBO, $^{*}p < 0.05$ vs. 5/6Nx + HSD + PBO

The discrepancy between changes in ACE2 and TMPRSS2 mRNA and protein expression was previously described in mouse and human studies [19, 36–38] indicating that the expression of ACE2 and TMPRSS2 is regulated at the post-transcriptional level. Recent studies demonstrated that post-transcriptional regulation of ACE2 can occur via microRNAs [39] or protein shedding [40]. Single-cell sequencing analysis revealed that *Ace2* is predominantly expressed in proximal tubules, whereas *Tmprss2* is predominantly expressed in the distal nephron [41, 42]. In heart tissue, Qi et al. showed that the cardiomyocytes contain 6% ACE2-expressing cells and 0.8% TMPRSS2-expressing cells [43] which might explain the absence of detectable cardiac *Tmprss2* mRNA levels in our study.

Our study revealed that only the expression of renin was affected by a more than tenfold suppressed level in placebo treated 5/6 Nx rats which has previously been described [44]. Telmisartan normalized the *Ren* mRNA level compared to linagliptin treated 5/6 Nx rats as detailed recently [45]. Also, empagliflozin restored the renin levels in 5/6 Nx rats. In a sub-study of a double-blind, randomized, placebo-controlled, multicentre study (EMPA-RESPONSE-AHF) empagliflozin treatment was associated with a significant increase in plasma renin compared to placebo treated patients [46]. In sham kidneys, abundant expression of *Ren* mRNA was noted in the juxtaglomerular apparatus and not in the tubular epithelium whereas subtotal nephrectomy (STNx) resulted in decreased renin level based on the loss of renal mass. Moreover, altered distribution of renin gene expression was detected in the kidney of nephrectomized rats resulted by de novo renin expression in renal tubular epithelial cells with minimal or absent expression in the juxtaglomerular apparatus [47]. In perindopril-treated STNx rats, areas distant from the infarct

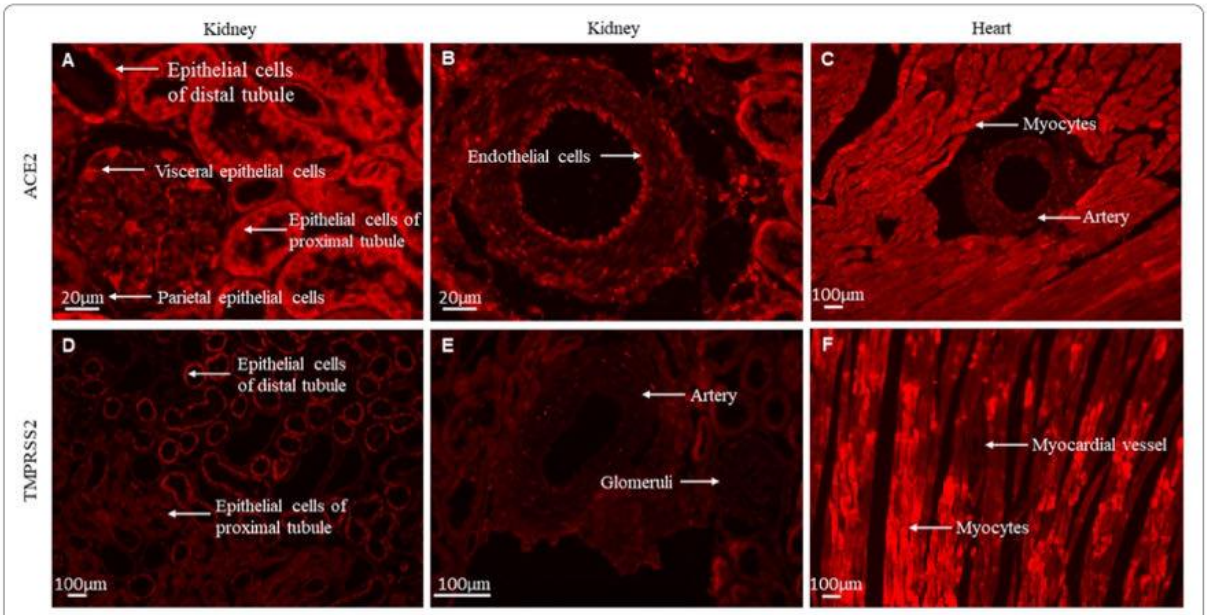

Fig. 3 Cellular localization of renal and cardiac ACE2 and TMPRSS2 expression. Photomicrographs of immunofluorescence-stained kidney and hearts. The red color indicates ACE2 and TMPRSS2. Magnification × 20. Upper panels show immunofluorescence staining of ACE2 in the glomerulus, proximal and distal tubules **(A)**, renal arterial endothelium **(B)**, cardiac myocytes and artery **(C)**. Lower panels show immunofluorescence staining of TMPRSS2 in proximal and distal tubules **(D)**, renal artery and glomerulus **(E)**, cardiac myocytes and myocardial vessel **(F)**

scar demonstrated a pattern of renin gene transcription similar to that of control animals which is in line with our findings observed in telmisartan and empagliflozin treated rats.

A recently conducted comprehensive meta-analysis reported that RAAS-blocking drugs are not associated with increased risk of severe outcomes in COVID-19 patients and may further decrease all-cause mortality in COVID-19 patients[1]. Furthermore, DPP4 plays a role in SARS-CoV-2 infection as a co-receptor, and sDPP4 levels are upregulated in obesity and T2DM, possibly complicating disease outcomes, if these patients acquire COVID-19. DPP-4 inhibitors are currently investigated as a therapeutic approach preventing cardiovascular complications in COVID-19 due to their anti-inflammatory effects at the vascular level. Several clinical studies are currently under investigation which use RAAS-blocking drugs (BRACE-CORONA (NCT04364893)), gliptins (SIDIACO (NCT04365517); linagliptin trials NCT04371978 & NCT04341935) and SGLT2 inhibitors (DARE-19 (NCT04350593) in COVID-19 patients.

Our study also has limitations. First, it must be shown that our data in a rat CKD model are transferable to humans. It is also important to investigate other animal models to verify whether our observations regarding the regulation of SARS CoV-2 host factors can also be found in other CKD animal models and thus be generalized. In particular, CKD animal models with diabetes would also be of interest.

Conclusion

Our study revealed that telmisartan, linagliptin and empagliflozin are not associated with a further increase in ACE2 and TMPRSS2 levels in kidney and heart tissue under high-salt condition compared to sham control and normal diet-fed 5/6 nephrectomy rats. The results obtained in a preclinical, experimental non-diabetic kidney failure model need confirmation in ongoing interventional clinical trials. Ongoing clinical trials with above mentioned drugs in the setting of COVID-19 will ultimately clarify their potential involvement.

Abbreviations

ACE2: Angiotensin-converting enzyme 2; ACR: Albumin-to-creatinine ratio; ARB: Angiotensin II receptor blockers; BNP45: Brain Natriuretic Peptide-45; BW: Body weight; CKD: Chronic kidney disease; DPP-4: Dipeptidyl peptidase-4; EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme-Linked Immunosorbent Assay; HSD: High salt-diet; ND: Normal diet; PBS-T: Phosphate-buffered saline/Tween 20; PBO: Placebo; RAAS: Renin-angiotensin-aldosterone system; SGLT2: Sodium-glucose Cotransporter-2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; SHR: Spontaneously hypertensive rats; TMPRSS2: Transmembrane protease, serine-subtype-2.

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Authors' contributions

BH, LY, TK, BKK and DD designed the study. YX, SZ, XC, CC and AAH carried out the experiments. YX, SZ, XC, CC, DD and AAH analyzed the results. YX, DD, LY and BH wrote the paper. YX, DD, SZ, XC, CC, AAH, BKK, TK, LY and BH revised the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The animal experiment was approved by laboratory animal ethics committee (20170904092822, Jinan University, Guangzhou, China) and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no.85–23, revised 1996); and in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, the Basel Declaration. Animals were euthanized according to the guidelines of the American Veterinary Medical Association (AVMA) Panel for euthanasia of animals (Ver. 2020).

Consent for publication

Not applicable.

Competing interests

Author Denis Delic and Thomas Klein are employed by Boehringer Ingelheim Pharma GmbH & Co. KG. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's Note

13. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

14. Publication list

1. **Xiong Y***, Delic D*, Zeng S, Chen X, Chu C, Hasan AA, Kramer BK, Klein T, Yin L, Hocher B. Regulation of SARS CoV-2 host factors in the kidney and heart in rats with 5/6 nephrectomy-effects of salt, ARB, DPP4 inhibitor and SGLT2 blocker. *BMC Nephrol* 2022, 23(1):117. DOI: 10.1186/s12882-022-02747-1 (IF 2.388)
2. Yaochen Cao*, **Yingquan Xiong***, Hongming Sun，Ziqiang Wang Neurorescuing effect of Cinacalcet against hypercalcemia‐induced nerve injury in chronic kidney disease via TRAF2/cIAP1/KLF2/SERPINA3 signal axis. Cell Biol Toxicol 2022, Doi: 10.1007/s10565-022-09717-1 (IF 6.819)
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