The differential effects of low dose sacubitril and/or valsartan on renal disease in salt-sensitive hypertension

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ABSTRACT

Diuretics and renin-angiotensin system (RAAS) blockers are often insufficient to control the blood pressure (BP) in salt-sensitive (SS) subjects. Abundant data support the proposal that level of Atrial Natriuretic Peptide (ANP) may correlate with pathogenesis of SS hypertension. We hypothesized here that increasing ANP level with sacubitril, combined with RAAS blockage by valsartan, can be beneficial for alleviation of renal damage in a model of SS hypertension, the Dahl SS rat. To induce a BP increase, the rats were challenged with a high salt 4% NaCl diet for 21 days, and chronic administration of vehicle or low dose sacubitril and/or valsartan (75 ug/day each) was performed. Urine flow, Na^+ excretion and water consumption were increased on HS diet compared to starting point (0.4% NaCl) in all groups, but remained similar among the groups at the end of the protocol. Upon salt challenge, we observed a mild decrease in systolic BP and urinary NGAL levels (indicative of alleviated tubular damage) in the valsartan-treated groups. Sacubitril, as well as sacubitril/valsartan, attenuated GFR decline induced by the salt. Alleviation of the protein casts formation and lower renal medullary fibrosis were observed in the sacubitril/valsartan and valsartan treated groups, but not when sacubitril alone was administered. Interestingly, proteinuria was mildly mitigated only in the rats receiving sacubitril/valsartan. Further studies of the effects of sacubitril/valsartan in the setting of SS hypertension, perhaps involving higher dose of the drug, are warranted to determine if it can interfere with the progression of the disease.

Keywords: salt-sensitive hypertension; atrial natriuretic peptide; valsartan; sacubitril.

INTRODUCTION

Hypertension is becoming more prevalent in the United States and the world, and thus increases the risk of cardiovascular complications such as heart disease and stroke in the population. According to CDC, one in every three Americans suffers from high blood pressure(74). The likelihood of hypertension increases with a consistent consumption of high salt(58). A specific subgroup of hypertensive individuals classified as “salt sensitive” (SS) exhibit significant changes in blood pressure in response to salt intake, and are at a higher risk for renal disease (14). For years, diuretics and the antagonism of the renin-angiotensin-aldosterone system (RAAS) have been recognized, although not universally effective, treatments for the SS hypertension (70). There is a pressing need to develop new, potent and multifarious treatments for the growing and diverse SS subpopulation.

One of the important factors that have been shown to play a role in SS hypertension is Atrial Natriuretic Peptide (ANP). ANP is an osmoregulatory protein that is encoded by *Nppa*; it has been associated with regulation of electrolyte homeostasis and blood pressure(43, 69). ANP lowers blood pressure by promoting salt excretion, and is generally considered a counteractant that keeps the RAAS in check(42). ANP is synthesized by atrial and, to a lower extent, ventricular cardiomyocytes in a 151 aa pre-pro-peptide form(6), which is proteolytically cleaved by corin to yield active 28 aa - long ANP(8, 12, 23, 49). Interestingly, corin knockout as well as ANP knockout mice appear hypertensive and salt-sensitive(5, 28, 71).

ANP is a particularly interesting hormone in the context of SS hypertension, as its plasma concentration correlates with the salt intake(2, 9, 47). Animal studies have shown that lack of ANP may result in SS hypertension, and also leads to biventricular hypertrophy and cardiomyocyte enlargement (independently of blood pressure increase)(39). Human studies revealed that in response to a high salt intake, secretion of ANP may be blunted in black SS subjects with hypertension(28, 59). There is abundant data supporting a pathogenic role for low level of ANP in salt-sensitivity; for instance, the Dallas Heart study showed that black individuals had significantly lower natriuretic peptide levels than white and Hispanic individuals, and concluded that this may lead to greater susceptibility to salt

retention and hypertension(18). Furthermore, a blunted ANP response to acute volume expansion was reported in SS subjects, particularly after a 5-day long HS diet(72). Most interestingly, information derived from the Framingham Offspring Cohort was able to predict SS hypertension by lower levels of circulating N-terminal ANP(31).

Therefore, ANP can be crucial for the condition of salt-sensitivity, and makes it a compelling therapeutic target(14). However, ANP cannot solely be used as treatment because of its short (<5 min) plasma half-life(50); thus, current ANP-related therapies are based on targeting the enzymes responsible for its degradation. Besides receptor-mediated degradation, ANP is cleared by extracellular proteolytic enzymes neprilysin (NEP) and insulin-degrading enzyme(1, 50). Combinations of neprilysin inhibitors (NEPi, such as sacubitril) and RAAS inhibitors (for instance, ARBs (angiotensin receptor blockers), such as losartan or valsartan), have been recently deemed successful in treating heart failure(19, 21). One of such medications, ENTRESTO® or LCZ-696 (1:1 combination of sacubitril and valsartan, also known as ARNi - angiotensin receptor-neprilysin inhibitor), is approved by FDA for heart failure treatment(15). It is important that NEPis should be administered together with a RAAS inhibitor: since NEPis increase circulating ANP and then lower blood pressure, this evokes a counteracting response from the RAAS, which needs to be prevented by an ARB or ACEi(21). Medications based on ARBs and NEPis show potential for patients with chronic kidney disease (CKD), and might have an effect on hypertension(21, 27, 29, 62). Therefore, there is reasonable evidence to justify testing potential beneficial effects of increasing the circulating ANP levels using NEPis in renal disease, and in especially SS hypertension, where existing medications are often insufficient to properly control the blood pressure. In this study, we focused on the effects of LCZ-696 on the development of renal damage in Dahl SS rats, an established rodent model mimicking major aspects of human SS hypertension.

MATERIALS AND METHODS

Animal procedures and experimental protocol. Male Dahl SS rats were obtained from Charles River Laboratories (USA) at 7 weeks of age, and were kept on a purified AIN-76A based 0.4% NaCl diet (NS, Dyets Inc, cat no 113755) for a week. In order to induce salt-sensitive hypertension at the age of 8 weeks they were switched to a purified AIN-76A based 4% NaCl diet (HS, Dyets Inc) for 21 days. **Figure 1A** shows the experimental protocol schematic. The prospective groups were administered vehicle, sacubitril, valsartan, or a 1:1 mix of sacubitril/valsartan at 75 µg/day (each) via an osmotic pump (Alzet 2ML4, 2.5 µl/hour) installed subcutaneously 3 days before the HS dietary challenge. Glomerular filtration rate (GFR) was measured a day before the osmotic pump surgery, and at day 20 of HS; urine was collected in metabolic cages (Lab Products Inc) for 24 hours before the GFR measurements (following 24 hours of metabolic cage adjustment). Animals were weighed the day of GFR measurements. All experimental procedures regarding the Dahl SS rats were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee, as well as adhering to the NIH Guide for Care and Use of Laboratory Animals.

Blood pressure measurements, kidney flush and tissue isolation. Blood pressure measurements via tail cuff plethysmography (IITC Life Science Inc, USA) were obtained from each rat immediately before the endpoint kidney flush. At the completion of the HS diet salt challenge for 21 days, rats were surgically prepared for a kidney flush and arterial blood collection. Briefly, the rats were anesthetized, and the descending aortas were catheterized as done previously(11). Kidneys were first flushed with PBS (2 mL/min/kidney) until blanched via a catheter in the abdominal aorta. The kidneys were then excised and decapsulated. The kidney tissues were then snap frozen in liquid nitrogen or stored in 10% formalin for histological assessment.

Glomerular filtration rate measurements. GFR was measured in unrestrained conscious rats using a high-throughput method featuring detection of fluorescent FITC-labeled inulin (TdB Consultancy AB, Uppsala, Sweden) clearance from blood. The method was adapted for rats from a protocol previously described for mice by Rieg(55) and published by us earlier(24). Pre-dialyzed 20 mg/mL of FITC-inulin

126 solution in saline (2 μL per 1 g of body weight) was administered by a bolus tail vein injection to rats
127 briefly anesthetized with isoflurane. Immediately after the injection anesthesia was discontinued, and
128 the animals were allowed to regain consciousness. Then, 10 μL of blood was collected 3, 5, 8, 16, 25,
129 40, 60, 80, 100, and 120 min after the injection by tail bleed. Next, plasma was separated, and inulin
130 clearance was quantified by FITC intensity. Fluorescence measurements were performed using a
131 NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Wilmington, DE, USA). GFR was then
132 calculated from the observed decrease in FITC fluorescence using a two-compartment model (the initial
133 fast decay representing the redistribution of FITC-inulin from the intravascular compartment to the
134 extracellular fluid, and the slower phase reflecting clearance from plasma). The GFR curves were
135 approximated with a bi-exponential decay function using OriginPro 9.0 (OriginLab, Northhampton, MA)
136 software, and GFR values in mL/min were obtained from the fitting parameters using the previously
137 described equation(24).

138 **Tissue processing, histological staining and analysis.** Rat kidneys were fixed in zinc formalin,
139 paraffin embedded, then sectioned, and mounted on slide, following standard procedures. The slides
140 were stained with Masson trichrome and imaged on a Nikon Eclipse Ti-2 microscope. The tissues were
141 randomized and coded before being submitted for blocking, sectioning, and staining. Nikon Plan Fluor
142 optical lens of 20X was used to assess glomeruli (0.50 NA, 2.1 WD). Glomeruli were blindly scored on
143 a 0 - 4 scale. A score of 0 represented a healthy glomerulus with no sclerosis. Score of 1 represented a
144 1- 25% of mesangial expansion and sclerosis compared to a score of 2 which represented 26- 50% of
145 mesangial expansion and sclerosis. A score of 3 was given if 51- 75% of mesangial expansion and
146 sclerosis and score 4 represented 76- 100% of glomerular mesangial expansion and sclerosis. For the
147 analysis of fibrosis, Picro Sirius Red stained kidneys were imaged with a Nikon Eclipse Ti-2 microscope
148 for further use in digital analysis. Fiji (NIH) software was used to determine the percentage of fibrosis:
149 the region of interest (ROI, whole kidney) was selected and the area of the ROI was measured. Then,
150 using Color Deconvolution Plugin in ImageJ, the area of fibrosis was identified using a Threshold tool,
151 and percentage of total area was calculated (n=5-7, 10x images from each kidney were used for the
152 analysis). Protein casts were assessed from Trichrome stained slides scanned with a Perkin Elmer

Vectra Polaris Automated Quantitative Pathology Imaging System Slide scanner, and then scored separately and blindly by two people, on a 0-4 scale. A score of 0 represented a healthy kidney samples with no protein casts visible. Score of 1 represented a kidney sample with <5% protein casts compared to a score of 2, which represented 5- 10% of the sample containing protein casts. A score of 3 was given to a sample with 11- 15% protein casts, and a score of 4 was given to a kidney with >20% protein casts. Picro Sirius Red (PSR) was used to stain for collagen. Slides were incubated in a solution of 0.2% phosphomolybdic acid (EMS, RT 26357-01) for 3 minutes. The slides were then rinsed and transferred to the solution containing 0.1% Sirius Red in saturated picric acid (EMS RT 26357-02) for 90 minutes. Slides were then immediately put into acidified water for 2 minutes. All images were acquired at 10X using a Nikon Ti-2 microscope equipped with NIS Elements software and DS-Fi3 camera, and analyzed using Fiji (NIH).

Urinalysis (electrolytes, creatinine, protein) and plasma analysis (electrolytes, BUN). For the assessment of proteinuria, urine samples were centrifuged at 100g for 3 min to remove debris, and supernatants were used for the estimation of proteinuria by SDS PAGE. Each urine sample (10 µL) was mixed with Laemmli buffer (4x) in a ratio of 1:1 and heated at 90 ºC for five minutes. Then, samples were loaded into wells of a Criterion 26-well gel (cat# 3450044) and run at 120V using a High Current Bio-Rad PowerPac electrophoresis power supply for one hour. BSA (5 µg) was used as a reference point. Then, the gel was stained with Coomassie blue solution (0.01% Coomassie brilliant blue R 250, 50% (v/v) methanol, and 10% (v/v) glacial acetic acid) for 30 minutes at room temperature, and an image was acquired with a LI-COR Odyssey imaging system. Analysis was performed in Fiji (NIH); values were adjusted for 24-hour urinary flow rate.

The urine and plasma electrolytes were evaluated with a Carelyte™ analyzer from Diamond Diagnostics (to separate plasma, blood samples obtained from the abdominal aorta before kidney flush were centrifuged immediately after collection at 9600 rpm for 5 minutes, then snap-frozen in LN2 and stored at -80C). Plasma creatinine levels were measured using the Quantichrom Creatinine Assay Kit (DICT-500). A standard curve was created from the stock 50mg/dL creatinine standard (6 mg/dL, 2mg/dL, 1mg/dL, 0.5 mg/dL and 0 mg/dL). Creatinine concentrations were determined by measuring

absorbance per the manufacturer's instructions. BUN and aldosterone levels were measured using a urea assay kit (Abnova, #KA1652) and aldosterone ELISA (Enzo Life Sci, #ADI900173), respectively, according to manufacturers' instructions.

Western blotting. After excision, kidneys were cut in 1-2 mm slices, and the cortical kidney pieces were pulse-sonicated in RIPA buffer containing a protease inhibitor cocktail (Roche) on ice, and then spin cleared at 10,000x g for 10 min. The resulting supernatant was subjected to SDS-PAGE, transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) for probing with antibodies, and subsequently visualized by enhanced chemiluminescence (ECL; Thermo Scientific, Waltham, MA, USA). The following antibodies were used: ANP antibody (Invitrogen, PA5-79758) Goat anti-Rabbit IgG secondary antibody, HRP (Invitrogen, 31460), Alpha-Smooth Muscle Actin Antibody (Invitrogen, 14-9760-82), and anti-Mouse IgG, HRP (Promega, W4021B). Western blot analysis was also performed to determine the presence of KIM-1 and NGAL in urine. Urine samples were prepared by mixing spin-cleared urine with 2x Laemmli buffer (with BME) 1:1; 15 µl of each sample were loaded on the gel. KIM-1 antibody (Invitrogen, PA5- 793452) or NGAL antibody (pro# PA5- 46938) were used followed by an HRP-conjugated secondary antibodies (Invitrogen, 31460).

Statistical Analysis. One-way ANOVA with Holm- Sidak test post-hoc and One-way repeated measures ANOVA Holm-Sidak test were used when applicable. Data is expressed as box plots, the whiskers being the SD, the box representing the SEM, and the line showing the median; values of $p < 0.05$ were considered statistically significant. Origin 2019b was used for all statistical analysis.

RESULTS

Assessment of basic renophysiological parameters post drug administration. **Figure 1A** shows the timeline of the experimental protocol (described in detail in the Methods section). Body weight was measured in all groups before the start of the high salt challenge (on NS), and after 21 days on the HS diet. Body weight increased significantly at the end of the experiment compared to the NS ($p< 0.05$ for all groups; **Suppl. Figure 1S**). Endpoint kidney to body weight, heart to body weight ratios and body weight were similar between groups (results reported in **Figure 1B** and supplementary **Figure 1S,** https://figshare.com/s/ffddf9a743e0166dd926; DOI: 10.6084/m9.figshare.12049134). Blood pressure was assessed at the end of the experimental protocol (**Figure 1C**), and we report a significant decrease in systolic blood pressure in the VAL group vs VEH (155.8±7.7 vs 176.0±6.9 mmHg, respectively). We confirmed a significant increase in ANP level in the animals treated with SAC and S/V (see **Figure 1D** for a Western blot for ANP conducted in heart tissue). Endpoint plasma electrolyte levels are reported in Supplementary **Figure 2S** (https://figshare.com/s/ffddf9a743e0166dd926; DOI: 10.6084/m9.figshare.12049134), and were found similar among the studied groups.

24-hour urine flow rate was obtained in metabolic cages (following a 24-hour adjustment period) 4 days prior to administration of the dietary challenge, as well as on day 21 of the HS diet. As expected, all groups showed a statistically significant increase in urine production when comparing the NS time point to the day 21 of the HS challenge, while endpoint values were similar among groups ($p< 0.001$ for all groups; **Figure 2A**). Daily water consumption recorded on day 21 of the HS diet was similar among the groups (**Figure 2B**). GFR (glomerular filtration rate) measured before the start of the dietary challenge (**Figure 2C**) was similar among the groups (0.88±0.02, 0.81±0.03, 0.83±0.05 and 0.79±0.01 mL/min/100g body weight in VEH, S/V, SAV and VAL groups, respectively). At the end of the protocol, we observed a decrease in GFR in the control group compared to NS diet ($p=0.048$). Hyperfiltration was noted in the groups which were administered SAC (with or without VAL, p-values vs control 0.002 and 0.017, respectively), which was attenuated in the VAL treated group (0.80±0.03, 1.03±0.05, 0.90±0.05 and 1.00±0.06 mL/min/100g body weight in VEH, S/V, SAV and VAL groups, respectively).

Urinary osmolar and electrolytes excretion. Urine samples obtained in metabolic cage studies were used to determine electrolyte and osmolar excretion over a 24-hour time period (**Figure 3A-D**). We report a significant increase in urinary Na^+, Cl^- , and osmoles excretion in all urine samples collected from rats fed a HS when compared to a paired point before the dietary salt challenge. Among the groups fed the same diets excretion values for Na^+, Cl^- , and osmoles were similar. Interestingly, urinary K^+ excretion increased after the HS challenge in S/V (p=0.01) and SAC groups vs vehicle (p=0.02) (**Figure 3C**).

Renal damage. Analysis of renal damage markers in endpoint urine samples using Western blots revealed interesting trends. We report a significant decrease in NGAL excretion from rats treated with S/V, SAC and VAL, vs VEH group (data was normalized to urine flow rate, **Figure 4A**), indicative of lower tubular damage in these groups. Furthermore, VAL treatment also decreased KIM-1 excretion compared to the VEH-treated group (p=0.02; **Figure 4B**). Endpoint tissues collected from all four groups (HS diet) were stained with Masson Trichrome to assess glomerular damage and protein cast formation. Blinded glomerular damage scoring revealed similar glomerular lesions across all groups (**Figure 5A, B**). Protein casts analysis showed a dramatic attenuation of medullary and cortical protein casts formation in S/V and VAL groups, compared to control animals (**Figure 5C**). Picro Sirius Red (PSR) staining (**Figure 6A**) revealed a significant decrease in medullary, but not cortical, fibrosis of the animals treated with S/V (p=0.013 vs VEH-treated group). Next, we tested αSMA levels in renal cortex, and the Western blot revealed a lot of variation in the treated groups vs control (**Figure 6B**), therefore, no statistical significance was recorded. In accordance with protein casts analysis, we observed an attenuation of endpoint proteinuria in the S/V group vs VEH (p = 0.062 in s/v vs veh, **Figure 7 A, B**). As shown in **Figure 8A**, creatinine excretion was elevated in all treated groups vs VEH, while no differences in BUN were recorded (**Figure 8D**); plasma creatinine was not different between the groups (**Figure 8B**). Urinary aldosterone/creatinine ratio was assessed, and the values were similar among groups (**Figure 8C**).

DISCUSSION

The renin-angiotensin-aldosterone system (RAAS) is a crucial factor for the development of hypertension, as indicated by the successful use of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) to decrease blood pressure(13). However, a blunted RAAS is an essential characteristic of SS hypertension, and one of the reasons why ARBs are considered inferior to other treatments, such as calcium channel blockers and diuretics, in the reduction of blood pressure in patients with this form of hypertension(51). Interestingly, the plasma concentration of ANP correlates with the salt intake(2, 9, 47). Further, animal studies have shown that a lack of ANP may result in SS hypertension(39), while human studies have revealed that in response to a high salt intake, secretion of ANP may be blunted in SS subjects with hypertension(28, 59). These observations clearly point to the fact that ANP plays a critical role in mitigating the development of SS hypertension(14). Taking into consideration the success of ARNi (ARB/NEPi) to treat various cardiovascular complications, it was compelling to test the effects of these drugs in kidney disease and SS hypertension. Interestingly, the recent UK HARP-III trial, which assessed if neprilysin inhibition improved kidney function in CKD in the short - to medium term, found no effect on renal function(21) (vs an ARB control). In this study, we compared the effects of sacubitril (NEPi), valsartan (ARB) or their combination (ARNi) in the Dahl SS rat, a well-established model of salt-sensitive hypertension and associated renal damage.

We picked a relatively low dose of drugs for this study, an average of ~0.3 mg/kg/day of each drug (0.6 mg/kg/daily total for drug combination) was given to animals throughout the protocol. For humans, recommended starting oral dose of LCZ-696 is 25 to 50 mg/twice daily, which translates into approximately 0.7 to 1.5 mg/kg/day for a 70 kg person. Furthermore, we dispensed the drugs continuously, via an osmotic pump implanted subcutaneously. Various regimens for ARB/NEPi dosing have been reported. For instance, sacubitril/valsartan combination was administered to Zucker Obese rats at 68 mg/kg/day (oral gavage for 10 weeks)(19) or Sprague Dawley rats which underwent a 5/6 nephrectomy at 60 mg/day (also by gavage)(27, 62). In a different subtotal nephrectomy study, Wistar rats received 30 mg/kg LCZ696, daily by gavage(64). In another study, a combination of irbesartan

(ARB) and thiorphan (NEPi) was given to diabetic rats via an osmotic pump at 0.1 mg/kg/day via an osmotic minipump(57). Therefore, the selected dose here is on the lower side of the range, although the route of administration should be taken into consideration.

We observed differential effects of ARB, NEPi and the combination of the two on renal function and overall physiology (major experimental outcomes are summarized in **Table 1**). Two main effects were largely driven by LCZ-696: reduction in proteinuria, and renal medullary fibrosis (however, renal protein casts formation was also found to be reduced in the valsartan-treated animals). These findings are in accordance with studies that showed a reduction in proteinuria when ARBs were used together with NEPi vs ARB alone in kidney disease(19, 27, 57). Interestingly, the UK-HARP-III trial demonstrated that over the 12-month period, sacubitril/valsartan had similar effects on albuminuria to irbesartan(20). In the present study, sacubitril/valsartan in combination were able to lower renal medullary, but not cortical, fibrosis (shown in PSR staining). This finding is in line with the data reported by others. In kidney disease, LCZ-696 has been reported to ameliorate oxidative stress, inflammation and fibrosis, beyond treatment with ARB alone(27). However, it is also possible that treatment with valsartan or sacubitril alone may attenuate renal fibrosis. In diabetic kidney disease, renal periarterial and tubulointerstitial fibrosis were reduced in all treatment groups (sacubitril/valsartan, valsartan, and an anti-hypertensive drug) to a similar extent(19). Although several studies have shown that LCZ-696 attenuates fibrosis in cardiac tissue(4, 36, 62), a recent commentary in J Am Coll Cardiol, following the manuscript by Zile et al(79), raised the question if LCZ-696 is truly anti-fibrotic(76), and suggests that the various markers of renal fibrosis might be affected differentially, depending on the severity of the damage and the underlying cause. In order to fully comprehend the mechanisms behind this complex clinical picture, a more thorough study focused on fibrosis-related outcomes is warranted.

Overall, we found that the majority of the outcomes were driven by valsartan. However, we observed a mild increase in potassium excretion compared to baseline, in the groups that were administered sacubitril, compared to the starting point (no drug). There are multiple factors that might have contributed to this phenomenon; firstly, it is important to mention that there was no difference in potassium excretion when the independent groups were compared, therefore, this might be an artifact

of the metabolic cage collections, especially since the rats are presumably in a steady state after 21 days of HS diet. On the one hand, there are known effects of ANP on sodium and potassium transport. The actions of ANP along the nephron include inhibiting the N+/K+-ATPase, reducing apical Na+, K+, and protein organic cation transporter in the proximal tubule, decreasing NKCC activity in the TAL (63), and decreasing ENaC activity (17). In addition, ANP has been shown to dramatically reduce salt appetite (3, 26, 61), which can in turn affect potassium excretion (75). In a study by Vormfelde et al, it was shown that carriers of low functional alleles of ANP excreted more potassium when given a diuretic, than carriers of the higher functional alleles (67). However, if this is the case and ENaC is being inhibited in the sacubitril-treated groups and there no other confounding factors, ANP increase should result in lower potassium excretion. We believe that further research is needed to explore the potentially exciting interaction between ANP and potassium transport in SS hypertension, in a study designed to specifically resolve this question in this setting.

When the effects of valsartan are concerned, first and foremost, systolic blood pressure was significantly reduced by valsartan only, largely unaffected by sacubitril, and was trending towards a decrease when sacubitril was administered together with valsartan. A study by Imanishi et al(25) demonstrated in diabetic patients, that ARBs reduce the salt sensitivity of blood pressure by decreasing renal oxidative stress. In UK-HARP-III trial in patients with CKD, compared with irbesartan, allocation to sacubitril/valsartan was able to reduce average systolic and diastolic blood pressure by 5.4 (95% CI, 3.4-7.4) and 2.1 (95% CI, 1.0-3.3) mmHg(20). This could be attributed to the overall higher effectiveness of valsartan vs irbesartan, since the UK-HARP-III trial did not include valsartan-only or sacubitril-only groups. Nixon et al reported that in patients with essential hypertension valsartan is more effective at lowering blood pressure than losartan and shows comparable efficacy to other ARBs(45). However, a study in salt-sensitive Asian subjects showed that sacubitril/valsartan resulted in significantly greater decreases in ambulatory BP values compared with valsartan(70). Since there are significant genetic variations in factors that predispose humans (and animals) to salt-sensitivity(14, 30, 33, 35, 44), the genetics must be taken into consideration when assessing the effectiveness of the drugs.

In addition to blood pressure, in our study, valsartan drove the alleviation of tubular damage, renal cortical fibrosis, and renal protein cast formation. Jing et al reported that in Sprague Dawley rats that underwent a 5/6 nephrectomy, the degree of tubulointerstitial injury and glomerulosclerosis in LCZ-696 treated animals was significantly less compared to both the valsartan-alone, and the untreated groups (27). In diabetic nephropathy, KIM-1 was found to be reduced in rats treated with sacubitril/valsartan vs valsartan alone (19). In contrast, we show that urinary NGAL was significantly reduced in all three treatment groups, while urinary KIM-1 excretion was only significantly lower in valsartan-treated animals. Although the present findings are generally in line with previously reported findings, we need to also assess it from the perspective of GFR and urinary flow. In humans with CKD, sacubitril/valsartan was shown to improve eGFR compared to baseline (52). Further, in a rat model of diabetic nephropathy sac/val prevented hyperfiltration compared to valsartan only (19). Our study demonstrates a sacubitril-driven improvement in GFR. While we see a typical renal damage-driven decrease in GFR in control animals fed a high salt diet (7), endpoint GFR is higher in sacubitril and sacubitril/valsartan groups, but not in valsartan-only group. We can assume that high salt diet would evoke faster filtration due to an increased salt load and water consumption, which later decreases due to renal tissue damage; thus, we can hypothesize that sacubitril attenuated the GFR decline evoked by a high salt diet.

The importance of ANP has been established in inflammation-associated conditions in kidney, heart, pancreas, and lungs (10, 22, 41, 46, 77). Among recent findings, it was shown that ANP could downregulate IL-1β release by inhibiting the NLRP3 inflammasome (37), and was able to attenuate inflammatory responses in an acute lung injury model (78). A linkage of ANP to the immune system, and later its role in innate immune functions as well as in the adaptive immune response was proposed (40, 65, 66). However, RAAS is also known to be an important regulator and effector of inflammation, and potential therapeutic use of RAAS inhibitors has been proposed in the treatment of inflammatory diseases (38, 48, 54, 60, 73). In SS hypertension, in particular, inflammation is a well-known player, and inhibition of angiotensin receptors has been repeatedly associated with decreased kidney inflammation in the setting (16, 32, 53, 56, 68). However, in a condition of hypernatremia, a widely used

ARB, losartan, was not able to decreases the overexpression of the inflammatory markers, while ANP was deemed as a useful tool to regulate the expression of key components of the tubulointerstitial inflammation in the renal medulla (10). We speculate that modifications of the immune system and renal inflammation are important factors that could contribute to the observed renal outcomes, and the differential effects of sacubitril/valsartan and valsartan are due to their effects on inflammation. More studies are required to support these speculations, and we believe that further research into the potential link between ANP and inflammation in the setting of SS hypertension will close an important gap in knowledge.

Interestingly, a recent manuscript by Lunder et al showed that very low-dose fluvastatin-valsartan combination decreases parameters of inflammation and oxidative stress in patients with type 1 diabetes (34). Our data show that low-dose administration of sacubitril, valsartan and combination drug LCZ-696 have mild beneficial, although differential effects on renal damage, fibrosis, proteinuria, tubular damage, and blood pressure. This shows the plausibility of repurposing LCZ-696 for treatment of renal damage induced by SS hypertension. Thus, our study opens new possibilities and sets the stage to explore if low dose combination treatment could have clinical benefits for SS individuals. However, there is a need for more research studies and higher dosing, in different animal models and diverse genetic backgrounds, in order to completely close the existing gap in knowledge.

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FIGURE LEGENDS.

Figure 1. **Experimental protocol and basic endpoint parameters**. (**A**) Schematic representation of the experimental protocol. NS – normal salt diet; HS – high salt diet; MC – metabolic cage collections; GFR – glomerular filtration rate measurements; BP- blood pressure measurement; OP – osmotic pump installation. (**B**) and (**C**): endpoint two-kidney to total body weight ratio (B) and systolic blood pressure value (C) in VEH, S/V, SAC and VAL groups. (**D**) Western blotting showing ANP expression in the endpoint-collected heart tissues from the VEH, S/V, SAC and VAL groups. Each point on the graphs denotes data obtained from one animal; one-way ANOVA with Holm-Sidak was used for significance comparisons; p-values are shown for comparisons where $p<0.05$.

Figure 2. **In vivo renal function comparison in experimental groups**. (**A**) 24-hour urine flow (normalized to body weight) obtained from experimental animals before (NS) and on day 20 after a switch to a HS diet and administration of VEH, S/V, SAC and VAL. (**B**) Body weight normalized endpoint water consumption in studied experimental groups. (**C**) Glomerular filtration rate measured in experimental animals before (NS) and on day 20 after a switch to a HS diet and administration of VEH, S/V, SAC and VAL. Representative curves of FITC-inulin elimination are on the left (VEH treated animal, on NS diet and at the end of the HS diet protocol). Summarizing graph is shown on the right panel. Each point on the graphs denotes data obtained from one animal; one-way ANOVA with Holm-Sidak was used for significance comparisons; p-values are shown for comparisons where $p<0.05$.

Figure 3. **Electrolyte and osmoles excretion**. Excretion of Na^+ (**A**), Cl^- (**B**), K^+ (**C**) and total osmoles (**D**) excretion measured in urine samples collected for 24 hours from experimental animals before (NS) and on day 20 after a switch to a HS diet and administration of VEH, S/V, SAC and VAL. Each point on the graphs denotes data obtained from one animal; one-way ANOVA with Holm-Sidak was used for significance comparisons. Paired data (before-after HS diet) was compared using Student paired T-test. P-values are shown for comparisons where $p<0.05$.

Figure 4. **Analysis of renal tubular damage markers in the urine.** Shown are Western blot analyses of urinary NGAL (**A**) and KIM-1 (**B**) levels obtained from experimental animals on day 20 after a switch

to a HS diet and administration of VEH, S/V, SAC and VAL. Each lane on the Western blot represents a separate experimental animal. Summaries of densitometry values (normalized to 24 hour urine flow) are shown on the right. One-way ANOVA with Holm-Sidak was used for significance comparisons; p-values are shown for comparisons where $p<0.05$.

Figure 5. **Histological characterization of renal damage with Masson Trichrome staining**. (**A**) Representative images of renal tissues from experimental rats isolated at the endpoint of the experimental protocol (HS diet, upon administration of VEH, S/V, SAC and VAL). Top row shows scans of the coronal mid-sections of the kidneys stained with Masson Trichrome (scale bar 2 mm). Middle panel and bottom row demonstrate representative 10x images taken in the cortical area (scale bar 100 μm), and enlarged images of glomeruli from the renal cortex (scale bar 50 μm), respectively. Also shown are graphs summarizing the analysis of glomerular damage scoring (**B**), and protein casts scoring (**C**). One-way ANOVA with Holm-Sidak was used for significance comparisons; p-values are shown if data was statistically significant. Each point on the graphs denotes data obtained from one animal, except from (**B**), where each point is an average of at least 100 glomeruli scored in renal tissue of individual animals. P-values are shown for comparisons where $p<0.05$.

Figure 6. **Renal fibrosis analysis in the experimental groups**. (**A**) Representative images of renal tissues stained with PSR. Shown are images of cortex and medulla (10x, scale bar 100 μm) obtained from rats at the endpoint of the experimental protocol (HS diet, upon administration of VEH, S/V, SAC and VAL). Analysis of the staining is shown on the right. (**B**) Expression of αSMA measured in the renal cortex of the animals at the endpoint of the experimental protocol. Each lane on the Western blot represents a separate experimental animal. Summaries of densitometry values are shown on the right, total protein staining (Ponceau) is below the Western image. One-way ANOVA with Holm-Sidak was used for significance comparisons. P-values are shown for comparisons where $p<0.05$.

Figure 7. **Endpoint quantification of proteinuria.** Shown are Western blots obtained on urinary samples collected from experimental animals before (NS) and on day 20 after a switch to a HS diet and administration of VEH, S/V, SAC and VAL. 5 μg BSA was used as a loading control (first lane).

Quantification shown on the bottom row was performed by normalizing endpoint proteinuria value (corrected for urine flow) to starting point. Each point on the graphs denotes data obtained from one animal; one-way ANOVA with Holm-Sidak post-hoc was used for significance comparisons among groups on the same salt diet. Paired data (before-after HS diet) was compared using Student paired T-test. P-values are shown for comparisons where $p<0.05$.

Figure 8. **Plasma and urinary creatinine, aldosterone and BUN levels**. Shown are creatinine excretion (**A**, normalized to urine flow), plasma creatinine level (**B**), aldosterone to creatinine ratio (in the urine, **C**), and BUN (blood urea nitrogen, **D**). All data was obtained at the endpoint of the experimental protocol. One-way ANOVA with Holm-Sidak post-hoc was used for significance comparisons among groups. Each point on the graphs denotes data obtained from one animal. P-values are shown for comparisons where $p<0.05$.

Table 1. Summary of the experimental outcomes. Summary of the experimental outcomes. Shown is a summary of significant outcomes of the study driven by sacubitril only, valsartan only, both sacubitril and valsartan, and their combination. Shown are outcomes with $p<0.05$, and important outcomes with $p>0.05$ (due to lower power). An increase, decrease and no change (vs a vehicle-treated group) are denoted ↑, ↓, and ↔, respectively.

REFERENCES CITED

1. **Ando K, Umetani N, Kurosawa T, Takeda S, Katoh Y, and Marumo F.** Atrial natriuretic peptide in human urine. *Klin Wochenschr* 66: 768-772, 1988.
2. **Angelis E, Tse MY, and Pang SC.** Interactions between atrial natriuretic peptide and the renin-angiotensin system during salt-sensitivity exhibited by the proANP gene-disrupted mouse. *Mol Cell Biochem* 276: 121-131, 2005.
3. **Blackburn RE, Samson WK, Fulton RJ, Stricker EM, and Verbalis JG.** Central oxytocin and ANP receptors mediate osmotic inhibition of salt appetite in rats. *Am J Physiol* 269: R245-251, 1995.
4. **Burke RM, Lighthouse JK, Mickelsen DM, and Small EM.** Sacubitril/Valsartan Decreases Cardiac Fibrosis in Left Ventricle Pressure Overload by Restoring PKG Signaling in Cardiac Fibroblasts. *Circ Heart Fail* 12: e005565, 2019.
5. **Chan JC, Knudson O, Wu F, Morser J, Dole WP, and Wu Q.** Hypertension in mice lacking the proatrial natriuretic peptide convertase corin. *Proc Natl Acad Sci U S A* 102: 785-790, 2005.
6. **Chopra S, Cherian D, Verghese PP, and Jacob JJ.** Physiology and clinical significance of natriuretic hormones. *Indian J Endocrinol Metab* 17: 83-90, 2013.
7. **Cowley AW, Jr., Ryan RP, Kurth T, Skelton MM, Schock-Kusch D, and Gretz N.** Progression of glomerular filtration rate reduction determined in conscious Dahl salt-sensitive hypertensive rats. *Hypertension* 62: 85-90, 2013.
8. **Crimmins DL and Kao JL.** A 68 residue N-terminal fragment of pro-atrial natriuretic peptide is a monomeric intrinsically unstructured protein. *J Biochem* 150: 157-163, 2011.
9. **Cuneo RC, Espiner EA, Crozier IG, Yandle TG, Nicholls MG, and Ikram H.** Chronic and acute volume expansion in normal man: effect on atrial diameter and plasma atrial natriuretic peptide. *Horm Metab Res* 21: 148-151, 1989.
10. **Della Penna SL, Roson MI, Toblli JE, and Fernandez BE.** Role of angiotensin II and oxidative stress in renal inflammation by hypernatremia: benefits of atrial natriuretic peptide, losartan, and tempol. *Free Radic Res* 49: 383-396, 2015.
11. **Domondon M, Polina I, Nikiforova AB, Sultanova RF, Kruger C, Vasileva VY, Fomin MV, Beeson GC, Nieminen AL, Smythe N, Maldonado EN, Stadler K, and Ilatovskaya DV.** Renal Glomerular Mitochondria Function in Salt-Sensitive Hypertension. *Front Physiol* 10: 1588, 2019.
12. **Dong L, Wang H, Dong N, Zhang C, Xue B, and Wu Q.** Localization of corin and atrial natriuretic peptide expression in human renal segments. *Clin Sci (Lond)* 130: 1655-1664, 2016.
13. **Drenjancevic-Peric I, Jelakovic B, Lombard JH, Kunert MP, Kibel A, and Gros M.** High-salt diet and hypertension: focus on the renin-angiotensin system. *Kidney Blood Press Res* 34: 1-11, 2011.
14. **Elijovich F, Weinberger MH, Anderson CA, Appel LJ, Bursztyn M, Cook NR, Dart RA, Newton-Cheh CH, Sacks FM, Laffer CL, American Heart Association P, Public Education Committee of the Council on H, Council on Functional G, Translational B, and Stroke C.** Salt Sensitivity of Blood Pressure: A Scientific Statement From the American Heart Association. *Hypertension* 68: e7-e46, 2016.
15. **Fala L.** Entresto (Sacubitril/Valsartan): First-in-Class Angiotensin Receptor Neprilysin Inhibitor FDA Approved for Patients with Heart Failure. *Am Health Drug Benefits* 8: 330-334, 2015.
16. **Franco M, Martinez F, Rodriguez-Iturbe B, Johnson RJ, Santamaria J, Montoya A, Nepomuceno T, Bautista R, Tapia E, and Herrera-Acosta J.** Angiotensin II, interstitial inflammation, and the pathogenesis of salt-sensitive hypertension. *Am J Physiol Renal Physiol* 291: F1281-1287, 2006.
17. **Guo LJ, Alli AA, Eaton DC, and Bao HF.** ENaC is regulated by natriuretic peptide receptor-dependent cGMP signaling. *Am J Physiol Renal Physiol* 304: F930-937, 2013.
18. **Gupta DK, de Lemos JA, Ayers CR, Berry JD, and Wang TJ.** Racial Differences in Natriuretic Peptide Levels: The Dallas Heart Study. *JACC Heart Fail* 3: 513-519, 2015.
19. **Habibi J, Aroor AR, Das NA, Manrique-Acevedo CM, Johnson MS, Hayden MR, Nistala R, Wiedmeyer C, Chandrasekar B, and DeMarco VG.** The combination of a neprilysin inhibitor

(sacubitril) and angiotensin-II receptor blocker (valsartan) attenuates glomerular and tubular injury in the Zucker Obese rat. *Cardiovasc Diabetol* 18: 40, 2019.

20. **Haynes R, Judge PK, Staplin N, Herrington WG, Storey BC, Bethel A, Bowman L, Brunskill N, Cockwell P, Hill M, Kalra PA, McMurray JJV, Taal M, Wheeler DC, Landray MJ, and Baigent C.** Effects of Sacubitril/Valsartan Versus Irbesartan in Patients With Chronic Kidney Disease. *Circulation* 138: 1505-1514, 2018.
21. **Haynes R, Zhu D, Judge PK, Herrington WG, Kalra PA, and Baigent C.** Chronic kidney disease, heart failure and neprilysin inhibition. *Nephrol Dial Transplant*, 2019.
22. **Houng AK, McNamee RA, Kerner A, Sharma P, Mohamad A, Tronolone J, and Reed GL.** Atrial natriuretic peptide increases inflammation, infarct size, and mortality after experimental coronary occlusion. *Am J Physiol Heart Circ Physiol* 296: H655-661, 2009.
23. **Ichiki T, Boerrigter G, Huntley BK, Sangaralingham SJ, McKie PM, Harty GJ, Harders GE, and Burnett JC, Jr.** Differential expression of the pro-natriuretic peptide convertases corin and furin in experimental heart failure and atrial fibrosis. *Am J Physiol Regul Integr Comp Physiol* 304: R102-109, 2013.
24. **Ilatovskaya DV, Levchenko V, Pavlov TS, Isaeva E, Klemens CA, Johnson J, Liu P, Kriegel AJ, and Staruschenko A.** Salt-deficient diet exacerbates cystogenesis in ARPKD via epithelial sodium channel (ENaC). *EBioMedicine* 40: 663-674, 2019.
25. **Imanishi M, Okada N, Konishi Y, Morikawa T, Maeda I, Kitabayashi C, Masada M, Shirahashi N, Wilcox CS, and Nishiyama A.** Angiotensin II receptor blockade reduces salt sensitivity of blood pressure through restoration of renal nitric oxide synthesis in patients with diabetic nephropathy. *J Renin Angiotensin Aldosterone Syst* 14: 67-73, 2013.
26. **Itoh H, Nakao K, Katsuura G, Morii N, Shiono S, Sakamoto M, Sugawara A, Yamada T, Saito Y, Matsushita A, and et al.** Centrally infused atrial natriuretic polypeptide attenuates exaggerated salt appetite in spontaneously hypertensive rats. *Circ Res* 59: 342-347, 1986.
27. **Jing W, Vaziri ND, Nunes A, Suematsu Y, Farzaneh T, Khazaeli M, and Moradi H.** LCZ696 (Sacubitril/valsartan) ameliorates oxidative stress, inflammation, fibrosis and improves renal function beyond angiotensin receptor blockade in CKD. *Am J Transl Res* 9: 5473-5484, 2017.
28. **John SW, Krege JH, Oliver PM, Hagaman JR, Hodgin JB, Pang SC, Flynn TG, and Smithies O.** Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *Science* 267: 679-681, 1995.
29. **Judge P, Haynes R, Landray MJ, and Baigent C.** Neprilysin inhibition in chronic kidney disease. *Nephrol Dial Transplant* 30: 738-743, 2015.
30. **Katsuya T, Ishikawa K, Sugimoto K, Rakugi H, and Ogihara T.** Salt sensitivity of Japanese from the viewpoint of gene polymorphism. *Hypertens Res* 26: 521-525, 2003.
31. **Lieb W, Pencina MJ, Jacques PF, Wang TJ, Larson MG, Levy D, Kannel WB, and Vasan RS.** Higher aldosterone and lower N-terminal proatrial natriuretic peptide as biomarkers of salt sensitivity in the community. *Eur J Cardiovasc Prev Rehabil* 18: 664-673, 2011.
32. **Lu X and Crowley SD.** Inflammation in Salt-Sensitive Hypertension and Renal Damage. *Curr Hypertens Rep* 20: 103, 2018.
33. **Luft FC.** Molecular genetics of salt-sensitivity and hypertension. *Drug Metab Dispos* 29: 500-504, 2001.
34. **Lunder M, Janic M, Savic V, Janez A, Kanc K, and Sabovic M.** Very low-dose fluvastatin-valsartan combination decreases parameters of inflammation and oxidative stress in patients with type 1 diabetes mellitus. *Diabetes Res Clin Pract* 127: 181-186, 2017.
35. **Luzardo L, Noboa O, and Boggia J.** Mechanisms of Salt-Sensitive Hypertension. *Curr Hypertens Rev* 11: 14-21, 2015.
36. **Maslov MY, Foianini S, Mayer D, Orlov MV, and Lovich MA.** Synergy between sacubitril and valsartan leads to hemodynamic, antifibrotic, and exercise tolerance benefits in rats with preexisting heart failure. *Am J Physiol Heart Circ Physiol* 316: H289-H297, 2019.
37. **Mezzasoma L, Antognelli C, and Talesa VN.** Atrial natriuretic peptide down-regulates LPS/ATP-mediated IL-1beta release by inhibiting NF-kB, NLRP3 inflammasome and caspase-1 activation in THP-1 cells. *Immunol Res* 64: 303-312, 2016.

38. **Milanesi S, Verzola D, Cappadona F, Bonino B, Murugavel A, Pontremoli R, Garibotto G, and Viazzi F.** Uric acid and angiotensin II additively promote inflammation and oxidative stress in human proximal tubule cells by activation of toll-like receptor 4. *J Cell Physiol* 234: 10868-10876, 2019.
39. **Mishra S, Ingole S, and Jain R.** Salt sensitivity and its implication in clinical practice. *Indian Heart J* 70: 556-564, 2018.
40. **Mohapatra SS.** Role of natriuretic peptide signaling in modulating asthma and inflammation. *Can J Physiol Pharmacol* 85: 754-759, 2007.
41. **Najenson AC, Courreges AP, Perazzo JC, Rubio MF, Vatta MS, and Bianciotti LG.** Atrial natriuretic peptide reduces inflammation and enhances apoptosis in rat acute pancreatitis. *Acta Physiol* 222, 2018.
42. **Nehme A, Zouein FA, Zayeri ZD, and Zibara K.** An Update on the Tissue Renin Angiotensin System and Its Role in Physiology and Pathology. *J Cardiovasc Dev Dis* 6, 2019.
43. **Newton-Cheh C, Johnson T, Gateva V, Tobin MD, Bochud M, Coin L, Najjar SS, Zhao JH, Heath SC, Eyheramendy S, Papadakis K, Voight BF, Scott LJ, Zhang F, Farrall M, Tanaka T, Wallace C, Chambers JC, Khaw KT, Nilsson P, van der Harst P, Polidoro S, Grobbee DE, Onland-Moret NC, Bots ML, Wain LV, Elliott KS, Teumer A, Luan J, Lucas G, Kuusisto J, Burton PR, Hadley D, McArdle WL, Wellcome Trust Case Control C, Brown M, Dominiczak A, Newhouse SJ, Samani NJ, Webster J, Zeggini E, Beckmann JS, Bergmann S, Lim N, Song K, Vollenweider P, Waeber G, Waterworth DM, Yuan X, Groop L, Orho-Melander M, Allione A, Di Gregorio A, Guarrera S, Panico S, Ricceri F, Romanazzi V, Sacerdote C, Vineis P, Barroso I, Sandhu MS, Luben RN, Crawford GJ, Jousilahti P, Perola M, Boehnke M, Bonnycastle LL, Collins FS, Jackson AU, Mohlke KL, Stringham HM, Valle TT, Willer CJ, Bergman RN, Morken MA, Doring A, Gieger C, Illig T, Meitinger T, Org E, Pfeufer A, Wichmann HE, Kathiresan S, Marrugat J, O'Donnell CJ, Schwartz SM, Siscovick DS, Subirana I, Freimer NB, Hartikainen AL, McCarthy MI, O'Reilly PF, Peltonen L, Pouta A, de Jong PE, Snieder H, van Gilst WH, Clarke R, Goel A, Hamsten A, et al.** Genome-wide association study identifies eight loci associated with blood pressure. *Nat Genet* 41: 666-676, 2009.
44. **Nierenberg JL, Li C, He J, Gu D, Chen J, Lu X, Li J, Wu X, Gu CC, Hixson JE, Rao DC, and Kelly TN.** Blood Pressure Genetic Risk Score Predicts Blood Pressure Responses to Dietary Sodium and Potassium: The GenSalt Study (Genetic Epidemiology Network of Salt Sensitivity). *Hypertension (Dallas, Tex : 1979)* 70: 1106-1112, 2017.
45. **Nixon RM, Muller E, Lowy A, and Falvey H.** Valsartan vs. other angiotensin II receptor blockers in the treatment of hypertension: a meta-analytical approach. *Int J Clin Pract* 63: 766-775, 2009.
46. **Nojiri T, Hosoda H, Tokudome T, Miura K, Ishikane S, Kimura T, Shintani Y, Inoue M, Sawabata N, Miyazato M, Okumura M, and Kangawa K.** Atrial natriuretic peptide inhibits lipopolysaccharide-induced acute lung injury. *Pulm Pharmacol Ther* 29: 24-30, 2014.
47. **Overlack A, Ruppert M, Kolloch R, Gobel B, Kraft K, Diehl J, Schmitt W, and Stumpe KO.** Divergent hemodynamic and hormonal responses to varying salt intake in normotensive subjects. *Hypertension* 22: 331-338, 1993.
48. **Patel S, Rauf A, Khan H, and Abu-Izneid T.** Renin-angiotensin-aldosterone (RAAS): The ubiquitous system for homeostasis and pathologies. *Biomed Pharmacother* 94: 317-325, 2017.
49. **Pemberton CJ, Siriwardena M, Kleffmann T, Ruygrok P, Palmer SC, Yandle TG, and Richards AM.** First identification of circulating prepro-A-type natriuretic peptide (preproANP) signal peptide fragments in humans: initial assessment as cardiovascular biomarkers. *Clin Chem* 58: 757-767, 2012.
50. **Potter LR.** Natriuretic peptide metabolism, clearance and degradation. *FEBS J* 278: 1808-1817, 2011.
51. **Qi H, Liu Z, Cao H, Sun WP, Peng WJ, Liu B, Dong SJ, Xiang YT, and Zhang L.** Comparative Efficacy of Antihypertensive Agents in Salt-Sensitive Hypertensive Patients: A Network Meta-Analysis. *Am J Hypertens* 31: 835-846, 2018.
52. **Quiroga B, de Santos A, Sapiencia D, Saharaui Y, and Alvarez-Chiva V.** Sacubitril/valsartan in chronic kidney disease, the nephrologist point of view. *Nefrologia* 39: 646-652, 2019.
53. **Quiroz Y, Pons H, Gordon KL, Rincon J, Chavez M, Parra G, Herrera-Acosta J, Gomez-Garre D, Largo R, Egido J, Johnson RJ, and Rodriguez-Iturbe B.** Mycophenolate mofetil prevents salt-

sensitive hypertension resulting from nitric oxide synthesis inhibition. *Am J Physiol Renal Physiol* 281: F38-47, 2001.
54. **Ranjbar R, Shafiee M, Hesari A, Ferns GA, Ghasemi F, and Avan A.** The potential therapeutic use of renin-angiotensin system inhibitors in the treatment of inflammatory diseases. *J Cell Physiol* 234: 2277-2295, 2019.
55. **Rieg T.** A High-throughput method for measurement of glomerular filtration rate in conscious mice. *J Vis Exp*: e50330, 2013.
56. **Rodriguez-Iturbe B, Pons H, Quiroz Y, Gordon K, Rincon J, Chavez M, Parra G, Herrera-Acosta J, Gomez-Garre D, Largo R, Egido J, and Johnson RJ.** Mycophenolate mofetil prevents salt-sensitive hypertension resulting from angiotensin II exposure. *Kidney Int* 59: 2222-2232, 2001.
57. **Roksnoer LC, van Veghel R, van Groningen MC, de Vries R, Garrelds IM, Bhaggoe UM, van Gool JM, Friesema EC, Leijten FP, Hoorn EJ, Danser AH, and Batenburg WW.** Blood pressure-independent renoprotection in diabetic rats treated with AT1 receptor-neprilysin inhibition compared with AT1 receptor blockade alone. *Clin Sci (Lond)* 130: 1209-1220, 2016.
58. **Rust P and Ekmekcioglu C.** Impact of Salt Intake on the Pathogenesis and Treatment of Hypertension. *Adv Exp Med Biol* 956: 61-84, 2017.
59. **Rutledge DR, Sun Y, and Ross EA.** Polymorphisms within the atrial natriuretic peptide gene in essential hypertension. *J Hypertens* 13: 953-955, 1995.
60. **Satou R, Penrose H, and Navar LG.** Inflammation as a Regulator of the Renin-Angiotensin System and Blood Pressure. *Curr Hypertens Rep* 20: 100, 2018.
61. **Stellar E and Epstein AN.** Neuroendocrine factors in salt appetite. *J Physiol Pharmacol* 42: 345-355, 1991.
62. **Suematsu Y, Jing W, Nunes A, Kashyap ML, Khazaeli M, Vaziri ND, and Moradi H.** LCZ696 (Sacubitril/Valsartan), an Angiotensin-Receptor Neprilysin Inhibitor, Attenuates Cardiac Hypertrophy, Fibrosis, and Vasculopathy in a Rat Model of Chronic Kidney Disease. *J Card Fail* 24: 266-275, 2018.
63. **Theilig F and Wu Q.** ANP-induced signaling cascade and its implications in renal pathophysiology. *Am J Physiol Renal Physiol* 308: F1047-1055, 2015.
64. **Ushijima K, Ando H, Arakawa Y, Aizawa K, Suzuki C, Shimada K, Tsuruoka SI, and Fujimura A.** Prevention against renal damage in rats with subtotal nephrectomy by sacubitril/valsartan (LCZ696), a dual-acting angiotensin receptor-neprilysin inhibitor. *Pharmacol Res Perspect* 5, 2017.
65. **Vollmar AM.** The role of atrial natriuretic peptide in the immune system. *Peptides* 26: 1086-1094, 2005.
66. **Vollmar AM, Lang RE, Hanze J, and Schulz R.** A possible linkage of atrial natriuretic peptide to the immune system. *Am J Hypertens* 3: 408-411, 1990.
67. **Vormfelde SV, Toliat MR, Nurnberg P, and Brockmoller J.** Atrial natriuretic peptide polymorphisms, hydrochlorothiazide and urinary potassium excretion. *Int J Cardiol* 144: 72-74, 2010.
68. **Wade B, Petrova G, and Mattson DL.** Role of immune factors in angiotensin II-induced hypertension and renal damage in Dahl salt-sensitive rats. *Am J Physiol Regul Integr Comp Physiol* 314: R323-R333, 2018.
69. **Wakui H, Tamura K, Masuda S, Tsurumi-Ikeya Y, Fujita M, Maeda A, Ohsawa M, Azushima K, Uneda K, Matsuda M, Kitamura K, Uchida S, Toya Y, Kobori H, Nagahama K, Yamashita A, and Umemura S.** Enhanced angiotensin receptor-associated protein in renal tubule suppresses angiotensin-dependent hypertension. *Hypertension (Dallas, Tex : 1979)* 61: 1203-1210, 2013.
70. **Wang TD, Tan RS, Lee HY, Ihm SH, Rhee MY, Tomlinson B, Pal P, Yang F, Hirschhorn E, Prescott MF, Hinder M, and Langenickel TH.** Effects of Sacubitril/Valsartan (LCZ696) on Natriuresis, Diuresis, Blood Pressures, and NT-proBNP in Salt-Sensitive Hypertension. *Hypertension (Dallas, Tex : 1979)* 69: 32-41, 2017.
71. **Wang W, Shen J, Cui Y, Jiang J, Chen S, Peng J, and Wu Q.** Impaired sodium excretion and salt-sensitive hypertension in corin-deficient mice. *Kidney Int* 82: 26-33, 2012.
72. **Widecka K, Krzyzanowska-Swiniarska B, Celibala R, Gruszczynska M, Gozdzik J, Ciechanowski K, and Czekalski S.** [Effect of intravenous sodium chloride load on levels of atrial natriuretic peptide (ANP) and 3'5' guanosine monophosphate (cGMP) in plasma of patients with

uncomplicated sodium-sensitive arterial hypertension maintained on different dietary sodium intake]. *Pol Arch Med Wewn* 89: 117-124, 1993.

73. **Xue B, Thunhorst RL, Yu Y, Guo F, Beltz TG, Felder RB, and Johnson AK.** Central Renin-Angiotensin System Activation and Inflammation Induced by High-Fat Diet Sensitize Angiotensin II-Elicited Hypertension. *Hypertension* 67: 163-170, 2016.
74. **Yoon SS, Gu Q, Nwankwo T, Wright JD, Hong Y, and Burt V.** Trends in blood pressure among adults with hypertension: United States, 2003 to 2012. *Hypertension (Dallas, Tex : 1979)* 65: 54-61, 2015.
75. **Young DB, Jackson TE, Tipayamontri U, and Scott RC.** Effects of sodium intake on steady-state potassium excretion. *Am J Physiol* 246: F772-778, 1984.
76. **Zannad F and Ferreira JP.** Is Sacubitril/Valsartan Antifibrotic? *Journal of the American College of Cardiology* 73: 807-809, 2019.
77. **Zhang J, Li M, Yang Y, Yan Y, Li J, Qu J, and Wang J.** NPR-A: A Therapeutic Target in Inflammation and Cancer. *Crit Rev Eukaryot Gene Expr* 25: 41-46, 2015.
78. **Zhu YB, Zhang YB, Liu DH, Li XF, Liu AJ, Fan XM, Qiao CH, Ling F, and Liu YL.** Atrial natriuretic peptide attenuates inflammatory responses on oleic acid-induced acute lung injury model in rats. *Chin Med J (Engl)* 126: 747-750, 2013.
79. **Zile MR, O'Meara E, Claggett B, Prescott MF, Solomon SD, Swedberg K, Packer M, McMurray JJV, Shi V, Lefkowitz M, and Rouleau J.** Effects of Sacubitril/Valsartan on Biomarkers of Extracellular Matrix Regulation in Patients With HFrEF. *Journal of the American College of Cardiology* 73: 795-806, 2019.

Figure 1

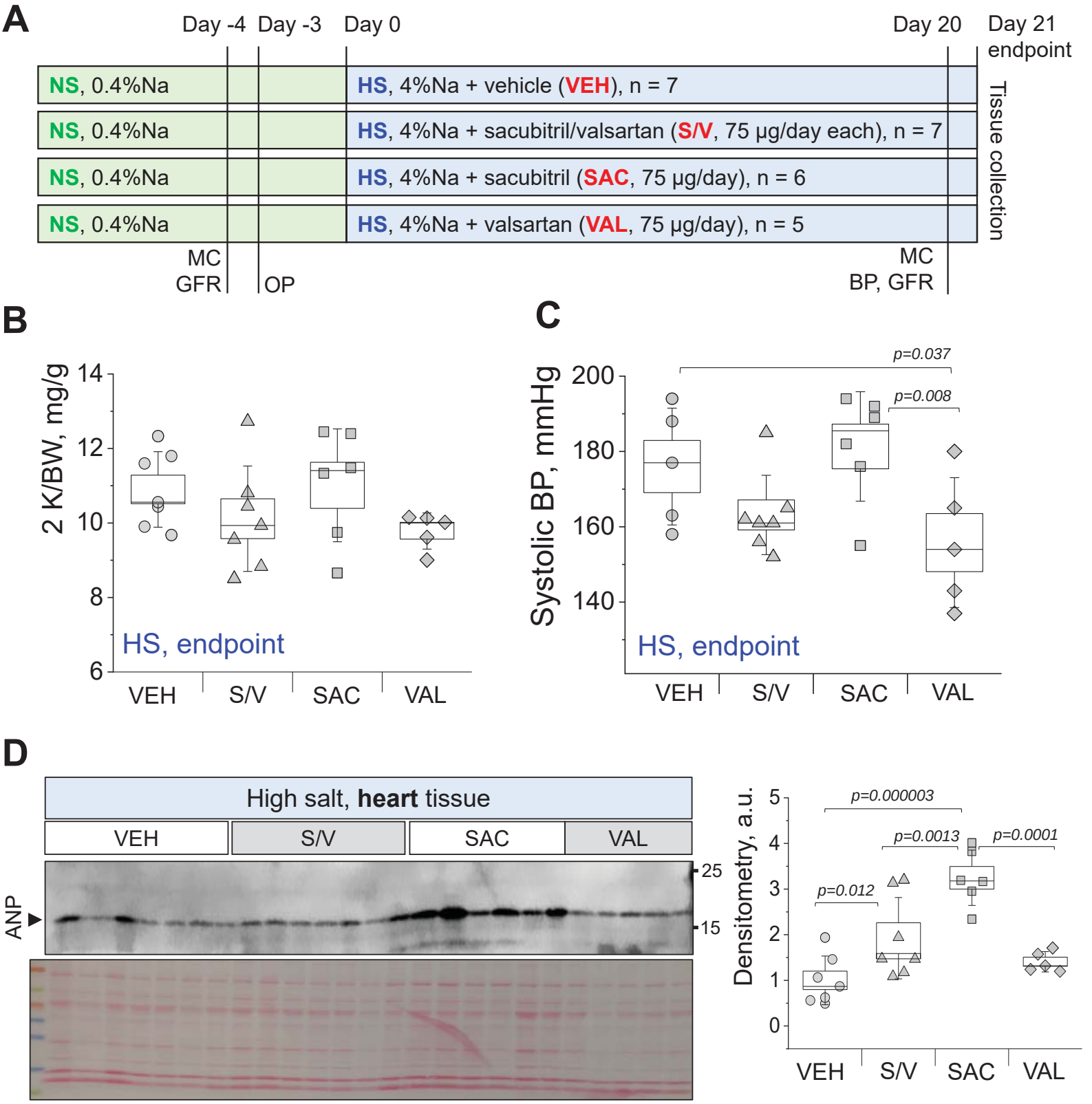


Figure 2

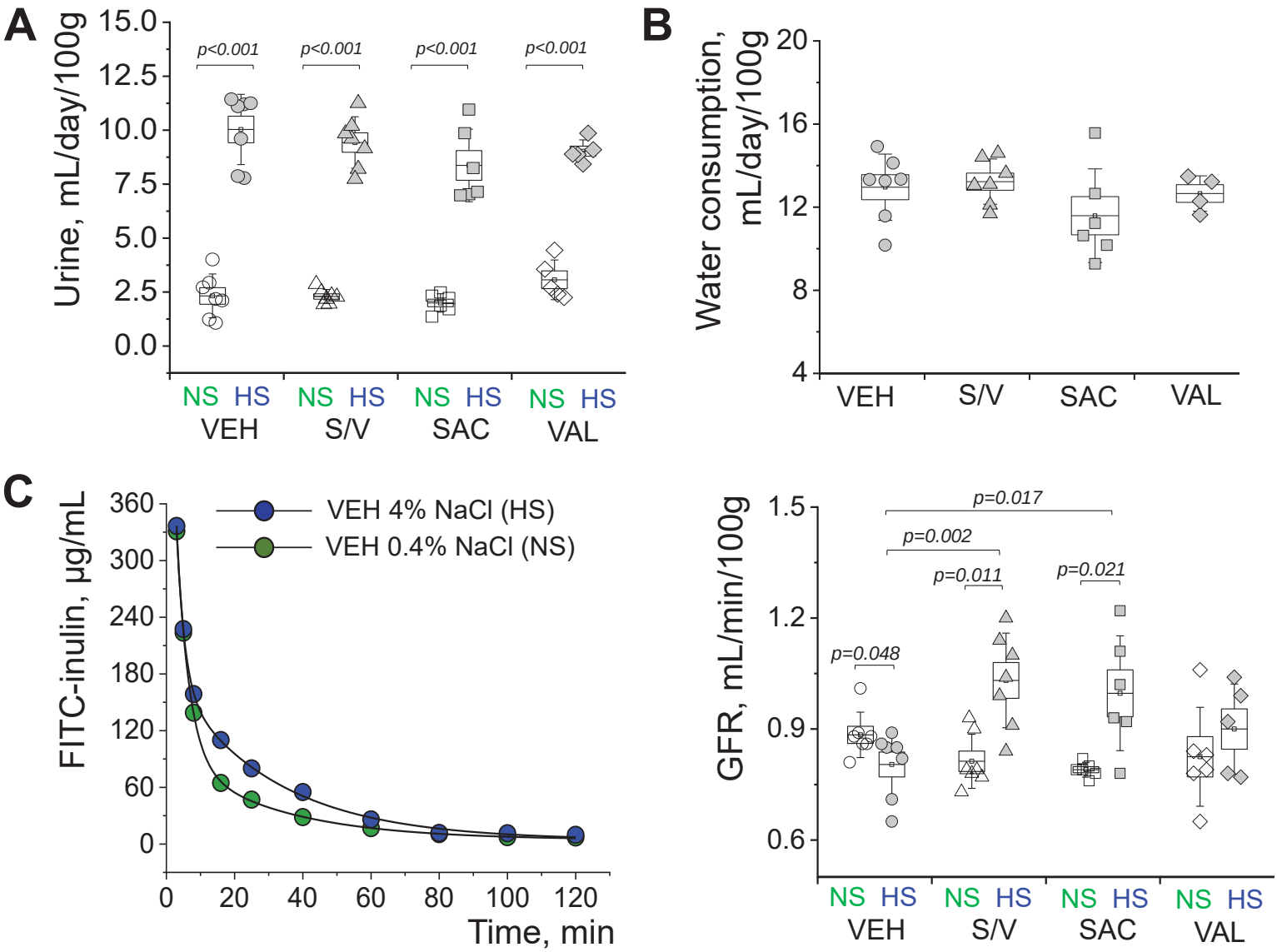


Figure 3

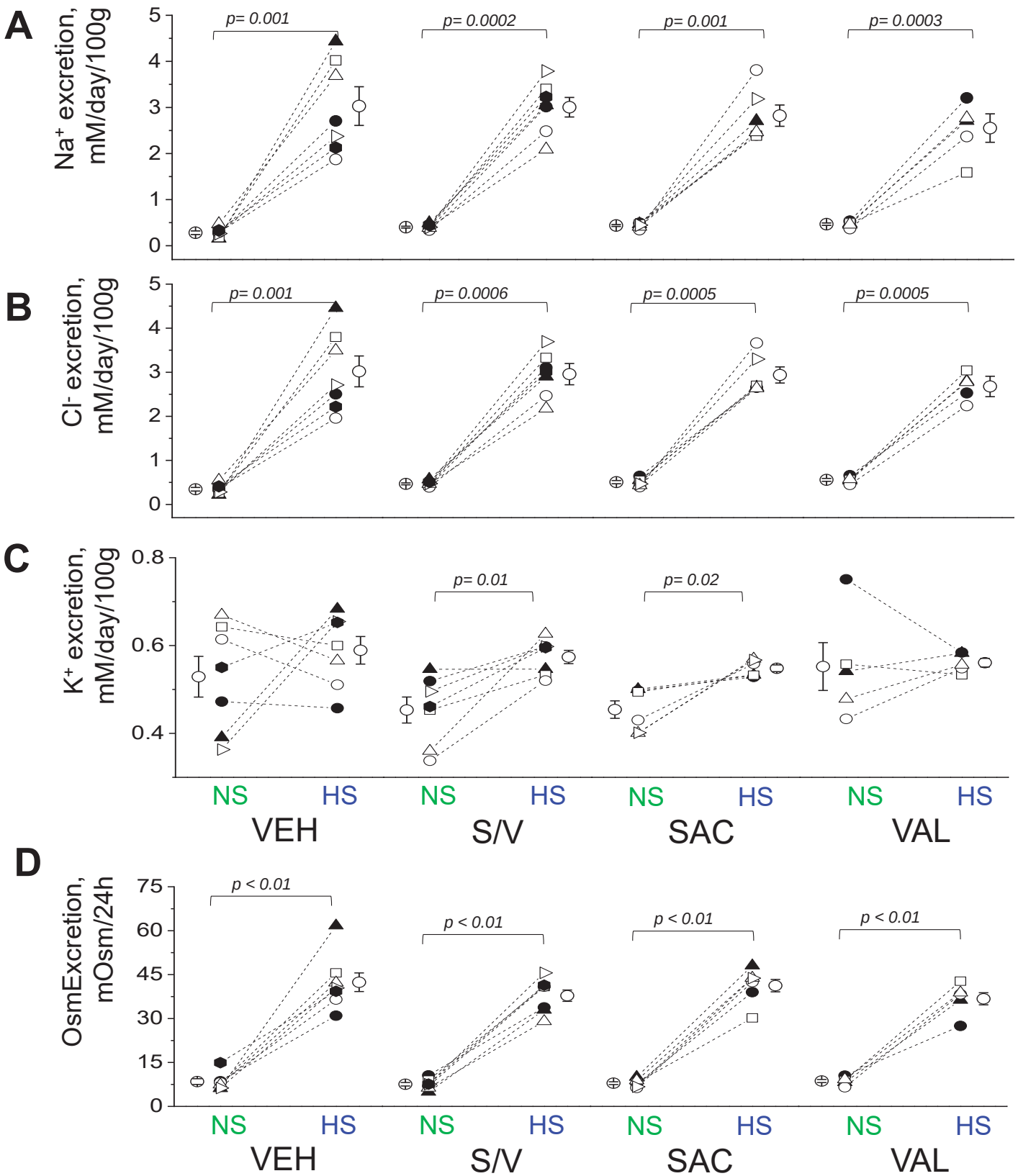
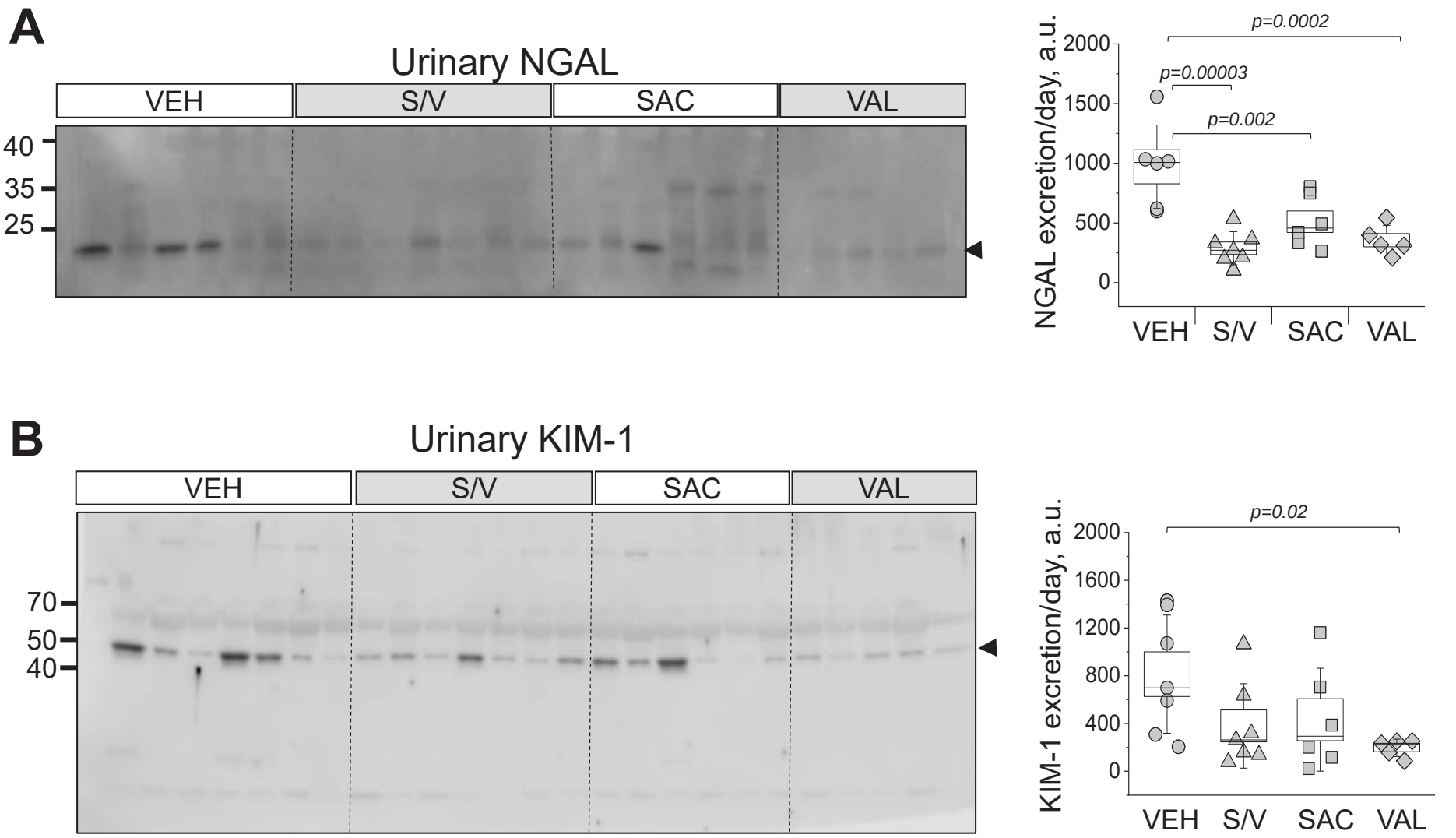


Figure 4



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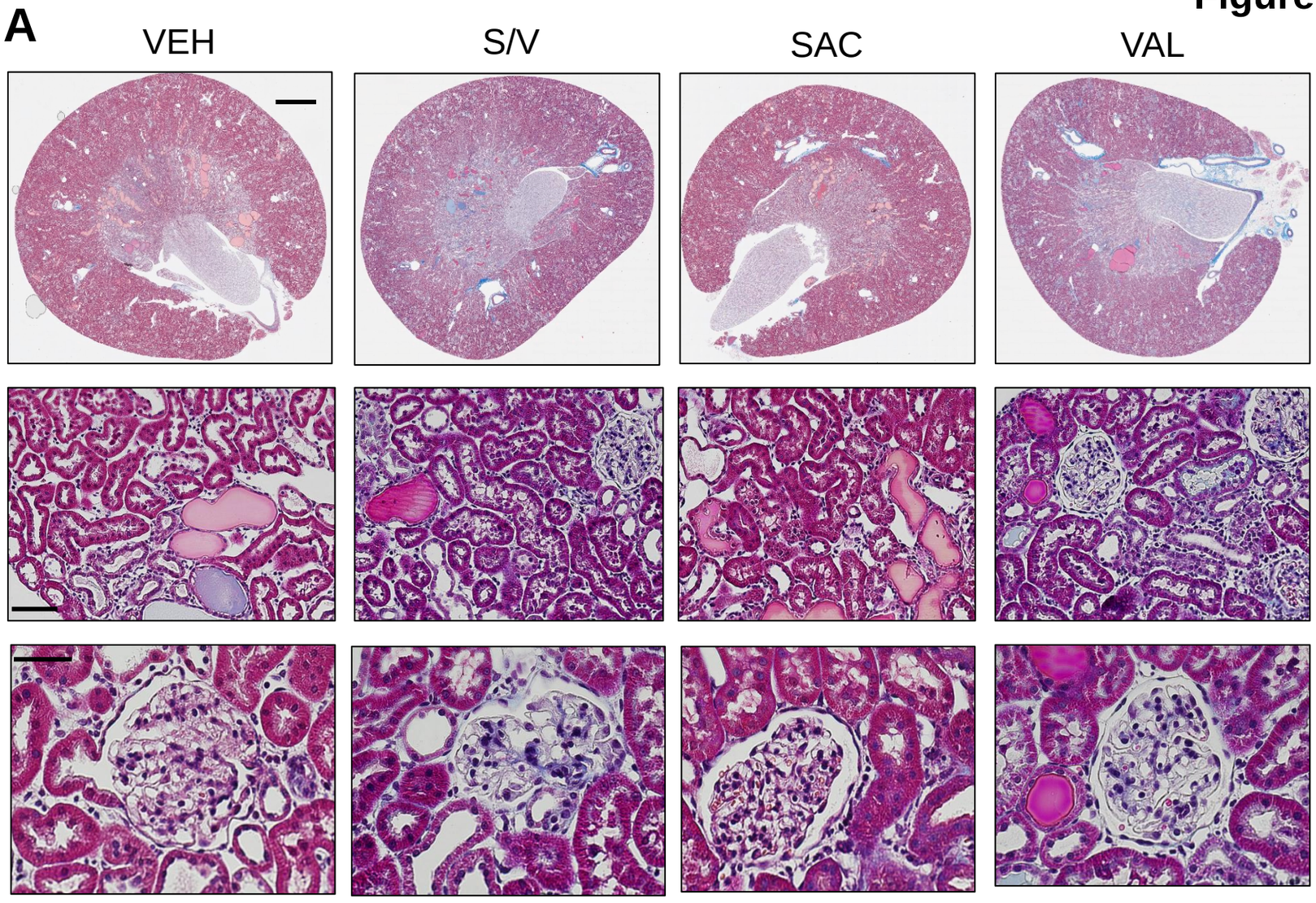
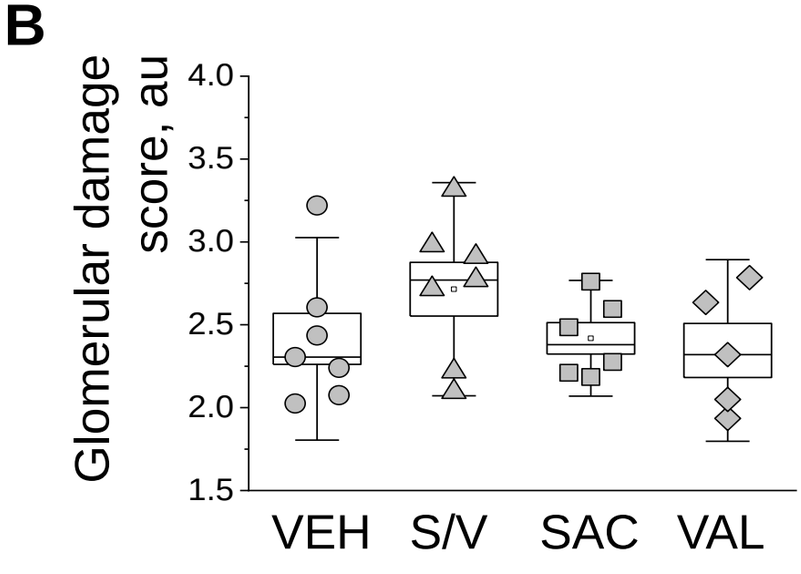




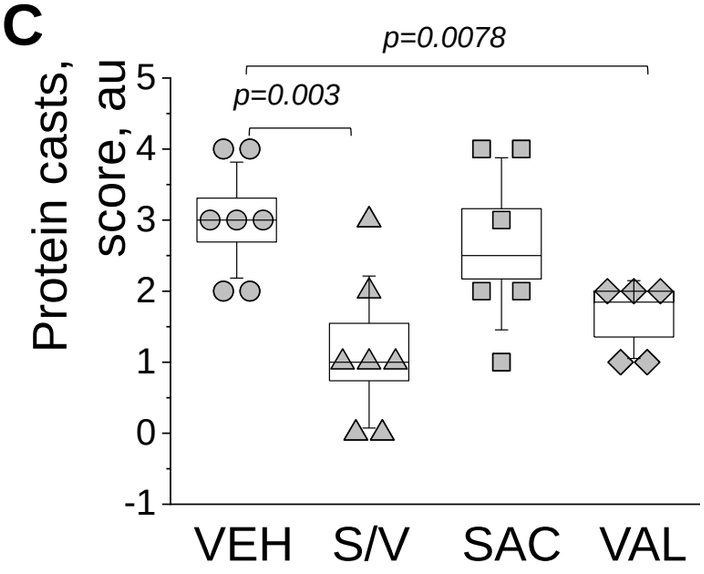
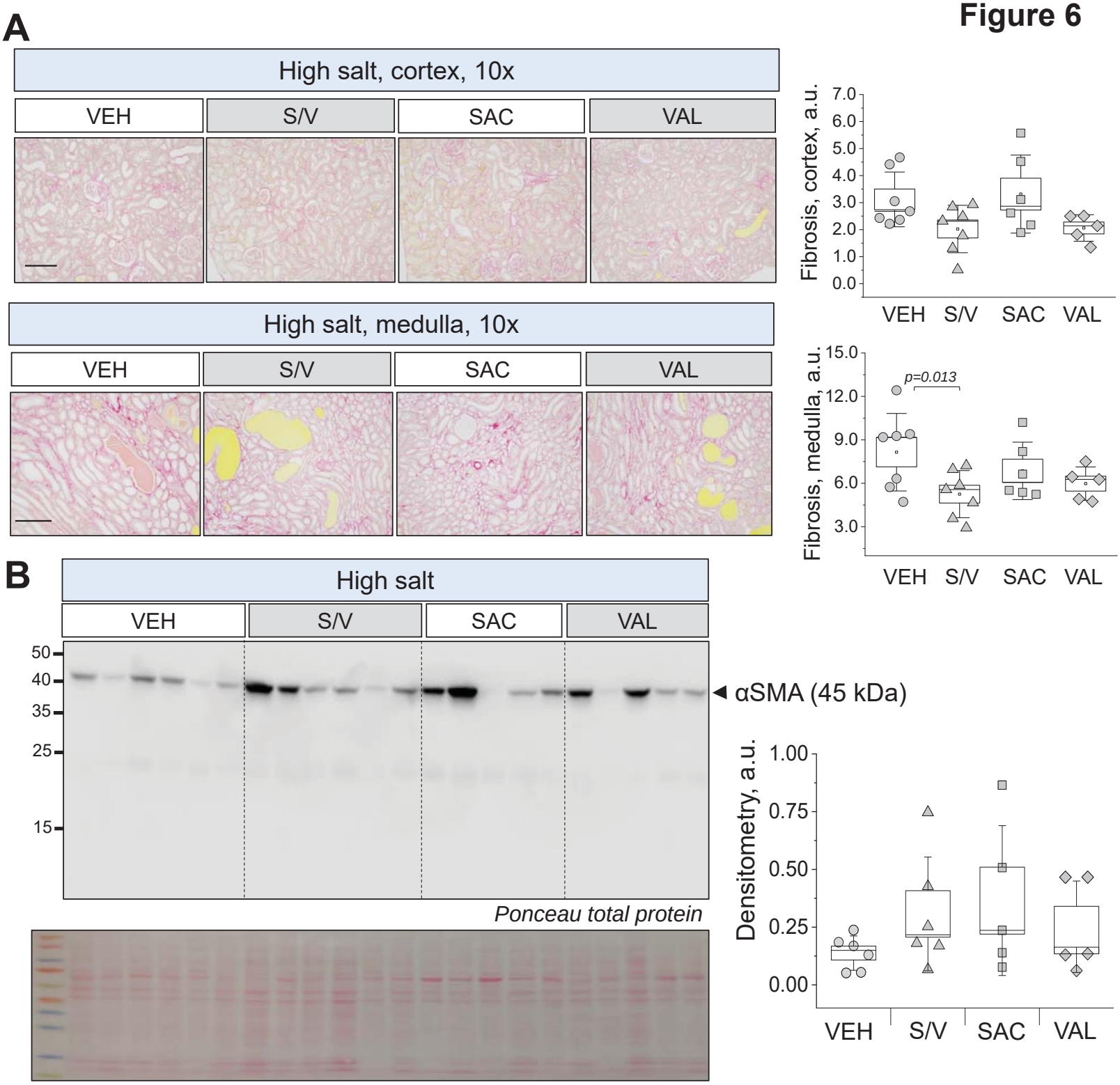


Figure 6



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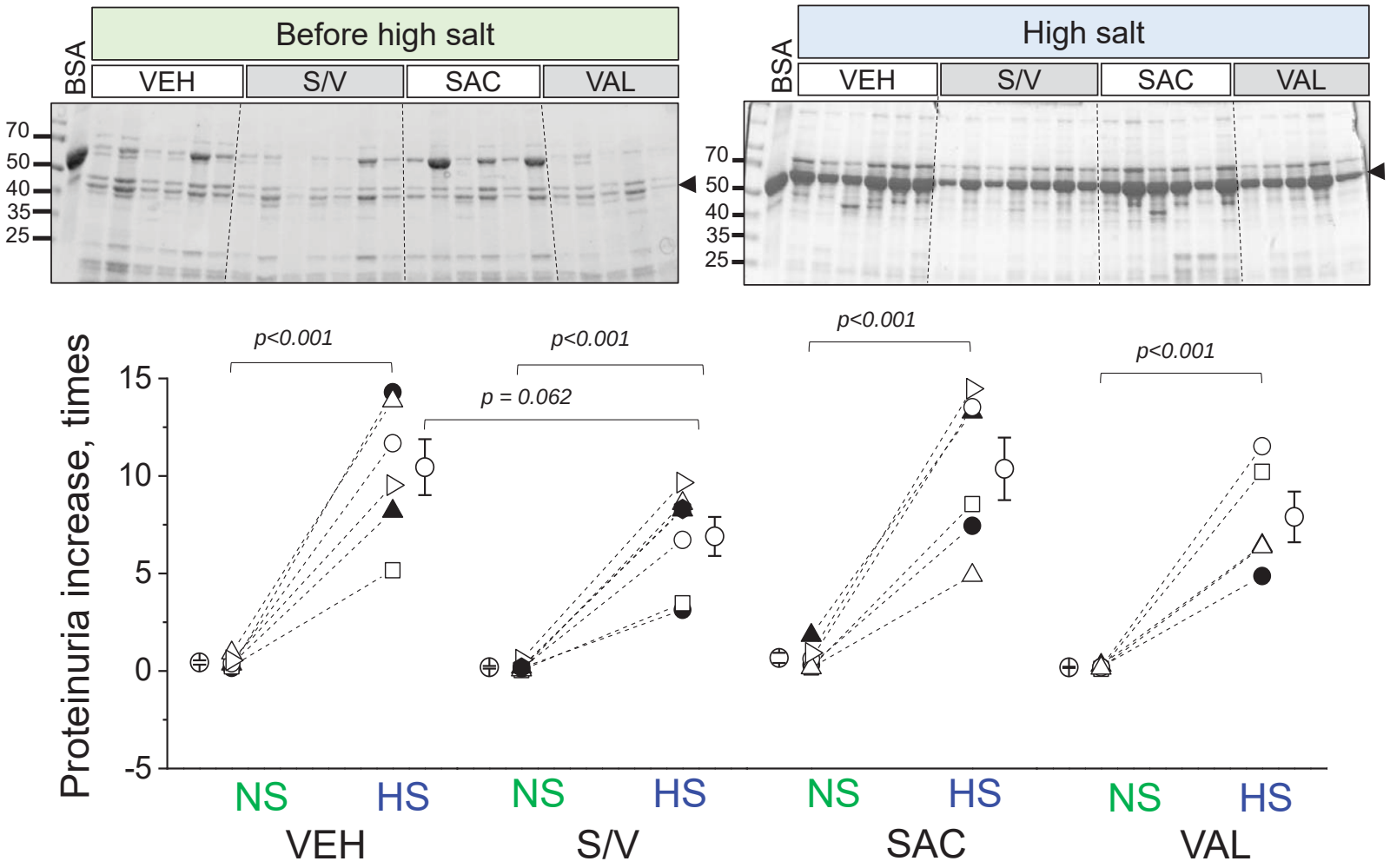
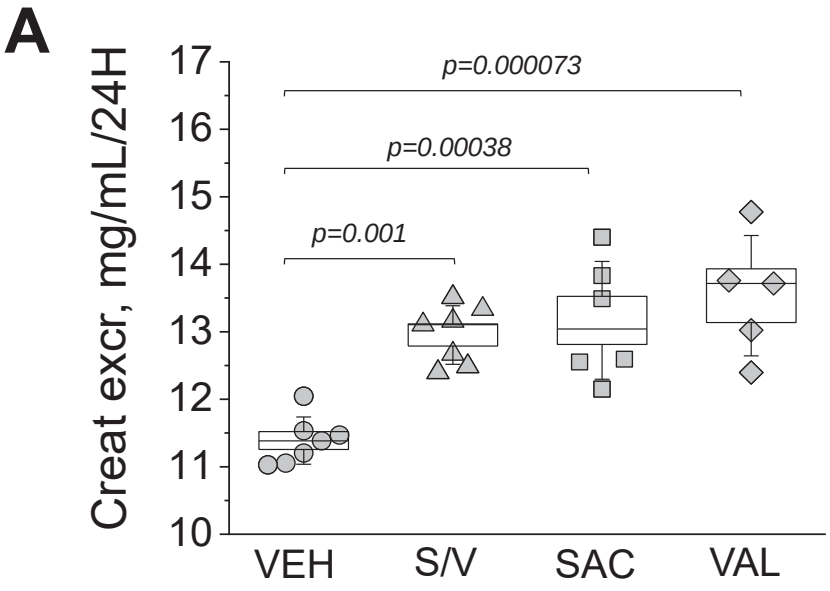
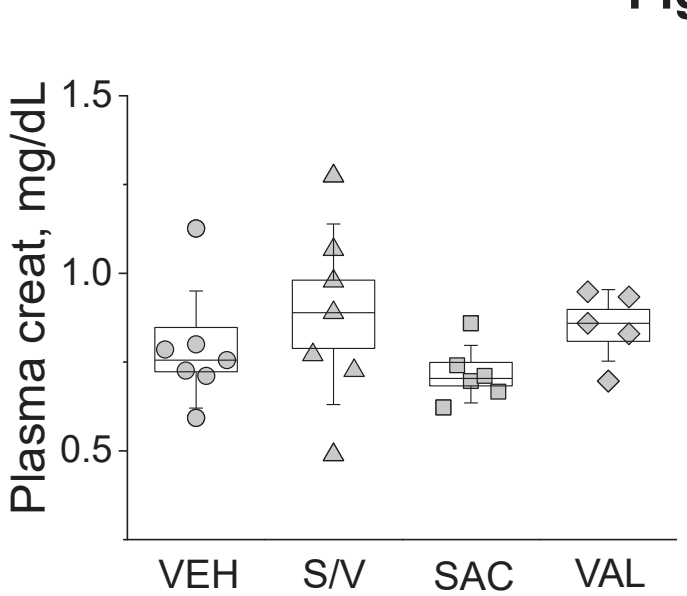
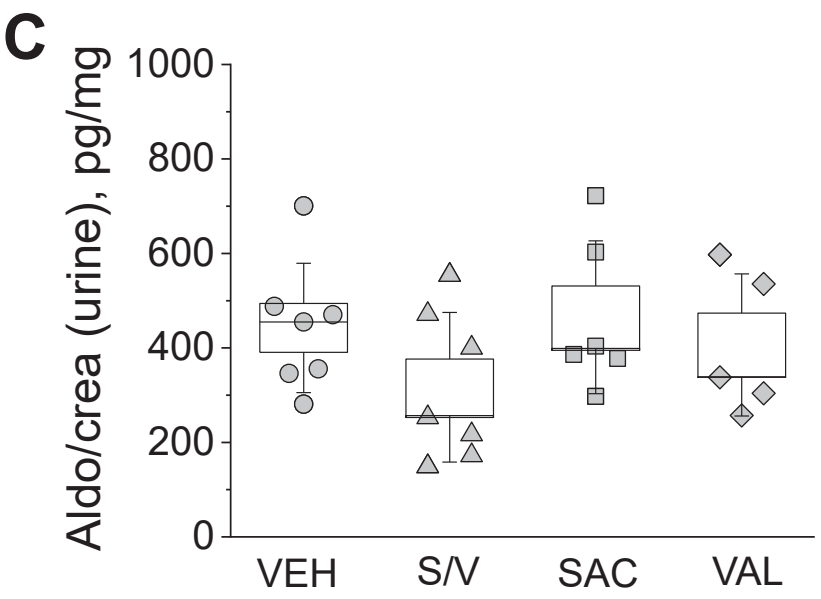


Figure 8







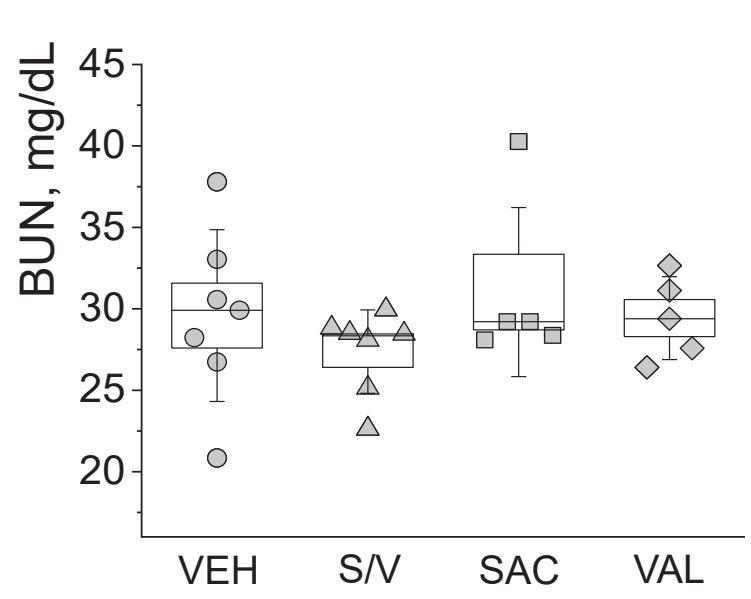


Table 1. Summary of the experimental outcomes. Shown is a summary of significant outcomes of the study driven by sacubitril only, valsartan only, both sacubitril and valsartan, and their combination. Shown are outcomes with p<0.05, and important outcomes with p>0.05 (due to lower power). An increase, decrease and no change (vs a vehicle-treated group) are denoted ↑, ↓, and ↔, respectively.

Parameter	Sacubitril and Valsartan	Sacubitril	Valsartan
Outcome, *vs vehicle* (endpoint, on HS). *indicates p<0.05			
Driven by Drug Combination			
Proteinuria	↓, *p = 0.061*	↔	↔
Renal medullary fibrosis	**↓, p = 0.013***	↔	↓, *p = 0.08*
Primarily Sacubitril Driven			
ANP level in heart tissue	**↑, p=0.012***	**↑, p=0.000003***	↔
Glomerular filtration rate	**↑, p=0.002***	**↑, p=0.017***	↔
Primarily Valsartan Driven			
Urinary KIM-1 excretion	↓, *p = 0.08*	↔	**↓, p = 0.02***
Systolic blood pressure	↔	↔	**↓, p = 0.008***
Renal protein casts	**↓, p = 0.003***	↔	**↓, p = 0.009***
Both Sacubitril and Valsartan Driven			
Urinary NGAL excretion	**↓, p = 0.00003***	**↓, p = 0.002***	**↓, p = 0.0002***
Creatinine excretion	**↑, p=0.001***	**↑, p=0.0004***	**↑, p=0.000073***