

Sacubitril/valsartan (LCZ696) Significantly Reduces Aldosterone and Increases cGMP Circulating Levels in a Canine Model of RAAS Activation

Jonathan P Mochel^{1,6*}, Chi Hse Teng², Mathieu Peyrou³, Jerome Giraudel³, Meindert Danhof⁴, Dean F Rigel⁵

¹Pharmacometrics, Novartis Pharma AG, Werk Saint Johann 4056 Basel Switzerland.

²Biostatistics NIBR, Novartis Institutes for BioMedical Research, 250 Massachusetts Ave., Cambridge, MA 02139, USA.

³Department of Research & Development, Elanco Animal Health, c/o Novartis Animal Health, 4002 Basel Switzerland.

⁴Department of Pharmacology, Leiden-Academic Centre for Drug Research, Pharmacology, 2300 Leiden The Netherlands.

⁵Novartis Institutes for BioMedical Research, East Hanover, NJ, USA.

⁶Department of Biomedical Sciences, Iowa State University College of Veterinary Medicine, 1800 Christensen Drive, 50010 Ames, USA

***Correspondence:**

Jonathan P. Mochel, DVM, MS, Ph.D, DECVPT

Associate Professor of Pharmacology

European Veterinary Specialist in Pharmacology and Toxicology

Iowa State University College of Vet. Medicine

2448 Lloyd, 1809 S Riverside Dr.

Ames, IA 50011-1250

Phone 515-294-7424

Email correspondence: jmochel@iastate.edu

2 **Abstract**

3 Simultaneous blockade of angiotensin receptors and enhancement of natriuretic peptides
4 (NP) by the first-in-class angiotensin receptor neprilysin (NEP) inhibitor sacubitril/valsartan
5 constitutes an effective approach to treating heart failure. This study examined the effects of
6 sacubitril/valsartan (225 and 675mg/day) vs. placebo, sacubitril (360mg/day), valsartan
7 (900mg/day), and benazepril (5mg/day) on the dynamics of the renin-angiotensin-
8 aldosterone system (RAAS) and the NP system in dogs. Beagle dogs (n=18) were fed a
9 low-salt diet (0.05% Na) for 15 days to model RAAS activation observed in clinical heart
10 failure. Drugs were administered once daily during the last 10 days, while the effects on the
11 RAAS and NPs were assessed on days 1, 5, and 10. Steady-state pharmacokinetics of the
12 test agents were evaluated on day 5. Compared with placebo, sacubitril/valsartan (675mg)
13 substantially increased cGMP circulating levels, while benazepril and valsartan showed no
14 effect. Additionally, sacubitril/valsartan (675mg) and valsartan significantly increased
15 plasma renin activity, angiotensin I and angiotensin II concentrations. Finally,
16 sacubitril/valsartan (both doses), and valsartan significantly decreased plasma aldosterone
17 vs. placebo. Systemic exposure to valsartan following sacubitril/valsartan 675mg
18 administration was similar to that observed with valsartan 900mg administration alone.
19 Sacubitril/valsartan favorably modulates the dynamics of the renin and NP cascades
20 through complementary NEP and RAAS inhibition.

21 **Keywords:** Angiotensin receptor neprilysin inhibitor, cGMP, plasma renin activity,
22 sacubitril/valsartan, renin angiotensin aldosterone inhibitor

23 1 Introduction

24 Chronic heart failure (HF) affects approximately 1–2% of the adult human population in
25 developed countries, with the prevalence rising to $\geq 10\%$ among persons 70 years of age or
26 older [1]. Importantly, the prognosis of chronic HF remains poor, even with effective
27 adherence to evidence-based pharmacological and non-pharmacological interventions [2, 3]
28 emphasizing the need for novel treatment strategies.

29 The renin-angiotensin-aldosterone system (RAAS) and natriuretic peptide (NP) cascade are
30 key counterregulatory mechanisms that play a critical role in cardiovascular (CV) physiology
31 and disease pathophysiology. Dysregulation of the RAAS leads to hemodynamic
32 perturbations and end-organ remodeling. Angiotensins I (Ang I) and II (Ang II) mediate
33 vasoconstriction, increase in blood pressure and sympathetic tone, sodium and water
34 retention, aldosterone release, fibrosis, and hypertrophy [4-6]. Furthermore, recent evidence
35 shows that elevated aldosterone levels are associated with reduced survival in patients with
36 hypertension and CV diseases [7], and are a significant prognostic marker in patients with
37 systolic HF [8-10]. In contrast, the NP system inhibits the RAAS and decreases sympathetic
38 activation through cyclic guanosine monophosphate (cGMP)-dependent pathways [11, 12].
39 Activation of NP receptors increases diuresis and natriuresis, decreases systemic vascular
40 resistance, and plays a protective role in the CV system by counteracting the effects of fluid
41 overload, as well as through anti-proliferative, anti-hypertrophic, and anti-fibrotic
42 mechanisms [11]. Advanced HF constitutes a state of NP deficiency [13] and is associated
43 with a prolonged activation of the RAAS [14, 15]. Although neprilysin (NEP) inhibitors are
44 capable of enhancing NP levels, they are in practice ineffective at lowering blood pressure
45 in hypertensive patients [16], probably due to a concomitant increase in vasoconstrictors
46 such as Ang II and endothelin (ET)-1 [17]. Therefore, simultaneous NEP and RAAS
47 inhibition offers a promising and innovative therapeutic approach in the management of HF.
48 Previous literature showed that concomitant angiotensin converting enzyme (ACE) and
49 NEP inhibition with omapatrilat tended to improve morbidity and mortality in chronic HF but
50 failed to demonstrate substantial benefit over enalapril alone [18]. In addition, omapatrilat
51 was withdrawn from development due to an unacceptably high rate of angioedema in
52 clinical trials [18, 19]. As clinical studies of sacubitril/valsartan (LCZ696) have shown,
53 replacing the ACE inhibitor with an angiotensin receptor blocker (ARB) minimizes the risk of

54 life-threatening angioedema while retaining the beneficial effects of combined NEP and
55 RAAS inhibition [20].

56 Sacubitril/valsartan is a first-in-class angiotensin receptor neprilysin inhibitor (ARNI), which
57 upon oral administration delivers systemic exposure to sacubitril (AHU377) and valsartan, a
58 well-established ARB recommended by established guidelines for the treatment of HF [1,
59 21, 22]. Sacubitril is an inactive prodrug that is rapidly hydrolyzed by carboxyl esterase 1 to
60 sacubitrilat, a pharmacologically active NEP inhibitor [23]. Phase II/III clinical trials with
61 sacubitril/valsartan have shown beneficial effects in patients with HF and reduced (HFrEF)
62 or preserved (HFpEF) ejection fraction [20, 24]. Sacubitril/valsartan has been approved in
63 many countries for the treatment of HFrEF and is recommended by European and
64 American HF guidelines [25, 26] for the treatment of chronic symptomatic HFrEF (New York
65 Heart Association Class II–IV).

66 Similar to humans, activation of the RAAS accompanies the reduced cardiac output
67 reported in canine chronic HF [27, 28], which motivated the choice of this animal species in
68 the present study [29, 30]. Likewise, ACE inhibitors are the standard of care for the
69 treatment of canine chronic HF, and the effects of the ACE inhibitor prodrug benazepril on
70 the renin cascade have been investigated following low-salt-diet activation of the RAAS in
71 dogs [31].

72 Although numerous studies have shown the positive hemodynamic and clinical effect of
73 sacubitril/valsartan, a comprehensive evaluation of the temporal effects of
74 sacubitril/valsartan on the dynamics of the RAAS and NP cascade is currently missing. The
75 objective of this pharmacology study was to report the pharmacodynamic effects of
76 sacubitril/valsartan on the renin-angiotensin system and cGMP in beagle dogs using a non-
77 invasive model of RAAS activation.

78 **2 Methods**

79 **2.1 Animals**

80 Beagle dogs (N = 18; 9 males and 9 females) from the Novartis Centre de Recherche Sante
81 Animale Test Facility colony (St-Aubin, Switzerland), aged 4–5 years, weighing 10–20 kg,
82 and that were deemed healthy by the study veterinarian, were included in the study.

83 Suitability for inclusion was evaluated by a physical examination and confirmed by
84 measuring selected hematological (red and white blood cells counts, hemoglobin,
85 hematocrit) and clinical chemistry (albumin, total protein, alanine aminotransferase,
86 aspartate aminotransferase, blood urea nitrogen, creatinine) parameters in blood. Prior to
87 the study start, dogs were acclimatized to the experimental facility for a week. Animals were
88 housed in pens (about 2 m²/animal) containing granulate bedding material and an
89 additional elevated platform for resting. The study rooms had natural daylight and additional
90 artificial light of similar intensity (400 lux) from 07:00 to 19:00 h. Room temperature and
91 relative humidity were within the target ranges of 17–23°C and 35–75%, respectively. The
92 quality of drinking water was compliant with the Swiss Federal Regulations on Foodstuff
93 and was offered *ad libitum*.

94 **2.2 Sample size**

95 In absence of preliminary data on the effect of sacubitril/valsartan on biomarkers of the
96 RAAS and cGMP in dogs, a formal sample size calculation with predefined power and type
97 1 error could not be performed. Instead, determination of the study sample size was based
98 on data evaluation of mean differences in plasma renin activity (PRA) between standard of
99 care benazepril and placebo from a previous experiment in 12 beagle dogs (N = 6 per
100 group, [32], for a type 1 error $\alpha = 0.05$ with statistical power = 0.80.

101 **2.3 Experimental model**

102 The non-invasive and fully reversible low-sodium diet (0.05% Na) was used to model
103 activation of the RAAS, as observed in the course of HF. The low-sodium diet has been
104 established as a reliable and reproducible model of RAAS activation to evaluate the effect
105 of RAAS inhibitors in multiple studies [6, 32, 33]. The experimental procedures were
106 performed in compliance with the registered permit number 10/09 covering animal
107 experiments for CV research in dogs, adopted and approved by the Cantonal Animal
108 Welfare Committee of Fribourg (Switzerland): '*Modèle de régime hyposodé pour les*
109 *maladies cardiovasculaires chez le chien*' in February 2009. The study protocol was
110 designed to use the fewest number of animals possible while being consistent with the
111 scientific needs of the study, and conformed to international ethical standards [34].

112

113 **2.4 Study design**

114 A 3-way partial crossover study design was chosen to examine the effect of the test and
115 reference treatment items over a period of 10 days (Figure 1). To achieve steady-state
116 activation of RAAS biomarkers, animals were fed a low-sodium diet for 5 days prior to the
117 oral administration of the study drugs: sacubitril (SAC) calcium salt at 360 mg (Period A); a
118 low sacubitril/valsartan tri-sodium hemipentahydrate salt dose (SVL) at 225 mg (period A); a
119 high sacubitril/valsartan dose (same formulation) (SVH) at 675 mg (Period B and C);
120 valsartan free acid (VAL) 900 mg (Period B and C); benazepril hydrochloride (BNZ) 5 mg
121 (Period C); and empty capsules as placebo (PBO; period A and B) (Figure 1). Dogs were
122 administered the appropriate treatment at 7:00 AM and were fed 12 hours thereafter
123 following withdrawal of the +12-hour blood sample. A 2-week washout period with the dogs
124 on normal chow was maintained between each successive treatment Period.

125 **2.5. Drug dose justification**

126 The selected nominal doses of the therapeutic drugs corresponded to SAC ~24 mg/kg, SVL
127 and SVH ~15 and ~45 mg/kg, VAL ~60 mg/kg, and BNZ ~0.33 mg/kg for an average 15 kg
128 body weight dog. The SVL and SVH doses were selected based on internal preliminary
129 efficacy/safety evaluation and drug metabolism and pharmacokinetics/safety studies in
130 dogs. The dose of VAL was selected to match the exposure of valsartan from the SVH
131 group. This was based on previous findings from Gu et al. [35], showing that the oral
132 bioavailability of VAL following LCZ696 administration was about 3-fold higher than that
133 observed following administration of approximately equimolar doses of VAL alone. The
134 dose of SAC was chosen as an approximately equimolar dose to the NEP inhibitor
135 delivered by the SVH dose. The 5 mg BNZ dose corresponds to the recommended dose in
136 dogs with chronic HF [36].

137 **2.5. Pharmacodynamic assessment**

138 Due to the known sensitivity of the renin-angiotensin cascade to posture and external
139 stimuli [37], specific precautions were taken: i) dogs were maintained in a standing position
140 during blood collection, ii) sampling was performed in a sound-protected room, and iii) low-
141 intensity lighting was used during withdrawal. Blood samples were collected from the *vena*
142 *jugularis* or exceptionally from the *vena cephalixa antebraichii* into 1.2 or 2.7 mL S-

143 Monovette® tubes (Sarstedt Inc. Newton, NC, USA) and kept on ice until centrifugation
144 under refrigeration ($2 \pm 1^\circ\text{C}$), as described by [6, 38, 39]. Samples were collected at pre-
145 dose, and at 1, 2, 4, 6, and 12 hours after oral administration on dosing days 1, 5, and 10
146 for pharmacodynamic assessment of PRA, plasma angiotensin I (Ang I) and II (Ang II),
147 ALD, and plasma cGMP (Periods B and C only). Plasma samples for cGMP determination
148 were not collected during period A and results are therefore not available for SAC and SVL.
149 Measurements of plasma ALD concentrations were carried out using validated high-
150 performance liquid chromatography-mass spectrometry (LC-MS/MS) method with a lower
151 limit of quantification (LLOQ) of 0.02 ng/mL. Plasma cGMP (enzyme immunoassay [EIA] kit,
152 Cayman Chemical Company, USA), Ang I (liquid solid extraction kit, Bachem S-1188,
153 Switzerland), Ang II (EIA kit, SPI BIO, France), and PRA (EIA kit, USCN Life Sciences Inc,
154 China) were performed using validated kits, as previously described. PRA was determined
155 by measuring the rate of Ang I formation after 2-hour incubation of endogenous renin and
156 angiotensinogen in plasma at 37°C and pH 7.2. The LLOQs were 30 pg/mL and 2 pg/mL for
157 Ang I and Ang II, respectively, and 0.05 pmol/mL for cGMP. Analyses were performed in
158 duplicates; values with a coefficient of variation below 25% were retained for statistical
159 evaluation.

160 **2.6. Pharmacokinetic assessment**

161 Pharmacokinetic measures were performed using blood collected at day 5 from the *vena*
162 *jugularis* (or exceptionally from the *vena cephalica antebrachii*) into 1.2 mL S-Monovette®
163 tubes (Sarstedt Inc. Newton, NC, USA). For sample collection of BNZ, heparin was used as
164 an anticoagulant, and for VAL, SVL/SVH, and SAC, EDTA was used. The tubes were gently
165 inverted 5 times and chilled in ice immediately, then centrifuged at 1600 g for 15 minutes at
166 1°C to obtain the plasma specimen. Plasma samples were frozen at -80°C until further
167 analysis. Plasma concentrations of sacubitrilat, benazeprilat, and VAL were analyzed in the
168 SAC-, SVL/SVH-, BNZ-, and VAL-treated dogs. Concentrations of sacubitrilat, benazeprilat,
169 and VAL were determined using validated high-performance LC-MS/MS methods. The
170 LLOQ of benazeprilat in plasma was 0.5 ng/mL, and 5 ng/mL for sacubitrilat and VAL.

171 Pharmacokinetic parameter estimates were derived from a statistical moment (non-
172 compartmental) analysis implemented in validated SAS macros (SAS® Version 9.1) and
173 consisted of the following:

- 174 1) the maximum concentration (C_{\max}),
175 2) the time to maximum concentration (T_{\max}), and
176 3) the area under the concentration–time curve ($AUC_{0-\text{last}}$).

177 Pre-dose time was specified with time 0 (hour) and corresponding values below LLOQ were
178 replaced by zero. Below LLOQ values at subsequent times were excluded from the
179 analysis. Summary statistics including geometric mean and range of values were provided
180 for all mentioned pharmacokinetic parameters.

181 **2.7. Safety evaluation**

182 Safety assessments included hematology, biochemistry, hemostasis, body weight, and
183 body condition scoring.

184 **2.8. Statistical analyses of biomarker data**

185 To anticipate plausible variations in biomarker levels across treatment days, data were
186 expressed as absolute change from baseline, defined as the individual biomarker
187 concentration at hour 0 (pre-dose), separately for day 1, day 5 and day 10. In accordance
188 with previous descriptions of the effect of sacubitril/valsartan on the RAAS in humans [35],
189 individual time-weighted average (TWA) change from baselines were estimated separately
190 for each day (D1, D5 and D10) in each period (A, B, C), and analyzed by random effect-
191 repeated measures analyses of variance (RRMANOVA), with fixed effect classification
192 variables PERIOD (A, B and C), TRT (treatment group with 6 levels: Placebo, SAC, SVL,
193 SVH, VAL, and BNZ), DAY (D1, D5, and D10) and the two-way interaction TRT by DAY.
194 ANIMAL (1 to 18) was included as a random effect in the model.

195 Finally, in order to leverage all available pharmacodynamic information and derive
196 meaningful and robust statistical comparisons, plasma biomarker data (both time courses
197 and TWAs) from days 1, 5, and 10 were pooled for each treatment and analyzed by the
198 RRMANOVA approach. All calculations were done using SAS[®] Version 9.2 by applying
199 univariate analysis for calculation of summary statistics. SAS[®] procedure was applied to
200 execute the analyses of variance. All tests were performed two-sided with a level of
201 significance α pre-defined at 0.05.

202

203 **3 Results**

204 All experimental animals were randomly assigned to three groups of 6 dogs each and were
205 available for pharmacodynamic, pharmacokinetic, and safety assessments. No statistical
206 difference in baseline characteristics were observed between study groups, based on
207 selected hematological and clinical chemistry parameters.

208 **3.1. Safety assessment**

209 All experimental animals completed the study without any incidence of adverse events with
210 any of the test drugs. All dogs returned to the maintenance facility at the end of the
211 experiment.

212 **3.2. Effect on plasma cGMP**

213 The typical baseline value for cGMP across treatment groups was ca. 15 pmol/mL.
214 RRMANOVA results showed significant increases in cGMP circulating levels within all
215 treatment groups, including PBO (Figure 2, Panels A and B), which are indicative of diurnal
216 variations of this biomarker in dogs. The TRT effect was found to be significant, but the TRT
217 by DAY interaction did not reach the level of statistical significance. The estimated
218 differences in TWA changes in cGMP were significant between the SVH group and the
219 other three treatment groups (Figure 2, Panel C). On an average SVH significantly
220 increased circulating cGMP levels by approximately 4 pmol/mL as compared with VAL,
221 BNZ, and PBO (Figure 2, Panel C). Conversely, no apparent differences were reported
222 between VAL, BNZ, and PBO treated dogs.

223 **3.3. Effect on PRA**

224 The typical baseline value for PRA across treatment groups was ca. 400 pg/mL/h. PRA
225 remained relatively stable over the 12-hour observation period in the PBO and SAC groups,
226 but appeared to increase with the remaining treatments, and especially with SVH and VAL
227 (Figure 3, Panel A). Results from the RRMANOVA showed that only the TRT effect was
228 significant. The PERIOD and DAY effect, and the TRT by DAY interaction were not
229 statistically significant. The effect of sacubitril/valsartan on PRA was dose-dependent, with
230 only SVH and VAL showing a significant TWA change from baseline (Figure 3, Panel B).
231 Likewise, both SVH and VAL achieved significantly greater elevation of PRA than PBO,

232 SAC, and BNZ (Figure 3, Panel C). Also, VAL showed significantly greater increase in PRA
233 than SVL and SVH (Figure 3, Panel C).

234 **3.4. Effect on Ang I and Ang II**

235 The time-course of response for Ang I and Ang II appeared seemingly consistent with that
236 of PRA. The typical baseline value under low-sodium diet was ca. 185 pg/mL and 15 pg/mL
237 for Ang I and Ang II, respectively. For both angiotensins, the results of the RRMANOVA
238 showed that only the TRT effect was significant. The PERIOD and DAY effect, and the TRT
239 by DAY interaction were not significant.

240 An apparent increase in Ang I was observed for all treatment groups but SAC and PBO,
241 with VAL and SVH showing the most pronounced effect overall (Figure 4, Panel A). Similar
242 to PRA, only SVH and VAL showed a significant TWA Ang I change from baseline (Figure
243 4, Panel B). Differences to PBO were highly significant for both sacubitril/valsartan dosing
244 groups. The effect of VAL was superior to that of all other treatment groups, while dosing
245 with SVH and BNZ yielded significant differences to sacubitril alone (Figure 4, Panel C).

246 All treatment groups but PBO and BNZ appeared to elevate Ang II, with sacubitril/valsartan
247 showing the most pronounced effect overall (Figure 5, Panel A). Consistent with PRA and
248 Ang I, only SVH and VAL demonstrated a significant TWA Ang II change from baseline
249 (Figure 5, Panel B). SVH and VAL significantly increased Ang II as compared with PBO
250 (estimated difference of 9.6 and 6.7 pg/mL, respectively) (Figure 5, Panel C). There was a
251 modest and non-significant increase in Ang II following SVL (+4.4 pg/mL vs. PBO), and
252 SAC treatment alone (+2.8 pg/mL vs. PBO).

253 **3.5. Effect on ALD**

254 The typical baseline value for ALD under low-sodium diet was ca. 0.25 ng/mL. Both
255 sacubitril/valsartan doses and VAL had an apparent effect on ALD plasma concentrations,
256 while only modest and non-significant changes were reported in the other treatment groups
257 (Figure 6, Panel A). Interestingly enough, the decrease of ALD in the sacubitril/valsartan
258 groups was not dose-dependent, and a rebound of ALD concentration was observed in the
259 SVH dosing group. Results from the RRMANOVA showed that only the TRT effect was
260 significant. The PERIOD and DAY effect, and the TRT by DAY interaction were not of

261 significance. SVH, SVL VAL and BNZ achieved a significant TWA change from baseline
262 (Figure 6, Panel B). In addition, differences to PBO were found to be statistically significant
263 for SVH, SVL and VAL, but not significant for BNZ (Figure 6, Panel C). There was a trend
264 towards a decrease of ALD with SAC, but the estimated difference to PBO (approximately
265 half of the reduction obtained with VAL) did not reach the level of statistical significance.

266 **3.6. Pharmacokinetics of the test drugs**

267 Plasma pharmacokinetics following oral dosing with sacubitril/valsartan, SAC, VAL, and
268 BNZ on Day 5 is presented in Table 1. SVH delivered comparable systemic exposure (as
269 defined by C_{max} , AUC_{0-last}) of sacubitrilat as the SAC 360 mg dose. Systemic exposure to
270 VAL was also similar between SVH and VAL 900 mg. Furthermore, there was an apparent
271 more than dose-proportional increase in exposure to sacubitrilat between the SVL (225 mg)
272 and SVH (675 mg) doses. However, the exposure increase of VAL was approximately
273 proportional with the dose between SVL and SVH. The T_{max} values of the
274 sacubitril/valsartan analytes were similar for both SVL and SVH. The time to maximum
275 sacubitrilat and VAL peak concentrations appeared to be slightly shorter in the
276 sacubitril/valsartan groups as compared with the SAC and VAL alone treatments.

277 **4 Discussion**

278 We report the results of the first comprehensive evaluation of the temporal effects of
279 sacubitril/valsartan on biomarkers of the RAAS and cGMP using an established canine
280 model of RAAS activation.

281 Consistent with previous findings from Gu et al., [35], our pharmacokinetic analysis showed
282 that systemic exposure to VAL (AUC_{0-last} and C_{max}) following sacubitril/valsartan oral
283 administration was about 3-fold higher than that observed after approximately equimolar
284 doses of VAL. Consequently, the exposure to VAL between the 900 mg VAL group and the
285 675 mg sacubitril/valsartan oral treatments was comparable, such that differences in RAAS
286 and cGMP biomarkers between these two groups can be attributed to NEP inhibition alone.
287 The large between-dog variation in VAL and sacubitrilat exposure was expected and is in
288 agreement with previous results from Gu et al. [35] who reported a coefficient of variation
289 between 50% and 100% in a preliminary pharmacokinetic study with 3 Beagle dogs. At this
290 time, the structural causes of such variability are unclear, yet it had apparent consequences

291 on the variations of the RAAS and cGMP biomarkers response to SVH, SVL and VAL,
292 limiting statistical comparisons between study groups. Finally, the pharmacokinetics of
293 benazeprilat following 5 mg oral dosing with BNZ is consistent with previous literature in
294 dogs [36, 40].

295 Inhibition of NEP is known to be associated with increased levels of NPs, which stimulate
296 synthesis of cGMP [35]. The observed trend towards plasma cGMP increase in the late
297 afternoon for all treatment groups is consistent with published literature in humans [41],
298 which is indicative of diurnal oscillations of this biomarker in dogs. While sacubitril/valsartan,
299 VAL, and BNZ modulated the renin cascade, the increased cGMP levels by
300 sacubitril/valsartan, but not VAL or BNZ, demonstrate activation of the NP system
301 attributable to sacubitrilat, the active metabolite of SAC [35, 42]. These changes could also
302 be mediated (at least in part) by variations in circulating nitric oxide (NO) levels as recent
303 studies in rats showed an increase in NO bioavailability consecutive to sacubitril/valsartan
304 treatment [43]. Although cGMP data for the SVL dose were not available in the present
305 study, previous publications in healthy humans have demonstrated dose-dependent
306 increases in circulating cGMP levels. In these experiments, plasma cGMP increased as
307 early as 4 hours following sacubitril/valsartan administration compared with placebo, with a
308 return to baseline level within 24 hours [35]. Similarly, in the same study, dose dependent
309 increases in RAAS biomarkers (PRA and Ang II) reached maximum within 4 hours of
310 sacubitril/valsartan dose in healthy human participants [35]. In patients with HF and left
311 ventricular ejection fraction $\leq 40\%$, sacubitril/valsartan 100 mg titrated to 200 mg twice daily
312 increased plasma cGMP (1.4 times baseline) and urinary ANP as a result of NEP inhibition
313 [44]. Likewise, significant reductions of plasma NT-pro brain NP in patients with HF_{rEF}
314 treated with sacubitril/valsartan 200 mg showed clinical benefits in the PARADIGM-HF
315 study, which correlated with risk reduction in CV mortality and HF hospitalizations compared
316 with the ACE inhibitor enalapril [20, 45].

317 SVH and VAL alone significantly increased PRA over the course of the study. Similar to
318 PRA, plasma Ang I levels increased in both groups, indicating that sacubitril/valsartan
319 blocks Ang II signaling through the Ang II type 1 (AT₁) receptor, causing the known
320 compensatory up-regulation of plasma renin and Ang I [46, 47]. In contrast, the ACE
321 inhibitor benazepril did not significantly increase PRA, which was unexpected and in

322 contradiction with previous literature reporting similar ranges of systemic exposure to
323 benazeprilat in dogs [6, 32].

324 Interestingly, VAL showed significantly greater elevation of PRA than SVL and SVH.
325 Likewise, SAC and SVL did not show a significant effect on the levels of Ang I, while the
326 effect of VAL on Ang I appeared to be significantly greater than the effect of SVH. These
327 observations are consistent with the known effect of atrial NP to suppress renin production
328 [48], thereby leading to a more pronounced effect of VAL on PRA and Ang I as compared
329 with SVH.

330 Overall, the increase in plasma Ang II levels in response to treatment was similar to the
331 changes observed for PRA and Ang I, except for the effect of SVH being somewhat more
332 pronounced than VAL at the early time-points. This is consistent with the inhibition of NEP,
333 an enzyme known to degrade plasma Ang II [49, 50]. In contrast, BNZ had no noticeable
334 effect on plasma Ang II levels, which is also in line with our findings on PRA and Ang I. This
335 upholds previous reports showing only partial reduction of Ang II in dogs receiving 10 mg
336 BNZ, and no decrease in circulating Ang II in 45% of canine patients with stable chronic HF
337 despite long-term ACE inhibitor use. One possible explanation is activation of alternative
338 biological pathways (e.g. chymase, cathepsin G and tonin) for Ang II production [6, 31, 32,
339 51].

340 Both sacubitril/valsartan doses and VAL significantly decreased ALD levels, with the
341 greatest decrease observed in the sacubitril/valsartan treated groups within the first 2 hours
342 after dosing. In addition, SAC showed a moderate (but non-significant) decrease in ALD
343 levels compared with placebo (reaching approximately half of the reduction in ALD
344 observed with VAL), indicating that simultaneous inhibition of NEP and blockade of the AT₁
345 receptor by sacubitril/valsartan could in theory be additive and lead to positive clinical
346 outcomes by decreasing a known prognostic marker of HF. Of note, oral dosing with SVH
347 and VAL did result in comparable reduction of ALD in dogs (while providing similar
348 exposure to VAL), which would indicate that the effect of sacubitril/valsartan on ALD is
349 mainly driven by VAL. In humans, the degree of ALD increase is related to the severity of
350 heart failure [52] and ALD is known to worsen Ang II tissue-damaging properties [53].
351 Therefore, elevated exposure to ALD has been associated with a poor prognosis in multiple
352 case studies [54, 55]. More precisely, Swedberg et al. [56] have found a positive correlation

353 between mortality and systemic levels of ALD ($p < 0.003$) in a group of severe HF patients.
354 In a report from Güder et al. [8], high ALD concentrations were found to be a predictor of
355 increased mortality risk that provides complementary prognostic value in a prospective
356 cohort experiment of 294 HF patients. Finally, and consistently with our observations in
357 dogs, the clinical relevance of RAAS inhibition and ALD reduction in patients under ARNI
358 therapy was demonstrated in a study by Jordaan et al [57].

359 Interestingly, a steep ALD return to baseline was noted in the SVH group between 6 and 12
360 hours after dosing, implicating a rebound phenomenon occurring at the higher
361 sacubitril/valsartan dose and suggesting optimum ALD inhibition being achieved at a lower
362 therapeutic dose. Similar rebound in ALD levels were observed after infusion of atrial NP in
363 patients with mild-to-moderate hypertension [58].

364 **4.1. Limitations**

365 Because of the small study size, the statistical significance of certain findings was
366 hampered by low statistical power. This is illustrated by the non-significance of the inhibitory
367 effect of SAC alone on ALD, PRA and Ang I, and its stimulatory effect on Ang II.
368 Conversely, the clinical significance of the reported statistical differences between study
369 groups remains unclear, although, these are consistent with clinical results from the
370 PARADIGM-HF study demonstrating superiority of sacubitril/valsartan over enalapril in
371 human patients with HF. In addition, the effect of low dose sacubitril/valsartan on plasma
372 cGMP could not be investigated in the present study, leaving it unclear whether sufficient
373 inhibition of NEP could be achieved with a 225 mg dose. Finally, results from our earlier
374 research [59] have shown an 8- to 10-fold rise in urinary ALD in 6 healthy beagle dogs fed a
375 low-salt diet (0.05% Na) for 10 days. While sodium restriction is a powerful stimulant of the
376 renin-angiotensin cascade, a detailed description of Ang II and ALD elevation in dogs
377 suffering from HF is currently missing. This would be an important step towards the formal
378 validation of the low-salt diet as a reliable model of HF-related RAAS activation. As such,
379 the positive pharmacological effects of sacubitril/valsartan reported in the present study
380 should be confirmed by additional clinical work in dogs with HF to evaluate the
381 hemodynamic effect of ARNI on disease modulation in canines.

382

383 **4.2. Conclusion**

384 In conclusion, the ARNI sacubitril/valsartan reduced ALD, a known risk factor of CV
385 mortality, and enhanced the NP system via cGMP-mediated pathways in a low-sodium diet
386 model of RAAS activation. The results presented herein provide further evidence that the
387 effects on the renin cascade extend to reduced ALD levels beyond that achieved with RAAS
388 blockade alone. These positive findings in dogs also suggest that sacubitril/valsartan is a
389 promising pharmacological candidate for increased survival in canine cardiovascular
390 diseases.

391 **5 Funding**

392 This study was supported by Novartis Pharma AG, Basel, Switzerland.

393 **6 Conflict of Interest**

394 With the exception of Prof. Meindert Danhof, the authors of the manuscript were Novartis
395 employees at the time the study was performed.

396 **7 Author Contributions**

397 JPM, MP and DFR conceived the experimental protocols. JG and MD contributed to the
398 development of the hypothesis and reviewed the study protocols. CHT was responsible for
399 the statistical analysis of the study results. All authors contributed to the preparation of the
400 manuscript.

401 **8 Acknowledgments**

402 All contributing authors had full access to the study data and agree with the publication of
403 the results.

404

405 References

406

- 407 1 McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, Falk V,
408 Filippatos G, Fonseca C, Gomez-Sanchez MA, Jaarsma T, Kober L, Lip GY,
409 Maggioni AP, Parkhomenko A, Pieske BM, Popescu BA, Ronnevik PK, Rutten FH,
410 Schwitter J, Seferovic P, Stepinska J, Trindade PT, Voors AA, Zannad F, Zeiher A,
411 Task Force for the D, Treatment of A, Chronic Heart Failure of the European Society
412 of C, Bax JJ, Baumgartner H, Ceconi C, Dean V, Deaton C, Fagard R, Funck-
413 Brentano C, Hasdai D, Hoes A, Kirchhof P, Knuuti J, Kolh P, McDonagh T, Moulin C,
414 Popescu BA, Reiner Z, Sechtem U, Sirnes PA, Tendera M, Torbicki A, Vahanian A,
415 Windecker S, McDonagh T, Sechtem U, Bonet LA, Avraamides P, Ben Lamin HA,
416 Brignole M, Coca A, Cowburn P, Dargie H, Elliott P, Flachskampf FA, Guida GF,
417 Hardman S, Iung B, Merkely B, Mueller C, Nanas JN, Nielsen OW, Orn S, Parissis
418 JT, Ponikowski P, Guidelines ESCCfP (2012) ESC guidelines for the diagnosis and
419 treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis
420 and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of
421 Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of
422 the ESC. *Eur J Heart Fail.* 14 (8): 803-69. <https://doi.org/10.1093/eurjhf/hfs105>
- 423 2 Azad N, Lemay G (2014) Management of chronic heart failure in the older
424 population. *J Geriatr Cardiol.* 11 (4): 329-37. <https://doi.org/10.11909/j.issn.1671-5411.2014.04.008>
- 426 3 Oldland E, Driscoll A, Currey J (2014) High complexity chronic heart failure
427 management programmes: programme characteristics and 12 month patient
428 outcomes. *Collegian.* 21 (4): 319-26
- 429 4 Hsueh WA, Wyne K (2011) Renin-Angiotensin-aldosterone system in diabetes and
430 hypertension. *J Clin Hypertens (Greenwich).* 13 (4): 224-
431 37. <https://doi.org/10.1111/j.1751-7176.2011.00449.x>
- 432 5 Mishra A, Srivastava A, Mittal T, Garg N, Mittal B (2012) Impact of renin-angiotensin-
433 aldosterone system gene polymorphisms on left ventricular dysfunction in coronary
434 artery disease patients. *Dis Markers.* 32 (1): 33-41. <https://doi.org/10.3233/DMA-2012-0858>
- 436 6 Mochel JP, Peyrou M, Fink M, Strehlau G, Mohamed R, Giraudel JM, Ploeger B,
437 Danhof M (2013) Capturing the dynamics of systemic Renin-Angiotensin-Aldosterone
438 System (RAAS) peptides heightens the understanding of the effect of benazepril in
439 dogs. *J Vet Pharmacol Ther.* 36 (2): 174-80. <https://doi.org/10.1111/j.1365-2885.2012.01406.x>
- 441 7 Pimenta E, Gordon RD, Stowasser M (2013) Salt, aldosterone and hypertension. *J*
442 *Hum Hypertens.* 27 (1): 1-6. <https://doi.org/10.1038/jhh.2012.27>
- 443 8 Guder G, Bauersachs J, Frantz S, Weismann D, Allolio B, Ertl G, Angermann CE,
444 Stork S (2007) Complementary and incremental mortality risk prediction by cortisol
445 and aldosterone in chronic heart failure. *Circulation.* 115 (13): 1754-
446 61. <https://doi.org/10.1161/CIRCULATIONAHA.106.653964>
- 447 9 Girerd N, Pang PS, Swedberg K, Fought A, Kwasny MJ, Subacius H, Konstam MA,
448 Maggioni A, Gheorghiade M, Zannad F, investigators E (2013) Serum aldosterone is

- 449 associated with mortality and re-hospitalization in patients with reduced ejection
450 fraction hospitalized for acute heart failure: analysis from the EVEREST trial. *Eur J*
451 *Heart Fail.* 15 (11): 1228-35. <https://doi.org/10.1093/eurjhf/hft100>
- 452 10 Guder G, Hammer F, Deutschbein T, Brenner S, Berliner D, Deubner N, Bidlingmaier
453 M, Ertl G, Allolio B, Angermann CE, Fassnacht M, Stork S (2015) Prognostic value of
454 aldosterone and cortisol in patients hospitalized for acutely decompensated chronic
455 heart failure with and without mineralocorticoid receptor antagonism. *J Card Fail.* 21
456 (3): 208-16. <https://doi.org/10.1016/j.cardfail.2014.12.011>
- 457 11 Volpe M (2014) Natriuretic peptides and cardio-renal disease. *Int J Cardiol.* 176 (3):
458 630-9. <https://doi.org/10.1016/j.ijcard.2014.08.032>
- 459 12 Volpe M, Carnovali M, Mastromarino V (2016) The natriuretic peptides system in the
460 pathophysiology of heart failure: from molecular basis to treatment. *Clin Sci (Lond).*
461 130 (2): 57-77. <https://doi.org/10.1042/CS20150469>
- 462 13 Mangiafico S, Costello-Boerrigter LC, Andersen IA, Cataliotti A, Burnett JC, Jr.
463 (2013) Neutral endopeptidase inhibition and the natriuretic peptide system: an
464 evolving strategy in cardiovascular therapeutics. *Eur Heart J.* 34 (12): 886-
465 93c. <https://doi.org/10.1093/eurheartj/ehs262>
- 466 14 Schrier RW, Abdallah JG, Weinberger HH, Abraham WT (2000) Therapy of heart
467 failure. *Kidney Int.* 57 (4): 1418-25. <https://doi.org/10.1046/j.1523-1755.2000.00986.x>
- 468 15 Brewster UC, Setaro JF, Perazella MA (2003) The renin-angiotensin-aldosterone
469 system: cardiorenal effects and implications for renal and cardiovascular disease
470 states. *Am J Med Sci.* 326 (1): 15-24
- 471 16 Bevan EG, Connell JM, Doyle J, Carmichael HA, Davies DL, Lorimer AR, McInnes
472 GT (1992) Candoxatril, a neutral endopeptidase inhibitor: efficacy and tolerability in
473 essential hypertension. *J Hypertens.* 10 (7): 607-13
- 474 17 Ferro CJ, Spratt JC, Haynes WG, Webb DJ (1998) Inhibition of neutral
475 endopeptidase causes vasoconstriction of human resistance vessels in vivo.
476 *Circulation.* 97 (23): 2323-30. <https://doi.org/10.1161/01.CIR.97.23.2323>
- 477 18 Packer M, Califf RM, Konstam MA, Krum H, McMurray JJ, Rouleau JL, Swedberg K
478 (2002) Comparison of omapatrilat and enalapril in patients with chronic heart failure:
479 the Omapatrilat Versus Enalapril Randomized Trial of Utility in Reducing Events
480 (OVERTURE). *Circulation.* 106 (8): 920-
481 6. <https://doi.org/10.1161/01.CIR.0000029801.86489.50>
- 482 19 Kostis JB, Packer M, Black HR, Schmieder R, Henry D, Levy E (2004) Omapatrilat
483 and enalapril in patients with hypertension: the Omapatrilat Cardiovascular
484 Treatment vs. Enalapril (OCTAVE) trial. *Am J Hypertens.* 17 (2): 103-
485 11. <https://doi.org/10.1016/j.amjhyper.2003.09.014>
- 486 20 McMurray JJ, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL,
487 Shi VC, Solomon SD, Swedberg K, Zile MR, Investigators P-H, Committees (2014)
488 Angiotensin-neprilysin inhibition versus enalapril in heart failure. *N Engl J Med.* 371
489 (11): 993-1004. <https://doi.org/10.1056/NEJMoa1409077>

- 490 21 Langenickel TH, Dole WP (2012) Angiotensin receptor-neprilysin inhibition with
491 LCZ696: a novel approach for the treatment of heart failure. . Drug Discovery Today:
492 Therapeutic Strategies. 9 (4): e131-e9.<https://doi.org/10.1016/j.ddstr.2013.11.002>
- 493 22 Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, Fonarow GC,
494 Geraci SA, Horwich T, Januzzi JL, Johnson MR, Kasper EK, Levy WC, Masoudi FA,
495 McBride PE, McMurray JJ, Mitchell JE, Peterson PN, Riegel B, Sam F, Stevenson
496 LW, Tang WH, Tsai EJ, Wilkoff BL, American College of Cardiology F, American
497 Heart Association Task Force on Practice G (2013) 2013 ACCF/AHA guideline for
498 the management of heart failure: a report of the American College of Cardiology
499 Foundation/American Heart Association Task Force on Practice Guidelines. J Am
500 Coll Cardiol. 62 (16): e147-239.<https://doi.org/10.1016/j.jacc.2013.05.019>
- 501 23 Shi J, Wang X, Nguyen J, Wu AH, Bleske BE, Zhu HJ (2016) Sacubitril Is Selectively
502 Activated by Carboxylesterase 1 (CES1) in the Liver and the Activation Is Affected by
503 CES1 Genetic Variation. Drug Metab Dispos. 44 (4): 554-
504 9.<https://doi.org/10.1124/dmd.115.068536>
- 505 24 Solomon SD, Zile M, Pieske B, Voors A, Shah A, Kraigher-Krainer E, Shi V,
506 Bransford T, Takeuchi M, Gong J, Lefkowitz M, Packer M, McMurray JJ, Prospective
507 comparison of AwARBoMOhfwpefl (2012) The angiotensin receptor neprilysin
508 inhibitor LCZ696 in heart failure with preserved ejection fraction: a phase 2 double-
509 blind randomised controlled trial. Lancet. 380 (9851): 1387-
510 95.[https://doi.org/10.1016/S0140-6736\(12\)61227-6](https://doi.org/10.1016/S0140-6736(12)61227-6)
- 511 25 Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JG, Coats AJ, Falk V,
512 Gonzalez-Juanatey JR, Harjola VP, Jankowska EA, Jessup M, Linde C,
513 Nihoyannopoulos P, Parissis JT, Pieske B, Riley JP, Rosano GM, Ruilope LM,
514 Ruschitzka F, Rutten FH, van der Meer P, Authors/Task Force M, Document R
515 (2016) 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic
516 heart failure: The Task Force for the diagnosis and treatment of acute and chronic
517 heart failure of the European Society of Cardiology (ESC). Developed with the
518 special contribution of the Heart Failure Association (HFA) of the ESC. Eur J Heart
519 Fail. 18 (8): 891-975.<https://doi.org/10.1002/ejhf.592>
- 520 26 Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Colvin MM, Drazner MH,
521 Filippatos G, Fonarow GC, Givertz MM, Hollenberg SM, Lindenfeld J, Masoudi FA,
522 McBride PE, Peterson PN, Stevenson LW, Westlake C (2016) 2016 ACC/AHA/HFSA
523 Focused Update on New Pharmacological Therapy for Heart Failure: An Update of
524 the 2013 ACCF/AHA Guideline for the Management of Heart Failure: A Report of the
525 American College of Cardiology/American Heart Association Task Force on Clinical
526 Practice Guidelines and the Heart Failure Society of America. J Am Coll Cardiol. 68
527 (13): 1476-88.<https://doi.org/10.1016/j.jacc.2016.05.011>
- 528 27 Sayer MB, Atkins CE, Fujii Y, Adams AK, DeFrancesco TC, Keene BW (2009) Acute
529 effect of pimobendan and furosemide on the circulating renin-angiotensin-
530 aldosterone system in healthy dogs. J Vet Intern Med. 23 (5): 1003-
531 6.<https://doi.org/10.1111/j.1939-1676.2009.0367.x>
- 532 28 Watkins L, Jr., Burton JA, Haber E, Cant JR, Smith FW, Barger AC (1976) The renin-
533 angiotensin-aldosterone system in congestive failure in conscious dogs. J Clin
534 Invest. 57 (6): 1606-17.<https://doi.org/10.1172/JCI108431>

- 535 29 Cowley AW, Jr., Guyton AC (1972) Quantification of intermediate steps in the renin-
536 angiotensin-vasoconstrictor feedback loop in the dog. *Circ Res.* 30 (5): 557-
537 [66.https://doi.org/10.1161/01.RES.30.5.557](https://doi.org/10.1161/01.RES.30.5.557)
- 538 30 Guyton AC, Coleman TG, Cowley AW, Jr., Liard JF, Norman RA, Jr., Manning RD,
539 Jr. (1972) Systems analysis of arterial pressure regulation and hypertension. *Ann*
540 *Biomed Eng.* 1 (2): 254-81. <https://doi.org/10.1007/BF02584211>
- 541 31 Mochel JP, Danhof M (2015) Chronobiology and Pharmacologic Modulation of the
542 Renin-Angiotensin-Aldosterone System in Dogs: What Have We Learned? *Rev*
543 *Physiol Biochem Pharmacol.* 169: 43-69. https://doi.org/10.1007/112_2015_27
- 544 32 Mochel JP, Fink M, Peyrou M, Soubret A, Giraudel JM, Danhof M (2015)
545 Pharmacokinetic/Pharmacodynamic Modeling of Renin-Angiotensin Aldosterone
546 Biomarkers Following Angiotensin-Converting Enzyme (ACE) Inhibition Therapy with
547 Benazepril in Dogs. *Pharm Res.* 32 (6): 1931-46. <https://doi.org/10.1007/s11095-014-1587-9>
548
- 549 33 Kjolby MJ, Kompanowska-Jeziarska E, Wamberg S, Bie P (2005) Effects of sodium
550 intake on plasma potassium and renin angiotensin aldosterone system in conscious
551 dogs. *Acta Physiol Scand.* 184 (3): 225-34. <https://doi.org/10.1111/j.1365-201X.2005.01452.x>
552
- 553 34 Portaluppi F, Smolensky MH, Touitou Y (2010) Ethics and methods for biological
554 rhythm research on animals and human beings. *Chronobiol Int.* 27 (9-10): 1911-
555 29. <https://doi.org/10.3109/07420528.2010.516381>
- 556 35 Gu J, Noe A, Chandra P, Al-Fayoumi S, Ligueros-Saylan M, Sarangapani R, Maahs
557 S, Ksander G, Rigel DF, Jeng AY, Lin TH, Zheng W, Dole WP (2010)
558 Pharmacokinetics and pharmacodynamics of LCZ696, a novel dual-acting
559 angiotensin receptor-neprilysin inhibitor (ARNi). *J Clin Pharmacol.* 50 (4): 401-
560 14. <https://doi.org/10.1177/0091270009343932>
- 561 36 King JN, Mauron C, Kaiser G (1995) Pharmacokinetics of the active metabolite of
562 benazepril, benazeprilat, and inhibition of plasma angiotensin-converting enzyme
563 activity after single and repeated administrations to dogs. *Am J Vet Res.* 56 (12):
564 1620-8
- 565 37 Muller AF, Manning EL, Riondel AM (1958) Influence of position and activity on the
566 secretion of aldosterone. *Lancet.* 1 (7023): 711-3. [https://doi.org/10.1016/S0140-6736\(58\)91137-1](https://doi.org/10.1016/S0140-6736(58)91137-1)
567
- 568 38 Mochel JP, Fink M, Peyrou M, Desevaux C, Deurinck M, Giraudel JM, Danhof M
569 (2013) Chronobiology of the renin-angiotensin-aldosterone system in dogs: relation
570 to blood pressure and renal physiology. *Chronobiol Int.* 30 (9): 1144-
571 59. <https://doi.org/10.3109/07420528.2013.807275>
- 572 39 Mochel JP, Fink M, Bon C, Peyrou M, Bieth B, Desevaux C, Deurinck M, Giraudel
573 JM, Danhof M (2014) Influence of feeding schedules on the chronobiology of renin
574 activity, urinary electrolytes and blood pressure in dogs. *Chronobiol Int.* 31 (5): 715-
575 30. <https://doi.org/10.3109/07420528.2014.897711>
- 576 40 King JN, Maurer M, Morrison CA, Mauron C, Kaiser G (1997) Pharmacokinetics of
577 the angiotensin-converting-enzyme inhibitor, benazepril, and its active metabolite,

- 578 benazeprilat, in dog. *Xenobiotica*. 27 (8): 819-
579 [29.https://doi.org/10.1080/004982597240181](https://doi.org/10.1080/004982597240181)
- 580 41 Zhdanova IV, Simmons M, Marcus JN, Busza AC, Leclair OU, Taylor JA (1999)
581 Nocturnal increase in plasma cGMP levels in humans. *J Biol Rhythms*. 14 (4): 307-
582 [13.https://doi.org/10.1177/074873099129000722](https://doi.org/10.1177/074873099129000722)
- 583 42 Azizi M, Menard J (2004) Combined blockade of the renin-angiotensin system with
584 angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor
585 antagonists. *Circulation*. 109 (21): 2492-
586 [9.https://doi.org/10.1161/01.CIR.0000131449.94713.AD](https://doi.org/10.1161/01.CIR.0000131449.94713.AD)
- 587 43 Trivedi RK, Polhemus DJ, Li Z, Yoo D, Koiwaya H, Scarborough A, Goodchild TT,
588 Lefer DJ (2018) Combined Angiotensin Receptor-Nepriylsin Inhibitors Improve
589 Cardiac and Vascular Function Via Increased NO Bioavailability in Heart Failure. *J*
590 *Am Heart Assoc*. 7 (5).<https://doi.org/10.1161/JAHA.117.008268>
- 591 44 Kobalava Z, Kotovskaya Y, Averkov O, Pavlikova E, Moiseev V, Albrecht D, Chandra
592 P, Ayalasomayajula S, Prescott MF, Pal P, Langenickel TH, Jordaan P, Rajman I
593 (2016) Pharmacodynamic and Pharmacokinetic Profiles of Sacubitril/Valsartan
594 (LCZ696) in Patients with Heart Failure and Reduced Ejection Fraction. *Cardiovasc*
595 *Ther*. 34 (4): 191-8.<https://doi.org/10.1111/1755-5922.12183>
- 596 45 Packer M, McMurray JJ, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL,
597 Shi VC, Solomon SD, Swedberg K, Zile M, Andersen K, Arango JL, Arnold JM,
598 Belohlavek J, Bohm M, Boytsov S, Burgess LJ, Cabrera W, Calvo C, Chen CH,
599 Dukat A, Duarte YC, Erglis A, Fu M, Gomez E, Gonzalez-Medina A, Hagege AA,
600 Huang J, Katova T, Kiatchoosakun S, Kim KS, Kozan O, Llamas EB, Martinez F,
601 Merkely B, Mendoza I, Mosterd A, Negrusz-Kawecka M, Peuhkurinen K, Ramires FJ,
602 Refsgaard J, Rosenthal A, Senni M, Sibulo AS, Jr., Silva-Cardoso J, Squire IB,
603 Starling RC, Teerlink JR, Vanhaecke J, Vinereanu D, Wong RC, Investigators P-H,
604 Coordinatorsdagger (2015) Angiotensin receptor neprilysin inhibition compared with
605 enalapril on the risk of clinical progression in surviving patients with heart failure.
606 *Circulation*. 131 (1): 54-61.<https://doi.org/10.1161/CIRCULATIONAHA.114.013748>
- 607 46 Johns DW, Peach MJ, Gomez RA, Inagami T, Carey RM (1990) Angiotensin II
608 regulates renin gene expression. *Am J Physiol*. 259 (6 Pt 2): F882-
609 [7.https://doi.org/10.1152/ajprenal.1990.259.6.F882](https://doi.org/10.1152/ajprenal.1990.259.6.F882)
- 610 47 Keeton TK, Campbell WB (1980) The pharmacologic alteration of renin release.
611 *Pharmacol Rev*. 32 (2): 81-227
- 612 48 Kurtz A, Della Bruna R, Pfeilschifter J, Taugner R, Bauer C (1986) Atrial natriuretic
613 peptide inhibits renin release from juxtaglomerular cells by a cGMP-mediated
614 process. *Proc Natl Acad Sci U S A*. 83 (13): 4769-
615 [73.https://doi.org/10.1073/pnas.83.13.4769](https://doi.org/10.1073/pnas.83.13.4769)
- 616 49 Erdos EG, Skidgel RA (1989) Neutral endopeptidase 24.11 (enkephalinase) and
617 related regulators of peptide hormones. *FASEB J*. 3 (2): 145-
618 [51.https://doi.org/10.1096/fasebj.3.2.2521610](https://doi.org/10.1096/fasebj.3.2.2521610)
- 619 50 von Lueder TG, Atar D, Krum H (2014) Current role of neprilysin inhibitors in
620 hypertension and heart failure. *Pharmacol Ther*. 144 (1): 41-
621 [9.https://doi.org/10.1016/j.pharmthera.2014.05.002](https://doi.org/10.1016/j.pharmthera.2014.05.002)

- 622 51 van de Wal RM, Plokker HW, Lok DJ, Boomsma F, van der Horst FA, van
623 Veldhuisen DJ, van Gilst WH, Voors AA (2006) Determinants of increased
624 angiotensin II levels in severe chronic heart failure patients despite ACE inhibition. *Int*
625 *J Cardiol.* 106 (3): 367-72. <https://doi.org/10.1016/j.ijcard.2005.02.016>
- 626 52 MacFadyen RJ, Lee AF, Morton JJ, Pringle SD, Struthers AD (1999) How often are
627 angiotensin II and aldosterone concentrations raised during chronic ACE inhibitor
628 treatment in cardiac failure? *Heart.* 82 (1): 57-61. <https://doi.org/10.1136/hrt.82.1.57>
- 629 53 Rocha R, Chander PN, Zuckerman A, Stier CT, Jr. (1999) Role of aldosterone in
630 renal vascular injury in stroke-prone hypertensive rats. *Hypertension.* 33 (1 Pt 2):
631 232-7. <https://doi.org/10.1161/01.HYP.33.1.232>
- 632 54 Latini R, Masson S, Anand I, Salio M, Hester A, Judd D, Barlera S, Maggioni AP,
633 Tognoni G, Cohn JN, Val-He FTI (2004) The comparative prognostic value of plasma
634 neurohormones at baseline in patients with heart failure enrolled in Val-HeFT. *Eur*
635 *Heart J.* 25 (4): 292-9. <https://doi.org/10.1016/j.ehj.2003.10.030>
- 636 55 Roig E, Perez-Villa F, Morales M, Jimenez W, Orus J, Heras M, Sanz G (2000)
637 Clinical implications of increased plasma angiotensin II despite ACE inhibitor therapy
638 in patients with congestive heart failure. *Eur Heart J.* 21 (1): 53-
639 7. <https://doi.org/10.1053/euhj.1999.1740>
- 640 56 Swedberg K, Eneroth P, Kjeksus J, Wilhelmsen L (1990) Hormones regulating
641 cardiovascular function in patients with severe congestive heart failure and their
642 relation to mortality. CONSENSUS Trial Study Group. *Circulation.* 82 (5): 1730-
643 6. <https://doi.org/10.1161/01.CIR.82.5.1730>
- 644 57 Jordaan P (2011) Changes in RAAS (renin angiotensin aldosterone system)
645 biomarkers in patients with stable chronic heart failure following short-term
646 angiotensin receptor neprilysin inhibitor (ARNI) treatment. *SA Heart.* 8: 236
- 647 58 Franco-Saenz R, Atarashi K, Takagi M, Takagi M, Mulrow PJ (1989) Effect of atrial
648 natriuretic factor on renin and aldosterone. *J Cardiovasc Pharmacol.* 13 Suppl 6:
649 S31-5
- 650 59 Mochel JP, Fink M (2012) Response to letter from Atkins et al. *J Vet Pharmacol Ther.*
651 35 (5): 516-8

652

Table 1. Pharmacokinetic parameters on Day 5 in beagle dogs on a low salt diet.

		Sacubitrilat	Valsartan	Benazeprilat
	Group (Dose)	Mean (\pm S.D)	Mean (\pm S.D)	Mean (\pm S.D)
C_{max} (ng/mL)	SAC (360 mg)	2686 (\pm 2734)	–	–
	SVL (225 mg)	348 (\pm 150)	917 (\pm 486)	–
	SVH (675 mg)	2103 (\pm 2461)	2288 (\pm 1582)	–
	VAL (900 mg)	–	2769 (\pm 2178)	–
	BNZ (5 mg)	–	–	24 (\pm 8)
T_{max} (hour)	SAC (360 mg)	3.0 (\pm 1.0)	–	–
	SVL (225 mg)	1.7 (\pm 1.0)	2.5 (\pm 2.0)	–
	SVH (675 mg)	2.0 (\pm 0.6)	2.8 (\pm 3.1)	–
	VAL (900 mg)	–	4.2 (\pm 3.8)	–
	BNZ (5 mg)	–	–	2.2 (\pm 1.2)
AUC_{0-last} (ng/mL*h)	SAC (360 mg)	9026 (9289)	–	–
	SVL (225 mg)	1062 (295)	4325 (\pm 1537)	–
	SVH (675 mg)	6244 (6311)	14483 (\pm 9363)	–
	VAL (900 mg)	–	17396 (\pm 11347)	–
	BNZ (5 mg)	–	–	132 (\pm 40)

655 **Figure Captions**

656 **Figure 1.** A 3-way partial crossover study design was chosen to examine the effect of
657 sacubitril/valsartan over 10 days. To achieve steady-state activation of RAAS biomarkers,
658 animals were fed a low-salt diet for 5 days prior to the oral administration of the study drugs:
659 sacubitril (SAC) calcium salt at 360 mg (Period A); a low sacubitril/valsartan tri-sodium
660 hemipentahydrate salt dose (SVL) at 225 mg (period A); a high sacubitril/valsartan dose
661 (same formulation) (SVH) at 675 mg (Period B and C); valsartan free acid (VAL) 900 mg
662 (Periods B and C); benazepril hydrochloride (BNZ) 5 mg (Period C); and empty capsules as
663 placebo (PBO; Periods A and B). Low-salt diet was continued throughout the 10 treatment
664 days. Two weeks of washout with the dogs on normal chow were incorporated between
665 each successive treatment Period.

666
667 **Figure 2.** Pharmacodynamics of sacubitril/valsartan (SVH: 675 mg) compared with
668 standard of care benazepril (BNZ: 5mg), sacubitril (SAC: 360 mg) and valsartan (VAL: 900
669 mg) alone on plasma cGMP. (A) Temporal (absolute) change from baseline (pmol/mL):
670 mean \pm S.E.M; (B) Time-weighted average (TWA, pmol/mL) change from baseline (Δ):
671 mean + 95% CI; (C) Between-group differences: mean + 95% CI. *0.01 \leq p < 0.05; **: 0.001
672 \leq p < 0.01; ***: p < 0.001.

673
674 **Figure 3.** Pharmacodynamics of sacubitril/valsartan (SVL: 225 mg; SVH: 675 mg)
675 compared with standard of care benazepril (BNZ: 5mg), sacubitril (SAC: 360 mg) and
676 valsartan (VAL: 900 mg) alone on plasma renin activity. (A) Temporal (absolute) change
677 from baseline (pg/mL/h): mean \pm S.E.M; (B) Time-weighted average (TWA, pg/mL/h)
678 change from baseline (Δ): mean + 95% CI; (C) Between-group differences: mean + 95% CI.
679 *0.01 \leq p < 0.05; **: 0.001 \leq p < 0.01; ***: p < 0.001.

680
681 **Figure 4.** Pharmacodynamics of sacubitril/valsartan (SVL: 225 mg; SVH: 675 mg)
682 compared with standard of care benazepril (BNZ: 5mg), sacubitril (SAC: 360 mg) and
683 valsartan (VAL: 900 mg) alone on plasma angiotensin I. (A) Temporal (absolute) change
684 from baseline (pg/mL): mean \pm S.E.M; (B) Time-weighted average (TWA, pg/mL) change

685 from baseline (Δ): mean + 95% CI; (C) Between-group differences: mean + 95% CI. *0.01 \leq
686 $p < 0.05$; **: 0.001 $\leq p < 0.01$; ***: $p < 0.001$.

687

688 **Figure 5.** Pharmacodynamics of sacubitril/valsartan (SVL: 225 mg; SVH: 675 mg)
689 compared with standard of care benazepril (BNZ: 5mg), sacubitril (SAC: 360 mg) and
690 valsartan (VAL: 900 mg) alone on plasma angiotensin II. (A) Temporal (absolute) change
691 from baseline (pg/mL): mean \pm S.E.M; (B) Time-weighted average (TWA, pg/mL) change
692 from baseline (Δ): mean + 95% CI; (C) Between-group differences: mean + 95% CI. *0.01 \leq
693 $p < 0.05$; **: 0.001 $\leq p < 0.01$; ***: $p < 0.001$.

694

695 **Figure 6.** Pharmacodynamics of sacubitril/valsartan (SVL: 225 mg; SVH: 675 mg)
696 compared with standard of care benazepril (BNZ: 5mg), sacubitril (SAC: 360 mg) and
697 valsartan (VAL: 900 mg) alone on plasma aldosterone. (A) Temporal (absolute) change
698 from baseline (ng/mL): mean \pm S.E.M; (B) Time-weighted average (TWA, ng/mL) change
699 from baseline (Δ): mean + 95% CI; (C) Between-group differences: mean + 95% CI. *0.01 \leq
700 $p < 0.05$; **: 0.001 $\leq p < 0.01$; ***: $p < 0.001$.

Figure 2

Plasma cGMP

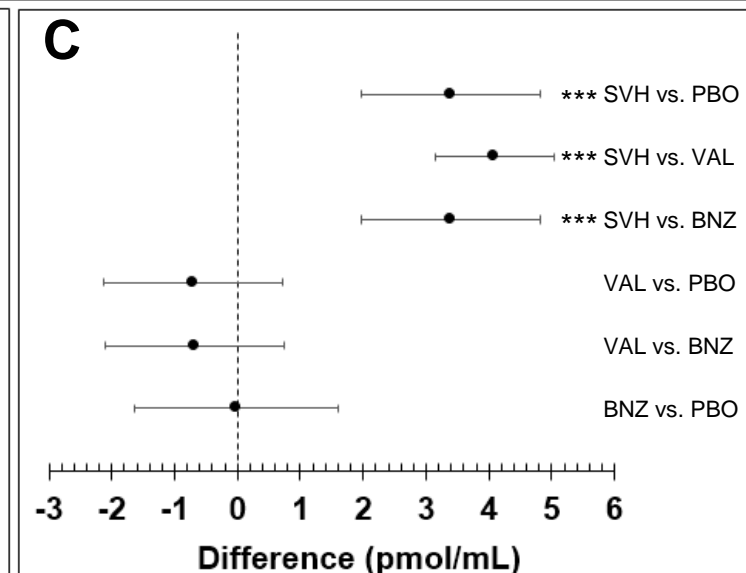
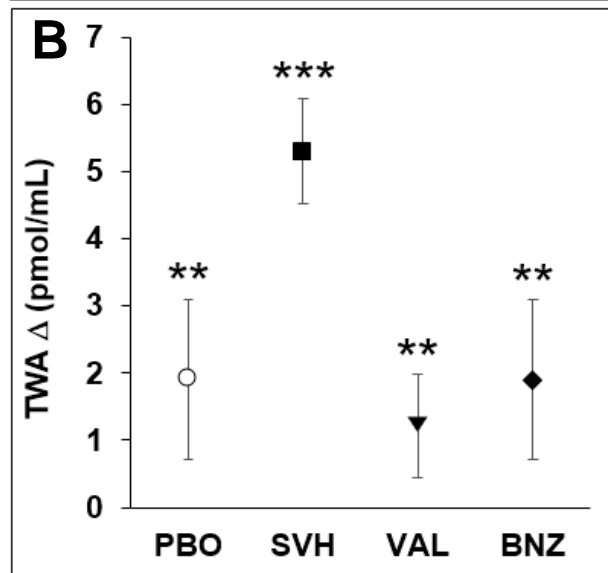
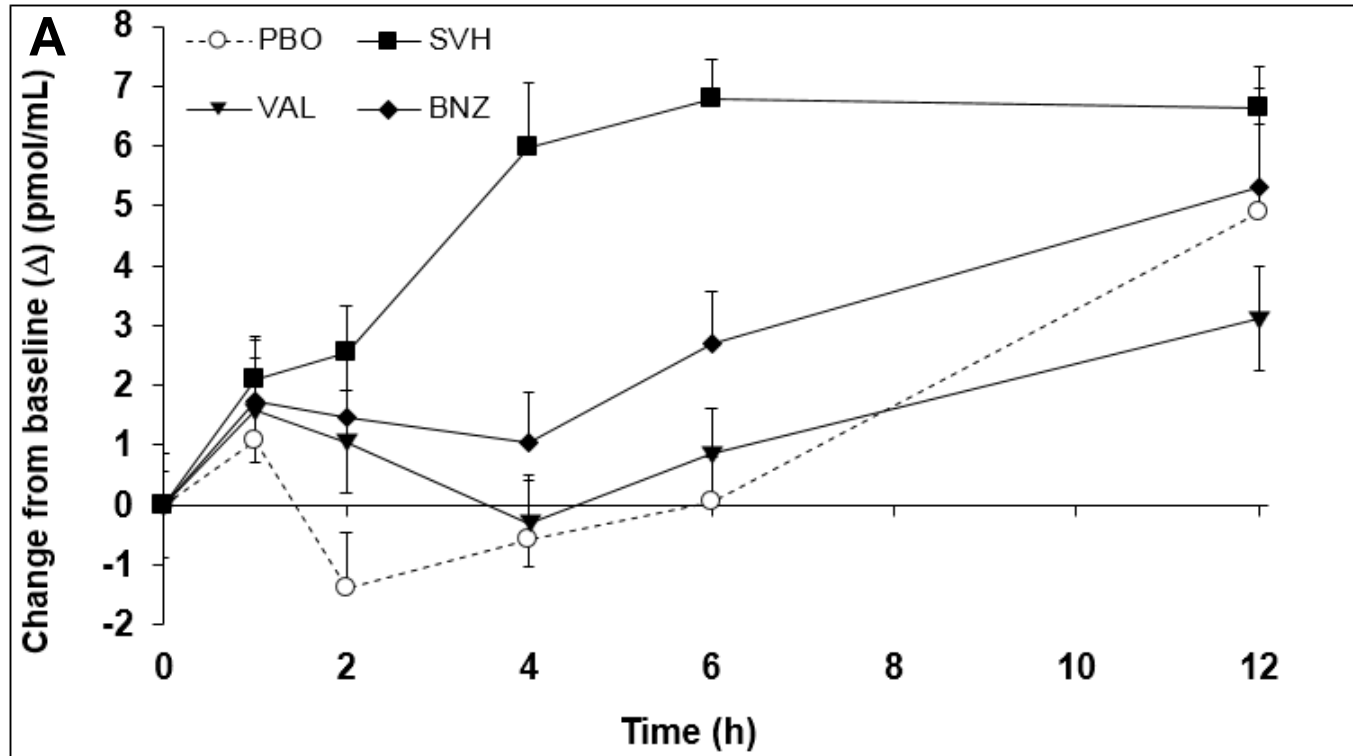


Figure 3

Plasma renin activity

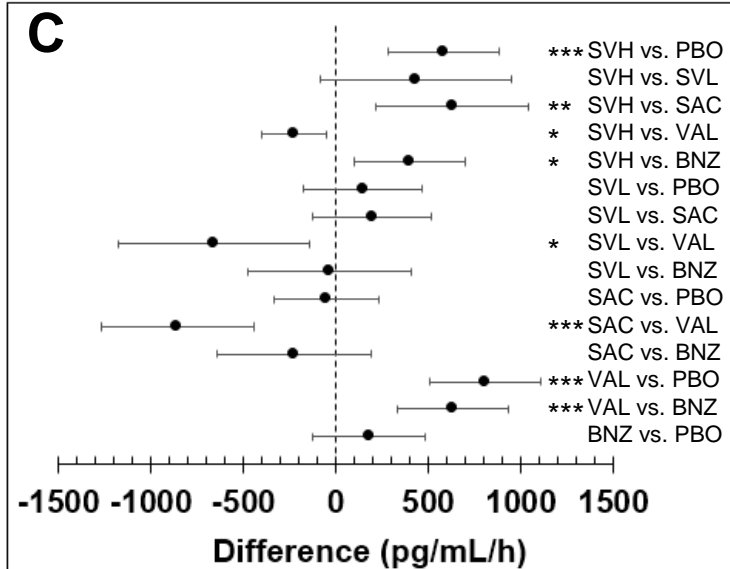
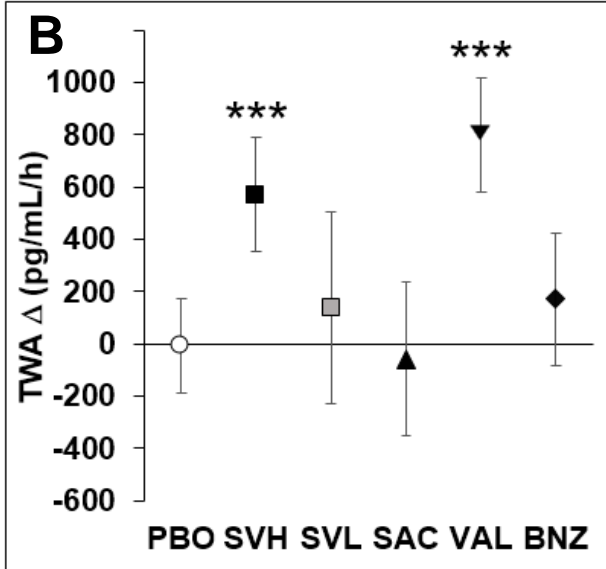
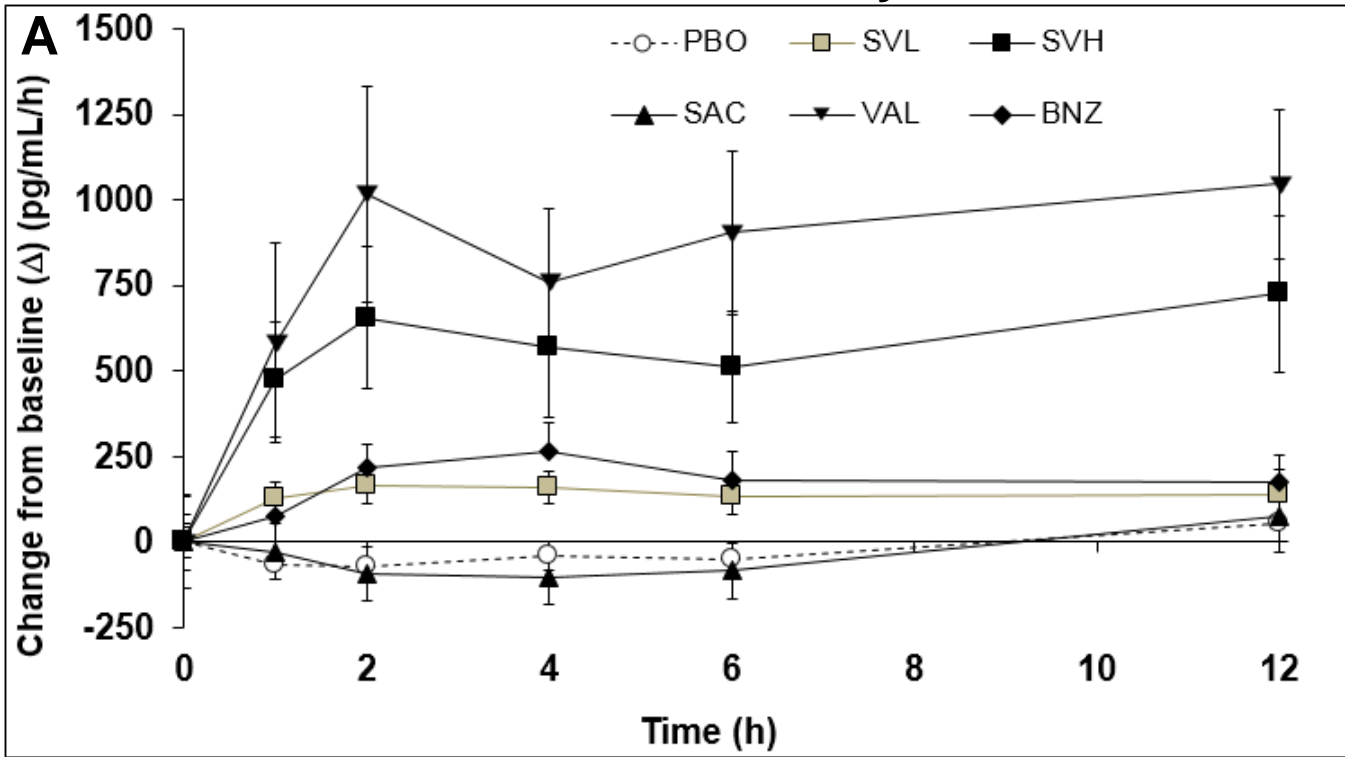


Figure 4

Plasma angiotensin I

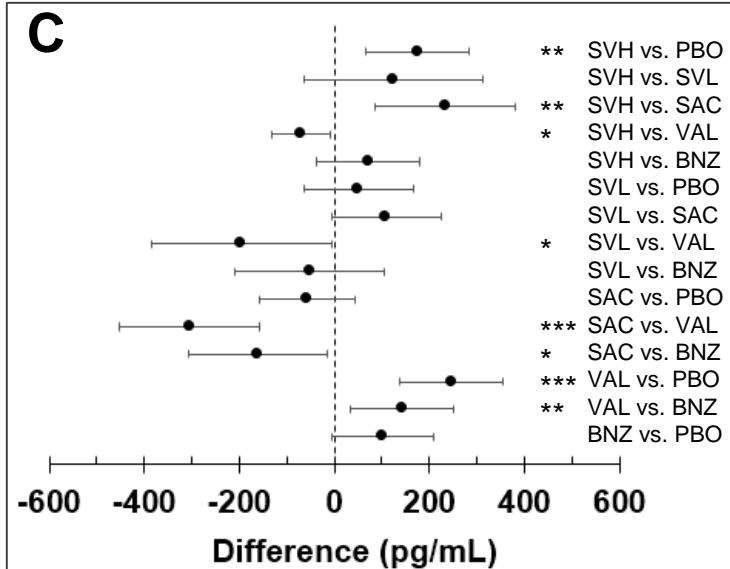
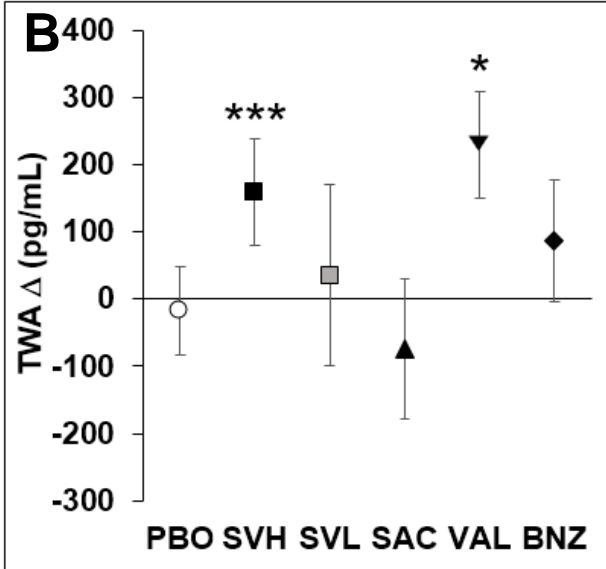
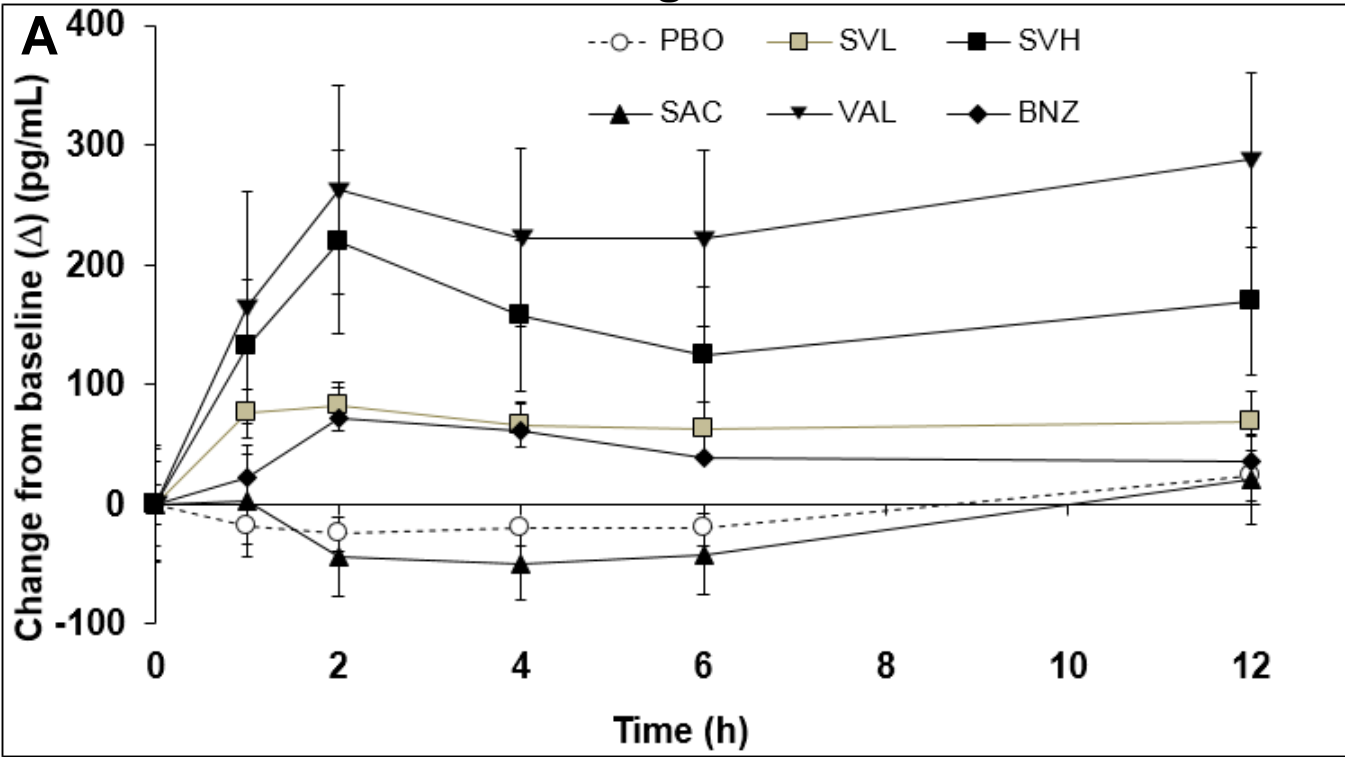


Figure 5

Plasma angiotensin II

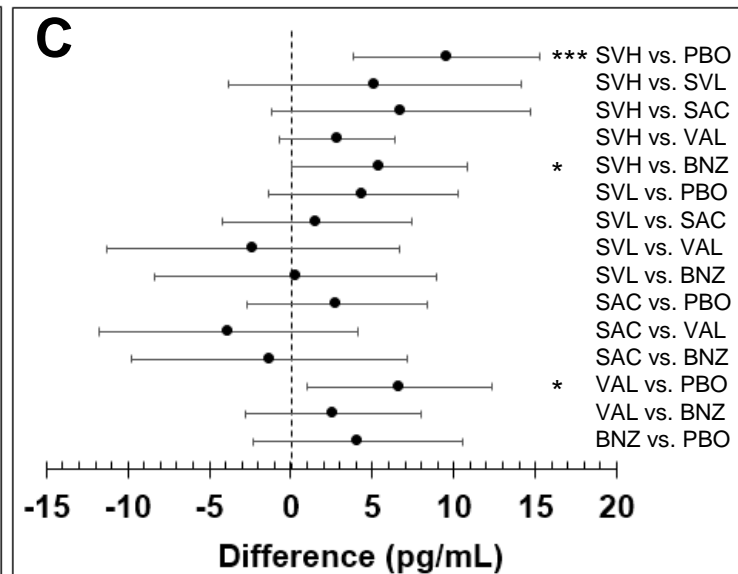
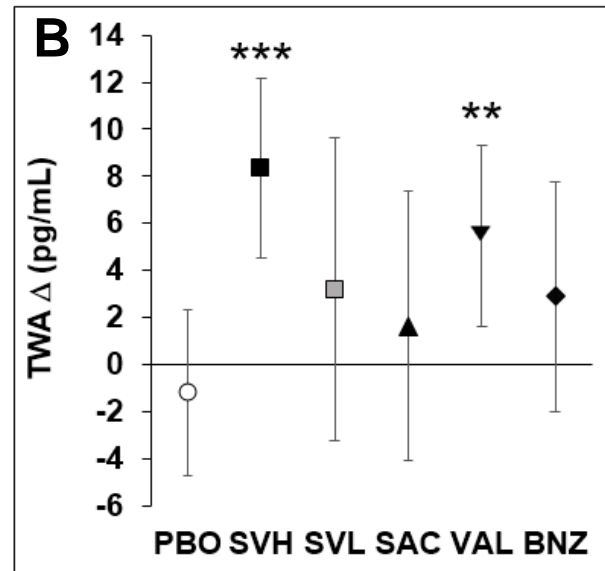
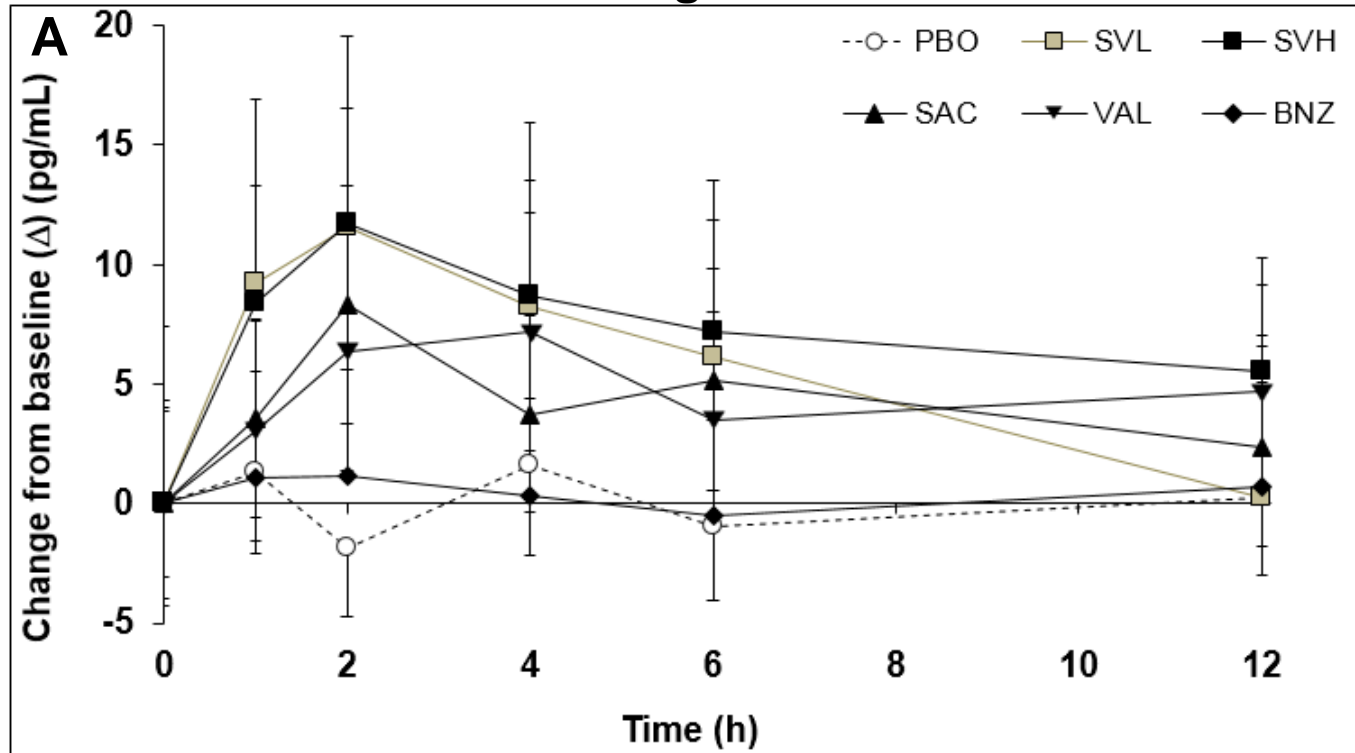


Figure 6

Plasma aldosterone

