



HAL
open science

Additive Protective Effects of Sacubitril/Valsartan and Bosentan on Vascular Remodeling in Experimental Pulmonary Hypertension

Marie-Camille Chaumais, Mohamed Reda Amar Djessas, Raphaël Thuillet, Amélie Cumont, Ly Tu, Guillaume Hébert, Pauline Gaignard, Alice Huertas, Laurent Savale, Marc Humbert, et al.

► To cite this version:

Marie-Camille Chaumais, Mohamed Reda Amar Djessas, Raphaël Thuillet, Amélie Cumont, Ly Tu, et al.. Additive Protective Effects of Sacubitril/Valsartan and Bosentan on Vascular Remodeling in Experimental Pulmonary Hypertension. *Cardiovascular Research*, 2020, pp.cvaa200. 10.1093/cvr/cvaa200 . inserm-02907132

HAL Id: inserm-02907132

<https://inserm.hal.science/inserm-02907132>

Submitted on 27 Jul 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Additive Protective Effects of Sacubitril/Valsartan and Bosentan on Vascular Remodeling in Experimental Pulmonary Hypertension

Short Title: Dual therapy with LCZ 696 plus bosentan in PH

Marie-Camille Chaumais^{1,2,3}, Mohamed Reda Amar Djessas^{1,2}, Raphaël Thuillet^{1,2}, Amélie Marie-Camille Chaumais^{1,2,3}, Mohamed Reda Amar Djessas^{1,4}, Raphaël Thuillet^{1,4}, Amélie Cumont^{1,4}, Ly Tu^{1,4}, Guillaume Hebert⁵, Pauline Gaignard^{6,7}, Alice Huertas^{1,4,7}, Laurent Savale^{1,4,7}, Marc Humbert^{1,4,7}, Christophe Guignabert^{1,4}

¹INSERM UMR_S 999 «Pulmonary Hypertension: Pathophysiology and Novel Therapies», Hôpital Marie Lannelongue, 92350 Le Plessis-Robinson, France; ² Université Paris-Saclay, Faculté de Pharmacie, 92290 Châtenay-Malabry, France; ³ Assistance Publique - Hôpitaux de Paris (AP-HP), Service de Pharmacie, Hôpital Bicêtre, 94270 Le Kremlin-Bicêtre, France ; ⁴ Université Paris-Saclay, Faculté de Médecine, 94270 Le Kremlin-Bicêtre, France; ⁵ Hôpital Marie Lannelongue, Service pharmacie, 92350 Le Plessis-Robinson, France ; ⁶ AP-HP, Laboratoire de biochimie, Hôpital Bicêtre, 94270 Le Kremlin-Bicêtre, France, ⁷ AP-HP, Service de Pneumologie et Soins Intensifs Respiratoires, Hôpital Bicêtre, 94270 Le Kremlin-Bicêtre, France;

Address correspondence to: Christophe Guignabert, PhD; INSERM UMR_S 999; 133, Avenue de la Résistance; 92350 Le Plessis-Robinson, France. Tel: +33-1-40948833; Fax: +33-1-40942522; E-mail: christophe.guignabert@inserm.fr

Manuscript word count: 4612

Scientific knowledge on the subject

- Although pulmonary arterial hypertension (PAH) is characterized by obliterative vascular remodeling in the lungs, severity of symptoms and survival are strongly associated with right ventricular function, and right heart failure is the main cause of death in patients with PAH.
- Sacubitril/valsartan (LCZ 696) has been shown to improve mortality and reduce hospitalizations in patients with heart failure (HF) with reduced ejection fraction (HFrEF).
- LCZ 696 alone reduces pulmonary pressures, vascular remodeling and right ventricular hypertrophy in the Sugen/Hypoxia (SuHx) rat model.

What this study adds to the field?

- LCZ 696 alone had also beneficial effects in the monocrotaline (MCT) rat model of severe pulmonary hypertension (PH).
- Combination therapy of LCZ 696 and bosentan had additive vascular protective effects against the pulmonary vascular remodeling and PH in the MCT and sugen/hypoxia (suHx) rat models of severe PH.
- LCZ 696 has anti-proliferative effect on cultured human pulmonary artery smooth muscle cells (PA-SMCs) derived from patients with idiopathic PAH, an effect that is more pronounced in presence of bosentan
- The beneficial effect of LCZ 696 (30mg/kg/day) and bosentan (100mg/kg/day) in the MCT and SuHx rat models is associated with an increased in the plasma levels of cyclic GMP (cGMP) and atrial natriuretic peptide (ANP)

1 **Abstract**

2 **Aims:** Although right ventricular (RV) function is an important determinant of morbidity and mortality in
3 patients with pulmonary arterial hypertension (PAH), there is no treatment targeting directly the RV. We
4 evaluate the efficacy of sacubitril/valsartan (LCZ 696) as add-on therapy to bosentan in rats with severe
5 pulmonary hypertension (PH).

6 **Methods and Results:** Combination therapy of LCZ 696 and bosentan has additive vascular protective
7 effects against the pulmonary vascular remodeling and PH in two preclinical models of severe PH.
8 Compared with monotherapy, co-treatment of LCZ 696 (30 or 68mg/kg/day for 2 weeks, *per os*) and
9 bosentan (100mg/kg/day for 2 weeks, *per os*) started 7-day after monocrotaline (MCT) injection
10 substantially reduces pulmonary pressures, vascular remodeling and RV hypertrophy and fibrosis in
11 rats. Consistent with these observations, cotreatment of rats with established PH induced by
12 sugen/hypoxia (SuHx) with LCZ 696 (30mg/kg/day for 3 weeks, *per os*) and bosentan (100mg/kg/day
13 for 3 weeks, *per os*) started 5 weeks after Sugén injection partially attenuate total pulmonary vascular
14 resistance and cardiovascular structures. We also obtained evidence showing that LCZ 696 has anti-
15 proliferative effect on cultured human pulmonary artery smooth muscle cells (PA-SMCs) derived from
16 patients with idiopathic PAH, an effect that is more pronounced in presence of bosentan. Finally, we
17 found that the plasma levels of ANP and cGMP are higher in rats co-treated with LCZ 696 (30mg/kg/day)
18 and bosentan (100mg/kg/day) than in MCT and SuHx rats treated with vehicle.

19 **Conclusion:** Dual therapy with LCZ 696 plus bosentan proved significantly superior beneficial effect to
20 LCZ 696 or bosentan alone on vascular remodeling and severity of experimental PH.

21

22 **Keywords:** *Pulmonary arterial hypertension • Pulmonary vascular remodeling • Cardiac dysfunction •*
23 *Entresto*

24

25 **Introduction:**

26 Pulmonary arterial hypertension (PAH) is a rare and devastating disease in which the progressive
27 increase in mean pulmonary artery pressure (mPAP) is due to an intense pulmonary vascular
28 remodelling, leading to right ventricular overload, hypertrophy, and ultimately right heart failure and
29 death ¹. Right heart function is the main determinant of prognosis in PAH; however, there is currently
30 no treatment available that directly target the right ventricle (RV).

31 LCZ 696 (sacubitril/valsartan) is a combination drug with a proven efficacy in chronic HF with reduced
32 ejection fraction in reducing mortality and hospitalization ². In this novel approach for the treatment of
33 heart failure, sacubitril is a pro-drug that upon activation acts as a potent inhibitor of neprilysin, an
34 endopeptidase that breaks down several vasoactive peptides including natriuretic peptides, bradykinin,
35 endothelin and angiotensin (Ang)-II. Since inhibition of neprilysin increases of plasmatic levels of both,
36 natriuretic peptides and Ang-II (with opposite biological effects), sacubitril is currently used in
37 combination with valsartan, an AT1 receptor blocker. Therefore, LCZ 696 by enhancing natriuretic
38 peptides ³ and suppressing the renin-angiotensin-aldosterone system (RAAS) ⁴ is a desirable
39 therapeutic goal to supplement the current PAH treatment options. Consistent with this notion, a
40 beneficial effect of LCZ 696 alone has been recently reported in the Sugen/Hypoxia (SuHx) rat model
41 of severe pulmonary hypertension (PH) ^{5,6}, but its efficacy as add-on therapy to a specific therapy used
42 in clinical practice for PAH management has not been studied yet.

43 The purpose of this study was to determine the potential benefit of combination therapy of LCZ 696 and
44 bosentan, a specific PAH treatment, in two complementary preclinical models [the monocrotaline (MCT)
45 and Sugen/Hypoxia (SuHx) rat models] of severe PH.

46

47 **Methods**

48 The authors declare that all supporting data are available within the article and its Online Data
49 Supplement. All animal procedures were designed to conform to the guidelines from Directive
50 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and
51 the protocols used were approved by the local Ethics Committee of the University Paris-Saclay

52 (CEEA26), Le Plessis-Robinson, France. All experimental studies using human samples comply with
53 the Declaration of Helsinki and were approved by the local ethics committee (Comité de Protection des
54 Personnes Ile-de-France VII). All patients gave informed consent before the study.

55

56 **1. Animals and *in vivo* treatment**

57 Young male Wistar rats (100 g, Janvier Labs, Saint Berthevin, France) were studied 3 weeks after a
58 single subcutaneous injection of MCT (60 mg/kg; Sigma-Aldrich) or vehicle (**Figure 1A**). Male rats were
59 used to minimize hormonal effects (e.g., of estrogen). At day-7, MCT-injected rats were randomly
60 divided into five groups and treated for 2 weeks with daily *per os* treatment with vehicle [drinking water],
61 or *via* oral gavage with LCZ 696 (68mg/kg/day)⁵, or bosentan (100mg/kg/day) alone⁷, or with the
62 combination of bosentan (100mg/kg/day) and LCZ 696 (30mg/kg/day or 68mg/kg/day). Two additional
63 groups of healthy rats were randomly divided into two groups and treated for 2 weeks with daily *per os*
64 treatment with vehicle [drinking water] or with the combination of LC Z696 (68mg/kg/day) and bosentan
65 (100mg/kg/day) [oral gavage].

66 To validate our findings obtained in the MCT rat model, a second rat model of severe PH was used
67 (**Figure 1B**). Briefly, young male Wistar rats received a single subcutaneous injection of SU5416
68 (20mg/kg) and were exposed to normobaric hypoxia for 3 weeks before to return to room air for 5 weeks.
69 At 5-weeks post-SU5416 injection (D0), pulsed-wave doppler during transthoracic echocardiography
70 was used to validate the presence of established PH by assessing pulmonary artery acceleration time
71 (AT) to right ventricular ejection time (ET) ratio, using Vivid E9 (GE Healthcare, Velizy-Villacoublay,
72 France). Then, SuHx rats were randomized to receive vehicle (drinking water), or the combination of
73 LCZ 696 (30mg/kg/day) and bosentan (100mg/kg/day) [oral gavage].

74 At the end of these protocols (D21), animals were anesthetized with isoflurane (2.0% in oxygen) and
75 transthoracic echocardiography was used to blindly determine the AT/ET and RV/(RV+LV) distance
76 ratio and evaluate right ventricular function by assessment of the tricuspid annular plane systolic
77 excursion (TAPSE). In addition, pulmonary pressures were measured blindly by closed chest right heart
78 catheterization, as previously described⁸⁻¹³. Briefly, a polyvinyl catheter was introduced into the right
79 jugular vein and pushed through the RV into the pulmonary artery. In parallel, a carotid artery was
80 cannulated for the measurement of systemic arterial pressure. Cardiac output (CO) in rats was
81 measured using the thermodilution method. Hemodynamic values were automatically calculated by the

82 physiological data acquisition system (LabChart 7 Software; ADInstruments Co., Shanghai, China).
83 After measurement of hemodynamic parameters, the thorax was opened and the left lung immediately
84 removed and frozen. The right lung was fixed in the distended state with formalin buffer. The right
85 ventricular hypertrophy was assessed by the Fulton index [weight ratio of RV and (LV + septum)] and
86 the percentage of wall thickness [(2 × medial wall thickness/ external diameter) × 100] and of
87 muscularized vessels were performed as previously described⁸⁻¹³.

88

89 **2. ELISA and Immunostaining**

90 Concentrations of cyclic guanosine monophosphate (cGMP) and atrial natriuretic peptide (ANP) in
91 plasma were evaluated using a rat cGMP ELISA Kits (KGE003, Bio-techne SAS, Noyal Châtillon sur
92 Seiche, France) and ANP ELISA Kits (ab108797, Abcam, Paris, France) according to the manufacturer
93 instructions. Immunohistochemistry staining for alpha-SMA (Sc32251), proliferating cell nuclear antigen
94 (PCNA, sc-56), and CD68 (sc-20060, Santa Cruz Biotechnology, Heidelberg, Germany) were
95 performed in rat lung paraffin sections^{5, 8, 10, 14, 15}. Briefly, lung sections were deparaffined and stained
96 with hematoxylin and eosin (H&E) (Sigma-Aldrich, Saint-Quentin Fallavier, France), picrosirius red, or
97 incubated with retrieval buffer. Then, sections were saturated with blocking buffer and incubated
98 overnight with specific antibodies, a vectastain ABC kit according to the manufacturer's instructions
99 (Abcys, Courtaboeuf, France) and counterstained with Hematoxylin (Sigma-Aldrich). Images were
100 taken using Eclipse 80i microscope (Nikon Instruments, Champigny-sur-Marne, France).

101

102 **3. Isolation, Culture, and Functional Analysis Human PA-SMCs**

103 Primary human pulmonary artery smooth muscle cells (PA-SMCs) were isolated using an explant-
104 outgrowth method and cultured as previously described^{9, 11, 12, 16}. Briefly, small pieces of freshly isolated
105 pulmonary arteries were cultured in Dulbecco modified Eagle medium supplemented with 15% fetal calf
106 serum (FCS), 2 mM L-glutamine, and antibiotics. The isolated pulmonary PA-SMCs were strongly
107 positive for α -smooth muscle actin, smooth muscle-specific SM22 protein, and calponin, and negative
108 for von Willebrand factor and CD31. Cells were routinely tested for mycoplasma and used at early
109 passages ≤ 7 . As previously described, PA-SMCs were seeded in 96-well plates at a density of 3000
110 cells/well and allowed to adhere. After being subjected to growth arrest for 48 hours in medium lacking
111 FCS, the PA-SMCs were treated with vehicle or bosentan (50 μ M, Selleckchem, Houston, TX, USA)¹⁷,

112 sildenafil (10 μ M, Selleckchem, Houston, TX, USA) ¹⁸ or to LCZ 696 (50 μ M, Selleckchem, Houston, TX,
113 USA) in presence of 5% FCS. Then, PA-SMC proliferation was assessed by measuring 5-bromo-2-
114 deoxyuridine incorporation using a DELFIA kit (Perkin Elmer) as recommended by the manufacturer.
115 BrdU incorporation was determined by measuring Eu-fluorescence in a time-resolved EnVision
116 Multilabel Reader (PerkinElmer, Waltham, MA, USA).

117

118 **4. Statistical analyses**

119 The data are expressed as mean \pm SD. Statistical significance was tested using one-way analysis of
120 variance (ANOVA) with post hoc testing for multiple comparisons by Bonferroni's test. To analyse and
121 compare the disease severity among the different groups, the mean of each groups was compared with
122 the mean of the control vehicle-treated rats. To analyse and compare the efficacy of the different
123 treatments on the physiological parameters and vascular remodeling induced by MCT, the mean of
124 each groups was compared with the mean of the MCT-injected rats. To analyse and compare the
125 efficacy of LCZ 696 alone or with bosentan at the two different doses on the physiological parameters
126 and vascular remodeling induced by MCT, the mean of these 4 groups of treated MCT rats was
127 analysed with post hoc testing for multiple comparisons by Bonferroni's test for multiple comparisons.
128 Significant difference was assumed at a p value of < 0.05.

129

130 **Results**

131 **1. Additive Beneficial Effects of LCZ 696 with Bosentan on the Progression of PH in the** 132 **MCT Rat Model**

133 First, we examined the effect of chronic treatment of LCZ 696 with bosentan against the progression of
134 PH induced in rats by a single subcutaneous injection of MCT. In the vehicle-treated MCT group, two
135 rats died within 21 days after monocrotaline treatment. By contrast, all rats in the other groups survived
136 the experiment. Therefore, the 21-day survival rates in the vehicle, bosentan, LCZ 696 (30mg/kg), LCZ
137 696 (68mg/kg), and LCZ 696 (68mg/kg) with bosentan (100mg/kg) groups were 84.6%, 100%, 100%,
138 100%, and 100%, respectively.

139 At day-21, a decrease in right ventricular function (assessed by TAPSE) and in the ratios of AT/ET were
140 found in vehicle-treated MCT rats compared with control rats, together with an increase in RV/(RV+LV)

141 distance (**Figure 2A**). Invasive hemodynamic using right heart catheterization confirmed the presence
142 of PH in vehicle-treated MCT rats as reflected by the increase in mPAP, total pulmonary vascular
143 resistance (TPVR) and a decrease in CO (**Figure 2B**). Consistent with these results, vehicle-treated
144 MCT rats exhibited also an increase in the percentages of medial wall thickness and of muscularized
145 distal pulmonary arteries (**Figure 3A**) together with higher numbers of CD68 and PCNA positive cells
146 (**Figure 3B**). Furthermore, right ventricular remodeling (Fulton index/body weight ratio) and increased
147 accumulation of collagen (stained with picrosirius red) in the RV were also found in vehicle-treated MCT
148 (**Figure 4**). MCT rats treated with the combination of LCZ 696 and bosentan exhibit higher TAPSE,
149 AT/ET, reduced RV/(RV+LV) ratio and attenuated mPAP, TPVR and decrease in CO compared to
150 vehicle-treated MCT rats (**Figure 2**). Consistent with these results the percentages of medial wall
151 thickness and of muscularized distal pulmonary arteries, numbers of CD68 and PCNA positive cells
152 were substantially reduced (**Figure 3A-B**) as well as the right ventricular hypertrophy (**Figure 4**). In
153 addition, we also noted a reduced accumulation of collagen in the RV of MCT rats treated with the
154 combination of LCZ 696 and bosentan compared to vehicle-treated MCT rats (**Figure 4**). Interestingly,
155 the administration of bosentan or LCZ 696 alone in MCT-injected rats also significantly reduced TPVR
156 compared to vehicle-treated MCT rats (733 ± 161 and 427 ± 113 versus 1139 ± 230 mmHg/mL/min,
157 respectively; $p < 0.001$), but at a lower level than the combination therapy (305 ± 155 and 285 ± 73
158 mmHg/mL/min at 30 and 68mg/kg/day, respectively; $p < 0.001$) (**Figure 2B**). In contrast to the others
159 groups of rats, MCT injected rats treated with bosentan and the highest dose of LCZ 696 tested
160 (68mg/kg/day) exhibited an ~20% reduction in mean systemic arterial blood pressure (mBP) (**Figure**
161 **2B**).

162

163 **2. Confirmation of the Efficacy of the Combination Therapy of LCZ 696 and Bosentan in the** 164 **Progression of PH in the Sugden/Hypoxia Model in Rats**

165 Since animal models mimic only parts of the human condition ¹⁹, we next tested the efficacy of dual
166 therapy with LCZ 969 plus bosentan in a second experimental model of severe PH, namely the SuHx
167 rat model. We evaluated the effects of a 3-week combination therapy of bosentan (100mg/kg/day) and
168 LCZ 696 (30mg/kg/day) against the progression of PH induced by a single subcutaneous injection of
169 Sugden (SU5416; 20 mg/kg) followed by 3 weeks of hypoxia (10% FiO₂) and 5 weeks of normoxia
170 (**Figure 1B**). Before treatment (D0), transthoracic echocardiography validated that SuHx rats exhibit

171 established PH, as reflected by lower values for TAPSE and AT/ET ratio and higher values of right
172 ventricular chamber dimension (RV/(RV+LV) ratio) (**Figure 5A**). Consistent with our observations
173 obtained in the previous model, rats treated with the combination of LCZ 696 and bosentan are
174 protected against the development of PH-induced by Sugden/Hypoxia (**Figure 5 and 6**). No rats died
175 during the studies. At 8-weeks post-SU5416 injection (D21), in SuHx rats treated with vehicle, a
176 decrease in TAPSE and AT/ET ratio were found together with an increased in RV/(RV+LV) by
177 transthoracic echocardiographic assessment compared with control rats (**Figure 5A**). Consistent with
178 these echocardiographic data, mPAP, TPVR (**Figure 5B**), percentages of medial wall thickness and of
179 muscularized distal pulmonary arteries (**Figure 6A**), and numbers of CD68 and PCNA positive cells
180 (**Figure 6B**) were increased in vehicle-treated SuHx rats compared to control animals. In addition,
181 values of Fulton index/body weight ratio and collagen accumulation in the RV were also increased in
182 vehicle-treated SuHx rats (**Figure 6C**). As shown in the rat model of MCT-induced PH, SuHx rats treated
183 with bosentan (100mg/kg/day) and LCZ 696 (30mg/kg/day) are also protected against the accumulation
184 of collagen in the RV compared to vehicle-treated SuHx rats (5.0 ± 2.4 versus 8.6 ± 0.3 , respectively;
185 $p < 0.01$) (**Figure 6C**). Of note, no differences were found in values of mBP (97 ± 7 versus 92 ± 8 ,
186 respectively, NS) between SuHx rats treated with bosentan (100mg/kg/day) and LCZ 696
187 (30mg/kg/day) and vehicle-treated SuHx or control rats (**Figure 5B**).

188

189 **3. Treatments with LCZ 696 (30mg/kg/d) and Bosentan (100mg/kg/d) significantly increased**
190 **cGMP and ANP circulating levels in preclinical models of PH**

191 cGMP is a central and critical second messenger that regulates contractility and accumulation of PA-
192 SMCs by modulating the activity of cGMP-dependent kinases, phosphodiesterases and ion channels
193 ²⁰. Compared with MCT or SuHx rats treated with vehicle, MCT- or SuHx-rats treated with the
194 combination of bosentan and LCZ 696 (30 mg/kg/d) exhibits increase in circulating cGMP levels (**Figure**
195 **7A**). Similarly, we found that MCT and SuHx rats treated with the combination of bosentan and LCZ
196 696 (30 mg/kg/d) exhibit higher levels of ANP in plasma as compared with MCT rats treated with vehicle
197 or bosentan- and vehicle-treated SuHx-rats (**Figure 7B**).

198

199 **4. LCZ 696 attenuates the pro-proliferative phenotype of cultured PA-SMCs derived from**
200 **idiopathic PAH (iPAH) patients**

201 PA-SMC proliferation contributes to the progression of PAH²¹, we thus examined the effect of LCZ 696
202 treatment on the proliferative potential of cultured PA-SMCs derived from idiopathic PAH patients. BrdU
203 incorporation assay showed that LCZ 696 has anti-proliferative effect on cultured human PA-SMCs
204 derived from patients with idiopathic PAH, an effect that is more pronounced in presence of bosentan
205 **(Figure 7C)** or of sildenafil **(Supplemental Fig. 1)**.

206

207

208 **Discussion**

209 To our knowledge this is the first study assessing benefit of LCZ 696 as an add-on therapy to bosentan,
210 a specific PAH treatment, in two experimental models of severe PH. Our data indicate that combination
211 therapy of LCZ 696 and bosentan attenuates right ventricular hypertrophy, pulmonary vascular
212 remodeling and decreases inflammatory cell infiltration in lungs of MCT or SuHx rats. We found that the
213 dual therapy had a more pronounced beneficial effect against the pulmonary vascular remodeling in
214 MCT rats than LCZ 696 or bosentan treatment used alone. These *in vivo* observations were replicated
215 *in vitro*, with a greater anti-proliferative effect for LCZ 696 + bosentan on PA-SMCs derived from
216 idiopathic PAH than LCZ 696 or bosentan used alone. We also report evidence that beneficial effect of
217 LCZ 696 (30mg/kg/day) and bosentan (100mg/kg/day) in the MCT and SuHx rat models is associated
218 with an increased in the plasma ANP and cGMP levels.

219 Previous animal studies have reported that LCZ 696 alone has beneficial effects in the SuHx rat model
220 of severe PH^{5,6}, but its efficacy in the MCT rat model and as an add-on therapy with other PAH-
221 targeted molecules have not been studied before. LCZ 696 treatment was found to be ineffective in a
222 rat model of pressure overload induced RV hypertrophy caused by pulmonary trunk banding (PTB)⁵,
223 suggesting a direct positive effect of LCZ 696 treatment against the remodeling of the pulmonary
224 vasculature. The lack of efficacy of valsartan alone in the SuHx rat model was also reported⁶,
225 supporting the notion that suppressing RAAS in experimental PH is more effective with a
226 pharmacological augmentation of natriuretic peptides. To test the efficacy of LCZ 696 as an add-on
227 therapy to other PAH-targeted molecules, we have chosen to use the dual endothelin-receptor
228 antagonist bosentan²² that is a well-known active substance with established efficacy and tolerability.
229 Of note, bosentan as the other current PAH-targeted molecules do not act directly by prevention of right

230 heart failure, but they mainly cause effective pulmonary vasodilation²²⁻²⁴. To induce experimental PH
231 and further validate the interest of LCZ 696 in PAH, we have chosen to use a different but
232 complementary animal model of severe PH induced by the administration of MCT that is a well-
233 established to cause PH and right heart failure¹⁹. Our data indicate that co-administration of bosentan
234 with LCZ 696 in MCT-injected rats is more efficient on TPVR, pulmonary vascular remodeling and
235 cardiac fibrosis than bosentan or LCZ 696 used alone. As frequently observed in several patients
236 treated with LCZ 696²⁵, the highest dose of LCZ 696 tested in our MCT-injected rats was associated
237 with a significant reduction in mBP. We found that LCZ 696 at a dose of 30mg/kg/day in association
238 with bosentan (100mg/kg/day) did not impact the mBP neither in MCT-injected rats nor in SuHx rats,
239 and thus represents the best efficacy-safety profile. Since no SuHx rats were treated with bosentan or
240 LCZ 696 alone, we cannot exclude that these monotherapies would have shown a more pronounced
241 positive effects in SuHx rats as compared to the dual therapy. Consistent with previous studies^{26, 27},
242 bosentan was only mildly effective as monotherapy at 100mg/kg/day in MCT rats. Indeed, bosentan
243 treatment decreased TPVR by improving cardiac output without changing mPAP. We also noted that
244 co-administration of bosentan with LCZ 696 has better efficacy in MCT-injected rats than in SuHx rats.
245 These differences could be partly explained by the fact that the SuHx rat model tends to progress more
246 slowly than the MCT rat model, together with a more compromised RV function. Because the MCT rat
247 model of PH is characterized by a high mortality in young rats that started at 21 days after injection and
248 also for ethical concerns, treatments were daily administered at D7 for 2 weeks, excluding any effect of
249 survivor bias. Therefore, these differences could also be due to the fact that MCT rats were treated 1-
250 week post-MCT injection just before the increase in pulmonary pressures, whereas SuHx rats were
251 treated when severe PH was already present. However, these differences could also be due to
252 differences in pathophysiology involved in these two animal models or in the age when the treatment
253 period is starting (1 week-post MCT injection *versus* 5 weeks-post SU5416 injection). In contrast to our
254 results obtained in adult rats with the combined use of a neprilysin inhibitor and an angiotensin receptor
255 blocker, mice deficient in neprilysin from birth are more prone to remodel their pulmonary vessels in
256 response to chronic hypoxia compared to their littermate controls²⁸. While some of this difference could
257 be explained by interspecies differences, or by the effects of knockout *versus* pharmacological
258 inhibition, the pleiotropic functions of ANP and other natriuretic peptides in the heart and in the vascular
259 system that are independent of blood pressure regulation could also explain this discrepancy²⁹.

260 Consistent with our findings showing that combination therapy of LCZ 696 and bosentan attenuates
261 right ventricular hypertrophy, pulmonary vascular remodeling and decreases inflammatory cell
262 infiltration in lungs of MCT or SuHx rats, higher levels of ANP and cGMP were found in plasma of rats
263 treated with the dual therapy. Elevation of circulating ANP in chronically hypoxic-rats treated with LCZ
264 696³⁰⁻³³ and in SuHx-rats treated with LCZ 696 was already reported⁶, but no data were available in
265 SuHx rats co-treated with bosentan or in MCT rats. Herein, our findings also indicated that combination
266 therapy of LCZ 696 and bosentan increased circulating cGMP levels. cGMP is a central and critical
267 second messenger generated in response to nitric oxide or natriuretic peptides^{34, 35} and regulates
268 contractility and accumulation of PA-SMCs by modulating the activity of cGMP dependent kinases,
269 phosphodiesterase and ion channels²⁰. Consistent with our findings, Clements and co-workers have
270 also reported that LC Z696 alone increased cGMP levels in SuHx rats⁶, suggesting that treatment
271 efficacy is at least partly related to cGMP and ANP signaling. The demonstration that LCZ 696 has anti-
272 proliferative effect that is more pronounced in presence of bosentan or sildenafil on human PA-SMCs
273 derived from patients with idiopathic PAH strongly support that LCZ 696 could be considered as a
274 potentially promising add-on therapy for PAH. Consistent with this notion, it has been reported that
275 patients taking the neprilysin inhibitor racecadotril exhibit a rapid increase in plasma cGMP levels within
276 the 6 hours following oral administration, a phenomenon associated with a decrease in PVR³⁶.
277 Unfortunately, the long-term impact of racecadotril was not assessed in this recent clinical trial.
278 However, LCZ 696 treatment was already reported to reduced pulmonary pressures in two cases of PH
279 patients due to left heart disease³⁷ and reverses PH in end-stage heart failure patients waiting
280 transplantation³⁸. Therefore, these results taken with our observations should encourage evaluation of
281 LCZ 696 or oether angiotensin receptor-neprilysin inhibitors in human PAH.

282 In summary, our study provides the first evidence that LCZ 696 could be used as an add-on therapy to
283 bosentan, to potentially prevent or even limit the remodeling of pulmonary blood vessels and to enhance
284 cardiac function in PH/PAH probably through elevation of ANP and cGMP. Although further experiments
285 are required to identify the exact mechanism underlying the beneficial effects of neprilysin inhibition in
286 these two preclinical models, we report evidence that dual therapy with LCZ 696 plus bosentan might
287 represent a promising therapeutic strategy for PAH patients in addition to the current available therapy.

288 **Acknowledgments:**

289 The authors thank Mina Ottaviani for her help with the immunohistochemical identification and
290 quantification of PCNA and CD68 positive cells in lung sections. The authors also thank Maria-Rosa
291 Ghigna, Caroline Communiaux and all technicians and pathologists from the Department of Pathology
292 at Marie Lannelongue hospital for their expertise and support. This research was supported by grants
293 from the French National Institute for Health and Medical Research (INSERM), the University Paris-
294 Saclay, the Marie Lannelongue Hospital, the French National Agency for Research (ANR) grant no.
295 ANR-16-CE17-0014 (TAMIRAH) and ANR-18-RHUS-0006 (RHU DESTINATION 2024), the Fondation
296 de la Recherche Médicale (FRM) grant no. DEQ20150331712 (Equipe FRM 2015), and in part by the
297 Département Hospitalo-Universitaire (DHU) Thorax Innovation (TORINO), the Assistance Publique-
298 Hôpitaux de Paris (AP-HP), Service de Pneumologie, Centre de Référence de l'Hypertension
299 Pulmonaire Sévère, the LabEx LERMIT (grant no ANR-10-LABX-0033), the French PAH patient
300 association (HTAP France) and the french Fonds de Dotation "Recherche en Santé Respiratoire" -
301 (FRSR) - Fondation du Souffle (FdS).

302

303 **Conflicts of interest:**

304 M.R.A.D., R.T., A.C., L.T., G.H., P.G., A.H. and C.G. have no conflict of interest to disclose. In the past
305 3 years, M.C.C reports nonfinancial support from Actelion, Bayer, Boehringer Ingelheim, and Novartis
306 outside of the submitted work. M.H. and L.S. report grants, personal fees, and nonfinancial support from
307 Actelion, Bayer, GlaxoSmithKline, and MSD, outside of the submitted work. M.H. is a member of the
308 Scientific Advisory Board of Morphogen-IX.

309

310 **Authors' contributions:**

311 Conception and design: MCC, LT, and CG; Analysis and interpretation: all; Drafting manuscript: MCC,
312 LT, and CG.

313 **References:**

- 314 1. Simonneau G, Galie N, Rubin LJ, Langleben D, Seeger W, Domenighetti G, Gibbs S, Lebrec D,
315 Speich R, Beghetti M, Rich S, Fishman A. Clinical classification of pulmonary hypertension. *J Am*
316 *Coll Cardiol* 2004;**43**:5S-12S.
- 317 2. McMurray JJ, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC,
318 Solomon SD, Swedberg K, Zile MR, Investigators P-H, Committees. Angiotensin-neprilysin
319 inhibition versus enalapril in heart failure. *N Engl J Med* 2014;**371**:993-1004.
- 320 3. Casserly B, Klinger JR. Brain natriuretic peptide in pulmonary arterial hypertension: biomarker and
321 potential therapeutic agent. *Drug Des Devel Ther* 2009;**3**:269-287.
- 322 4. Guignabert C, de Man F, Lombes M. ACE2 as therapy for pulmonary arterial hypertension: the
323 good outweighs the bad. *Eur Respir J* 2018;**51**.
- 324 5. Andersen S, Axelsen JB, Ringgaard S, Nyengaard JR, Hyldebrandt JA, Bogaard HJ, de Man FS,
325 Nielsen-Kudsk JE, Andersen A. Effects of combined angiotensin II receptor antagonism and
326 neprilysin inhibition in experimental pulmonary hypertension and right ventricular failure. *Int J*
327 *Cardiol* 2019;**293**:203-210.
- 328 6. Clements RT, Vang A, Fernandez-Nicolas A, Kue NR, Mancini TJ, Morrison AR, Mallem K,
329 McCullough DJ, Choudhary G. Treatment of Pulmonary Hypertension With Angiotensin II Receptor
330 Blocker and Neprilysin Inhibitor Sacubitril/Valsartan. *Circ Heart Fail* 2019;**12**:e005819.
- 331 7. Abdelsaid M, Kaczmarek J, Coucha M, Ergul A. Dual endothelin receptor antagonism with
332 bosentan reverses established vascular remodeling and dysfunctional angiogenesis in diabetic
333 rats: relevance to glycemic control. *Life Sci* 2014;**118**:268-273.
- 334 8. Bordenave J, Thuillet R, Tu L, Phan C, Cumont A, Marsol C, Huertas A, Savale L, Hibert M, Galzi
335 JL, Bonnet D, Humbert M, Frossard N, Guignabert C. Neutralization of CXCL12 attenuates
336 established pulmonary hypertension in rats. *Cardiovasc Res* 2019.
- 337 9. Tu L, Desroches-Castan A, Mallet C, Guyon L, Cumont A, Phan C, Robert F, Thuillet R, Bordenave
338 J, Sekine A, Huertas A, Ritvos O, Savale L, Feige JJ, Humbert M, Bailly S, Guignabert C. Selective
339 BMP-9 Inhibition Partially Protects Against Experimental Pulmonary Hypertension. *Circ Res*
340 2019;**124**:846-855.
- 341 10. Poble PB, Phan C, Quatremare T, Bordenave J, Thuillet R, Cumont A, Huertas A, Tu L, Dorfmueller
342 P, Humbert M, Ghigna MR, Savale L, Guignabert C. Therapeutic effect of pirfenidone in the
343 sugen/hypoxia rat model of severe pulmonary hypertension. *FASEB J* 2019;**33**:3670-3679.
- 344 11. Tamura Y, Phan C, Tu L, Le Hiress M, Thuillet R, Jutant EM, Fadel E, Savale L, Huertas A,
345 Humbert M, Guignabert C. Ectopic upregulation of membrane-bound IL6R drives vascular
346 remodeling in pulmonary arterial hypertension. *J Clin Invest* 2018;**128**:1956-1970.

- 347 12. Guignabert C, Phan C, Seferian A, Huertas A, Tu L, Thuillet R, Sattler C, Le Hiress M, Tamura Y,
348 Jutant EM, Chaumais MC, Bouchet S, Maneglier B, Molimard M, Rousselot P, Sitbon O,
349 Simonneau G, Montani D, Humbert M. Dasatinib induces lung vascular toxicity and predisposes
350 to pulmonary hypertension. *J Clin Invest* 2016;**126**:3207-3218.
- 351 13. Tu L, Desroches-Castan A, Mallet C, Guyon L, Cumont A, Phan C, Robert F, Thuillet R, Bordenave
352 J, Sekine A, Huertas A, Ritvos O, Savale L, Feige JJ, Humbert M, Bailly S, Guignabert C. Selective
353 BMP-9 Inhibition Partially Protects Against Experimental Pulmonary Hypertension. *Circ Res* 2019.
- 354 14. Tamura Y, Phan C, Tu L, Le Hiress M, Thuillet R, Jutant E-M, Fadel E, Savale L, Huertas A,
355 Humbert M, Guignabert C. Ectopic upregulation of the Membrane-bound IL6R Drives the Vascular
356 Remodeling in Pulmonary Arterial Hypertension. *J Clin Invest* 2018.
- 357 15. Aiello RJ, Bourassa PA, Zhang Q, Dubins J, Goldberg DR, De Lombaert S, Humbert M, Guignabert
358 C, Cavasin MA, McKinsey TA, Paralkar V. Tryptophan hydroxylase 1 Inhibition Impacts Pulmonary
359 Vascular Remodeling in Two Rat Models of Pulmonary Hypertension. *J Pharmacol Exp Ther*
360 2017;**360**:267-279.
- 361 16. Tu L, De Man FS, Girerd B, Huertas A, Chaumais MC, Lecerf F, Francois C, Perros F, Dorfmuller
362 P, Fadel E, Montani D, Eddahibi S, Humbert M, Guignabert C. A critical role for p130Cas in the
363 progression of pulmonary hypertension in humans and rodents. *Am J Respir Crit Care Med*
364 2012;**186**:666-676.
- 365 17. Kunichika N, Landsberg JW, Yu Y, Kunichika H, Thistlethwaite PA, Rubin LJ, Yuan JX. Bosentan
366 inhibits transient receptor potential channel expression in pulmonary vascular myocytes. *Am J*
367 *Respir Crit Care Med* 2004;**170**:1101-1107.
- 368 18. Wang C, Wang J, Zhao L, Wang Y, Liu J, Shi L, Xu M, Wang C. Sildenafil inhibits human pulmonary
369 artery smooth muscle cell proliferation by decreasing capacitative Ca²⁺ entry. *J Pharmacol Sci*
370 2008;**108**:71-78.
- 371 19. Bonniaud P, Fabre A, Frossard N, Guignabert C, Inman M, Kuebler WM, Maes T, Shi W, Stampfli
372 M, Uhlig S, White E, Witzenrath M, Bellaye PS, Crestani B, Eickelberg O, Fehrenbach H, Guenther
373 A, Jenkins G, Joos G, Magnan A, Maitre B, Maus UA, Reinhold P, Vernooy JHJ, Richeldi L, Kolb
374 M. Optimising experimental research in respiratory diseases: an ERS statement. *Eur Respir J*
375 2018;**51**.
- 376 20. Koress C, Swan K, Kadowitz P. Soluble Guanylate Cyclase Stimulators and Activators: Novel
377 Therapies for Pulmonary Vascular Disease or a Different Method of Increasing cGMP? *Curr*
378 *Hypertens Rep* 2016;**18**:42.
- 379 21. Humbert M, Guignabert C, Bonnet S, Dorfmuller P, Klinger JR, Nicolls MR, Olschewski AJ,
380 Pullamsetti SS, Schermuly RT, Stenmark KR, Rabinovitch M. Pathology and pathobiology of
381 pulmonary hypertension: state of the art and research perspectives. *Eur Respir J* 2019;**53**.

- 382 22. Chaumais MC, Guignabert C, Savale L, Jais X, Boucly A, Montani D, Simonneau G, Humbert M,
383 Sitbon O. Clinical pharmacology of endothelin receptor antagonists used in the treatment of
384 pulmonary arterial hypertension. *Am J Cardiovasc Drugs* 2015;**15**:13-26.
- 385 23. Humbert M, Sitbon O, Simonneau G. Treatment of pulmonary arterial hypertension. *N Engl J Med*
386 2004;**351**:1425-1436.
- 387 24. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk
388 Noordegraaf A, Beghetti M, Ghofrani A, Gomez Sanchez MA, Hansmann G, Klepetko W,
389 Lancellotti P, Matucci M, McDonagh T, Pierard LA, Trindade PT, Zompatori M, Hoeper M. 2015
390 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task
391 Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of
392 Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for
393 European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung
394 Transplantation (ISHLT). *Eur Respir J* 2015;**46**:903-975.
- 395 25. Nielsen PM, Grimm D, Wehland M, Simonsen U, Kruger M. The Combination of Valsartan and
396 Sacubitril in the Treatment of Hypertension and Heart Failure - an Update. *Basic Clin Pharmacol*
397 *Toxicol* 2018;**122**:9-18.
- 398 26. Mouchaers KT, Schalij I, de Boer MA, Postmus PE, van Hinsbergh VW, van Nieuw Amerongen
399 GP, Vonk Noordegraaf A, van der Laarse WJ. Fasudil reduces monocrotaline-induced pulmonary
400 arterial hypertension: comparison with bosentan and sildenafil. *Eur Respir J* 2010;**36**:800-807.
- 401 27. Mouchaers KT, Schalij I, Versteilen AM, Hadi AM, van Nieuw Amerongen GP, van Hinsbergh VW,
402 Postmus PE, van der Laarse WJ, Vonk-Noordegraaf A. Endothelin receptor blockade combined
403 with phosphodiesterase-5 inhibition increases right ventricular mitochondrial capacity in pulmonary
404 arterial hypertension. *Am J Physiol Heart Circ Physiol* 2009;**297**:H200-207.
- 405 28. Dempsey EC, Wick MJ, Karoor V, Barr EJ, Tallman DW, Wehling CA, Walchak SJ, Laudi S, Le M,
406 Oka M, Majka S, Cool CD, Fagan KA, Klemm DJ, Hersh LB, Gerard NP, Gerard C, Miller YE.
407 Nprilysin null mice develop exaggerated pulmonary vascular remodeling in response to chronic
408 hypoxia. *Am J Pathol* 2009;**174**:782-796.
- 409 29. Liczek M, Panek I, Damianski P, Jeczen O, Jazwiec J, Kuna P, Panek M. Nprilysin inhibitors as
410 a new approach in the treatment of right heart failure in the course of chronic obstructive pulmonary
411 disease. *Adv Respir Med* 2018.
- 412 30. Klinger JR, Petit RD, Warburton RR, Wrenn DS, Arnal F, Hill NS. Neutral endopeptidase inhibition
413 attenuates development of hypoxic pulmonary hypertension in rats. *J Appl Physiol (1985)*
414 1993;**75**:1615-1623.

- 415 31. Winter RJ, Zhao L, Krausz T, Hughes JM. Neutral endopeptidase 24.11 inhibition reduces
416 pulmonary vascular remodeling in rats exposed to chronic hypoxia. *Am Rev Respir Dis*
417 1991;**144**:1342-1346.
- 418 32. Zhao L, Hughes JM, Winter RJ. Reversal of pulmonary vascular remodelling following hypoxic
419 exposure: no effect of infusion of atrial natriuretic factor and neutral endopeptidase inhibitor.
420 *Cardiovasc Res* 1994;**28**:519-523.
- 421 33. Thompson JS, Sheedy W, Morice AH. Neutral endopeptidase (NEP) inhibition in rats with
422 established pulmonary hypertension secondary to chronic hypoxia. *Br J Pharmacol*
423 1994;**113**:1121-1126.
- 424 34. Krawutschke C, Koesling D, Russwurm M. Cyclic GMP in Vascular Relaxation: Export Is of Similar
425 Importance as Degradation. *Arterioscler Thromb Vasc Biol* 2015;**35**:2011-2019.
- 426 35. Lehnert M, Dobrowinski H, Feil S, Feil R. cGMP Signaling and Vascular Smooth Muscle Cell
427 Plasticity. *J Cardiovasc Dev Dis* 2018;**5**.
- 428 36. Hobbs AJ, Moyes AJ, Baliga RS, Ghedia D, Ochiel R, Sylvestre Y, Dore CJ, Chowdhury K,
429 Maclagan K, Quartly HL, Sofat R, Smit A, Schreiber BE, Coghlan GJ, MacAllister RJ. Neprilysin
430 inhibition for pulmonary arterial hypertension: a randomized, double-blind, placebo-controlled,
431 proof-of-concept trial. *Br J Pharmacol* 2019;**176**:1251-1267.
- 432 37. De Simone V, Guarise P, Zanotto G, Morando G. Reduction in pulmonary artery pressures with
433 use of sacubitril/valsartan. *J Cardiol Cases* 2019;**20**:187-190.
- 434 38. Zern EK, Cheng S, Wolfson AM, Hamilton MA, Zile MR, Solomon SD, Kittleson MM. Angiotensin
435 Receptor-Neprilysin Inhibitor Therapy Reverses Pulmonary Hypertension in End-Stage Heart
436 Failure Patients Awaiting Transplantation. *Circ Heart Fail* 2020;**13**:e006696.
- 437

438 **Figure Legend:**

439 **Figure 1: Study design used to test the efficacy of LCZ 696 alone or in combination with**
440 **bosentan (100mg/kg/day) (A) in the monocrotaline (MCT) (B) and in Sugden Hypoxia (SuHx) rat**
441 **models.**

442
443 **Figure 2: Chronic treatments with LCZ 696 and bosentan partially attenuate PH in MCT-injected**
444 **rats. (A) TAPSE, AT/ET ratio, RV/(RV/LV) distance ratio (n=6-12, biological replicates). (B) mPAP, CO,**
445 **TPVR, mBP (n=5-10, biological replicates). Values are means±SD. Comparisons were made using the**
446 **one-way ANOVA with Bonferroni's post hoc tests. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus**
447 **control rats; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.001 versus MCT rats treated with vehicle;**
448 **δp<0.05, δδp<0.01, δδδp<0.001, δδδδp<0.001 versus MCT rats treated with bosentan alone.**

449
450 **Figure 3: Chronic treatments with LCZ 696 and bosentan attenuate pulmonary vascular**
451 **remodeling in MCT-injected rats. (A) Representative images of H&E and α-smooth muscle actin (α-**
452 **SMA) immunostaining, and quantification of the percentage of wall thickness and of muscularized distal**
453 **pulmonary arteries (n=6-11, biological replicates). (B) Representative images and quantifications of**
454 **CD68 and PCNA positive cells per pulmonary vessels (n=4-6, biological replicates). Values are**
455 **means±SD. Comparisons were made using the one-way ANOVA with Bonferroni's post hoc tests. Scale**
456 **bar = 50 μm in all sections. **p<0.01, ***p<0.001, ****p<0.0001 versus control rats; #p<0.05, ##p<0.01,**
457 **####p<0.001 versus MCT rats treated with vehicle; δδp<0.01, δδδp<0.001 versus MCT rats treated**
458 **with bosentan alone.**

459
460 **Figure 4: Chronic treatments with LCZ 696 and bosentan attenuate remodeling of the right**
461 **ventricular wall in MCT-injected rats. Upper left panel: Values of Fulton index/body weight (n=6-10,**
462 **biological replicates). Upper right and bottom panels: Representative images and quantifications of**
463 **picrosirius staining of RV myocardium tissues (n=6-10, biological replicates). Values are means±SD.**
464 **Comparisons were made using the one-way ANOVA with Bonferroni's post hoc tests. Scale bar = 50**
465 **μm in all sections. *p<0.05, ****p<0.0001 versus control rats; #p<0.05, ###p<0.001, ####p<0.001**
466 **versus vehicle-treated MCT rats; δp<0.05, δδp<0.01 versus MCT rats treated with bosentan alone.**

467

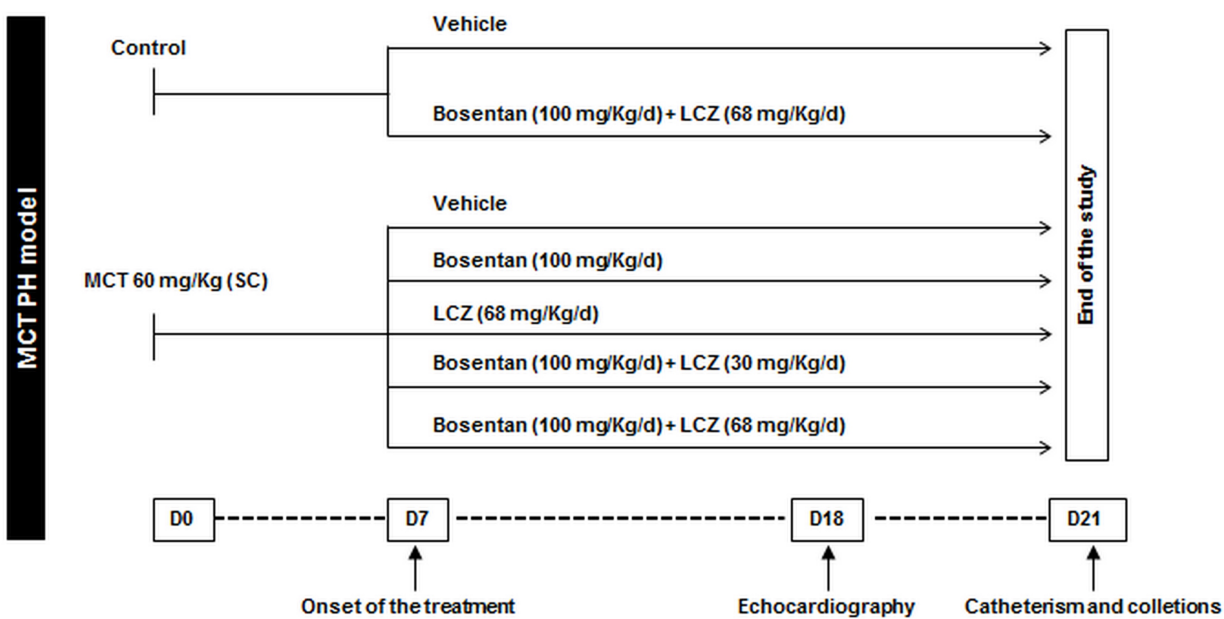
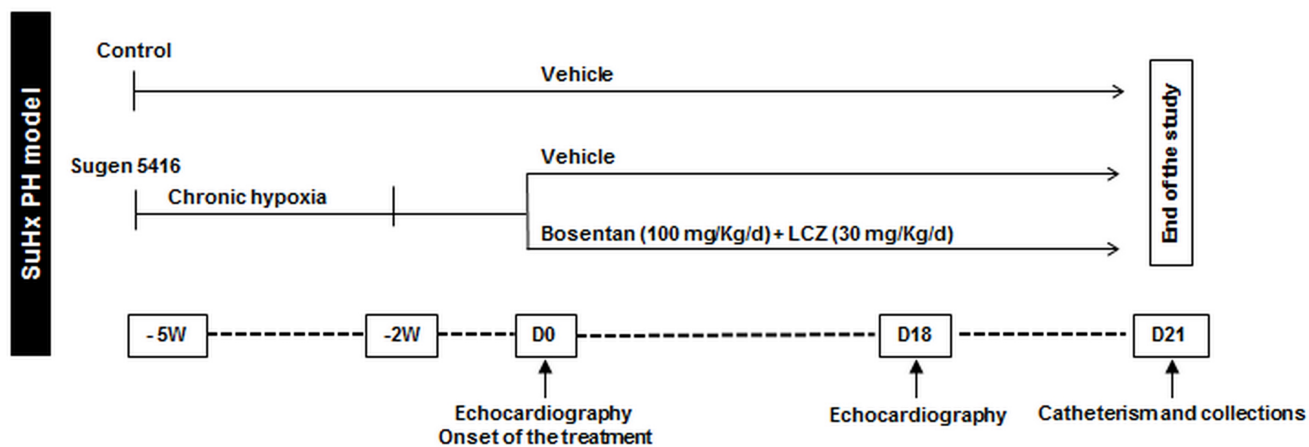
468 **Figure 5: Chronic treatments with LCZ 696 (30mg/kg/d) and Bosentan (100mg/kg) partially**
469 **reverse PH in SuHx rats. (A)** TAPSE, AT/ET ratio, RV/(RV/LV) distance ratio (n=6-12, biological
470 replicates). **(B)** mPAP, CO, TPVR, mBP (n=4-7, biological replicates). Values are means±SD.
471 Comparisons were made using the one-way ANOVA with Bonferroni's post hoc tests. *p<0.05,
472 **p<0.01, ***p<0.001, ****p<0.0001 *versus* control rats; ##p<0.01, ###p<0.001 *versus* SuHx rats treated
473 with vehicle. AU = arbitrary unit.

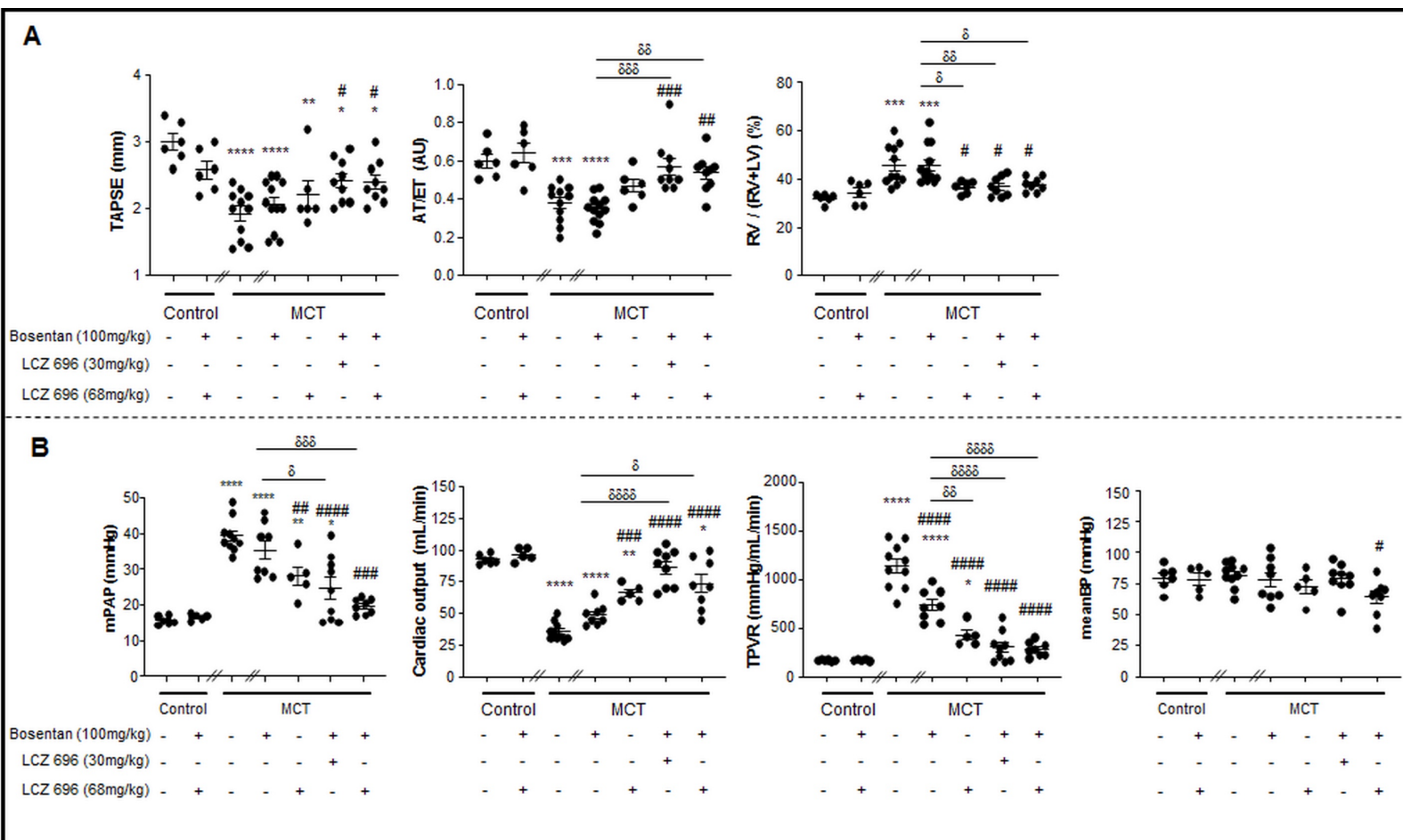
474

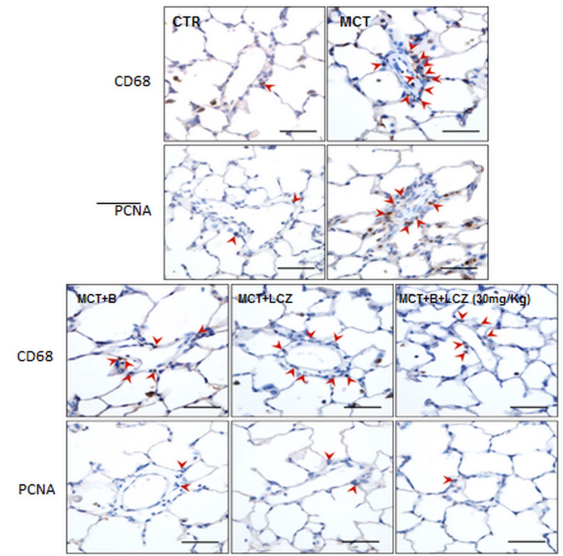
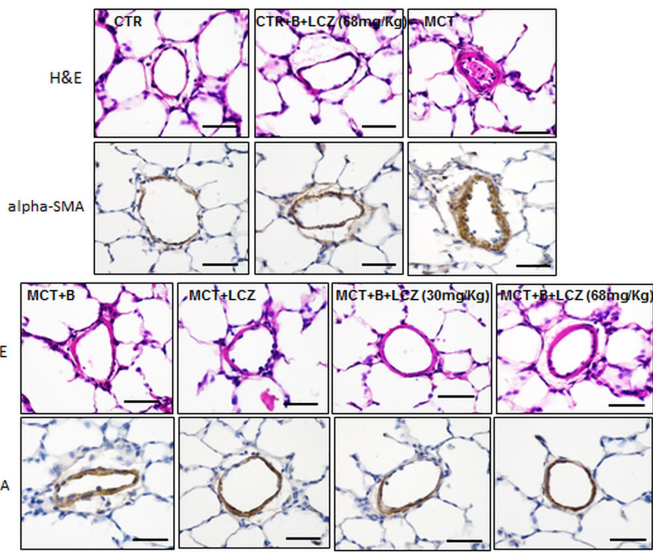
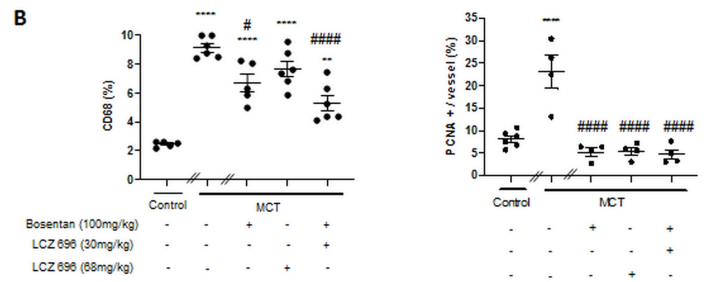
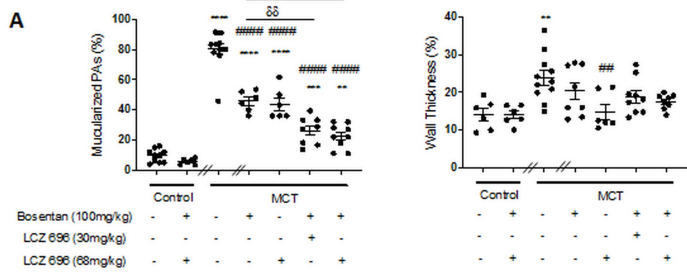
475 **Figure 6: Chronic treatments with LCZ 696 (30mg/kg/d) and Bosentan (100mg/kg) attenuate**
476 **remodeling of the right ventricular wall in the SuHx rats. (A)** Representative images of H&E and α -
477 smooth muscle actin (α -SMA) immunostaining, and quantification of the percentage of wall thickness
478 and of muscularized distal pulmonary arteries in lungs of control and MCT-injected rats treated with
479 LCZ 696 alone or in combination with bosentan at the indicated doses (n=3-7, biological replicates). **(B)**
480 Representative images and quantifications of CD68 and PCNA positive cells per pulmonary vessels
481 (n=4-5, biological replicates). **(C)** Values of Fulton index/body weight. Representative images and
482 quantifications of picrosirius staining of RV myocardium tissues (n=4-7, biological replicates). Values
483 are means±SD. Comparisons were made using the one-way ANOVA with Bonferroni's post hoc tests.
484 Scale bar = 50 μ m in all sections. *p<0.05, ***p<0.001, ****p<0.0001 *versus* control rats; #p<0.05,
485 ###p<0.001, ####p<0.001 *versus* SuHx rats treated with vehicle. ns = non significant.

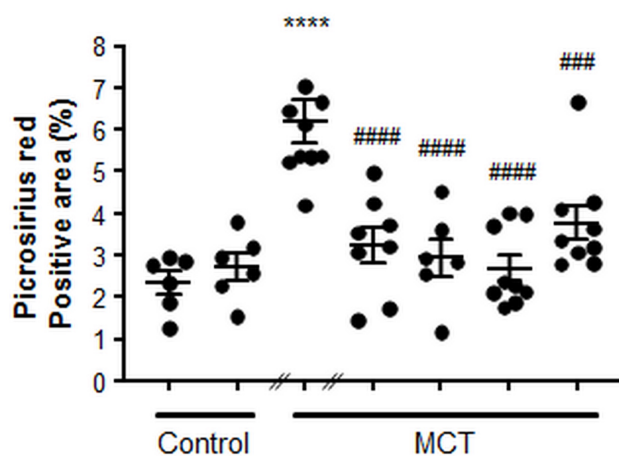
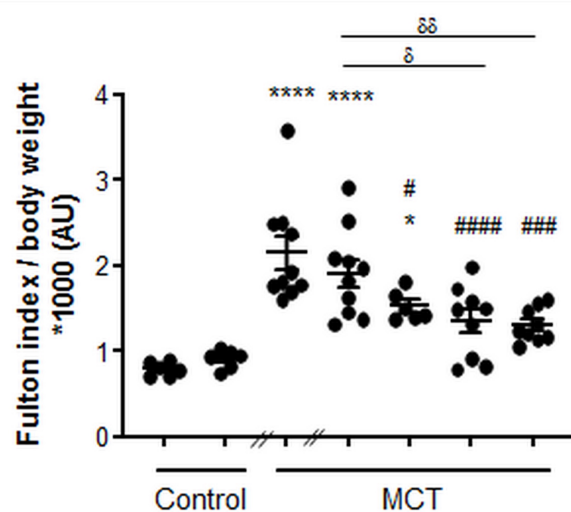
486

487 **Figure 7: Effects of LCZ 696 alone or in presence of bosentan on circulating levels of ANP and**
488 **cGMP in MCT- and SuHx-rats and on the pro-proliferative phenotype of cultured PA-SMCs**
489 **derived from idiopathic PAH patients.** Quantification of plasma levels of cGMP **(A)** and **(B)** ANP
490 (n=7-10, biological replicates). **(C)** 5-bromo-2-deoxyuridine (BrdU) incorporation in PA-SMCs derived
491 from 2 patients with idiopathic PAH under basal condition (0% fetal calf serum or FCS) or in response
492 to LCZ 69 with or without bosentan in presence of 5% FCS (n=8, technical replicates). Values are
493 means±SD. Comparisons were made using the one-way ANOVA with Bonferroni's post hoc tests.
494 *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 *versus* control rats or Vehicle. ##p<0.01, ###p<0.01,
495 ####p<0.0001 *versus* MCT or SuHx rats treated with vehicle or Vehicle in presence of 5% FCS.

A**B**







	Control		MCT				
Bosentan (100mg/kg)	-	+	-	+	-	+	+
LCZ 696 (30mg/kg)	-	-	-	-	-	+	-
LCZ 696 (68mg/kg)	-	+	-	-	+	-	+

	Control		MCT				
Bosentan (100mg/kg)	-	+	-	+	-	+	+
LCZ 696 (30mg/kg)	-	-	-	-	-	+	-
LCZ 696 (68mg/kg)	-	+	-	-	+	-	+

