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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

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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)

Abstract

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

Methods and Results: Combination therapy of LCZ 696 and bosentan has additive vascular protective effects against the pulmonary vascular remodeling and PH in two preclinical models of severe PH. Compared with monotherapy, co-treatment of LCZ 696 (30 or 68mg/kg/day for 2 weeks, *per os*) and bosentan (100mg/kg/day for 2 weeks, *per os*) started 7-day after monocrotaline (MCT) injection substantially reduces pulmonary pressures, vascular remodeling and RV hypertrophy and fibrosis in rats. Consistent with these observations, cotreatment of rats with established PH induced by sugen/hypoxia (SuHx) with LCZ 696 (30mg/kg/day for 3 weeks, *per os*) and bosentan (100mg/kg/day for 3 weeks, *per os*) started 5 weeks after Sugen injection partially attenuate total pulmonary vascular resistance and cardiovascular structures. We also obtained evidence showing that 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. Finally, we found that the plasma levels of ANP and cGMP are higher in rats co-treated with LCZ 696 (30mg/kg/day) and bosentan (100mg/kg/day) than in MCT and SuHx rats treated with vehicle.

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

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

Introduction:

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

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

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

Methods

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

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

1. Animals and *in vivo* treatment

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

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

At the end of these protocols (D21), animals were anesthetized with isoflurane (2.0% in oxygen) and transthoracic echocardiography was used to blindly determine the AT/ET and RV/(RV+LV) distance ratio and evaluate right ventricular function by assessment of the tricuspid annular plane systolic excursion (TAPSE). In addition, pulmonary pressures were measured blindly by closed chest right heart catheterization, as previously described [8-13]. Briefly, a polyvinyl catheter was introduced into the right jugular vein and pushed through the RV into the pulmonary artery. In parallel, a carotid artery was cannulated for the measurement of systemic arterial pressure. Cardiac output (CO) in rats was measured using the thermodilution method. Hemodynamic values were automatically calculated by the

physiological data acquisition system (LabChart 7 Software; ADInstruments Co., Shanghai, China). After measurement of hemodynamic parameters, the thorax was opened and the left lung immediately removed and frozen. The right lung was fixed in the distended state with formalin buffer. The right ventricular hypertrophy was assessed by the Fulton index [weight ratio of RV and (LV + septum)] and the percentage of wall thickness [(2 × medial wall thickness/ external diameter) × 100] and of muscularized vessels were performed as previously described [8-13].

2. ELISA and Immunostaining

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

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

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

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

4. Statistical analyses

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

Results

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

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

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

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

2. Confirmation of the Efficacy of the Combination Therapy of LCZ 696 and Bosentan in the Progression of PH in the Sugen/Hypoxia Model in Rats

Since animal models mimic only parts of the human condition [19], we next tested the efficacy of dual therapy with LCZ 969 plus bosentan in a second experimental model of severe PH, namely the SuHx rat model. We evaluated the effects of a 3-week combination therapy of bosentan (100mg/kg/day) and LCZ 696 (30mg/kg/day) against the progression of PH induced by a single subcutaneous injection of Sugen (SU5416; 20 mg/kg) followed by 3 weeks of hypoxia (10% FiO_2) and 5 weeks of normoxia (**Figure 1B**). Before treatment (D0), transthoracic echocardiography validated that SuHx rats exhibit

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

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

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

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

PA-SMC proliferation contributes to the progression of PAH [21], we thus examined the effect of LCZ 696 treatment on the proliferative potential of cultured PA-SMCs derived from idiopathic PAH patients. BrdU incorporation assay showed that LCZ 696 has anti-proliferative effect on cultured human PA-SMCs derived from patients with idiopathic PAH, an effect that is more pronounced in presence of bosentan (**Figure 7C**) or of sildenafil (**Supplemental Fig. 1**).

Discussion

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

Previous animal studies have reported that LCZ 696 alone has beneficial effects in the SuHx rat model of severe PH [5, 6], but its efficacy in the MCT rat model and as an add-on therapy with other PAH-targeted molecules have not been studied before. LCZ 696 treatment was found to be ineffective in a rat model of pressure overload induced RV hypertrophy caused by pulmonary trunk banding (PTB) [5], suggesting a direct positive effect of LCZ 696 treatment against the remodeling of the pulmonary vasculature. The lack of efficacy of valsartan alone in the SuHx rat model was also reported [6], supporting the notion that suppressing RAAS in experimental PH is more effective with a pharmacological augmentation of natriuretic peptides. To test the efficacy of LCZ 696 as an add-on therapy to other PAH-targeted molecules, we have chosen to use the dual endothelin-receptor antagonist bosentan [22] that is a well-known active substance with established efficacy and tolerability. Of note, bosentan as the other current PAH-targeted molecules do not act directly by prevention of right

heart failure, but they mainly cause effective pulmonary vasodilation [22-24]. To induce experimental PH and further validate the interest of LCZ 696 in PAH, we have chosen to use a different but complementary animal model of severe PH induced by the administration of MCT that is a well-established to cause PH and right heart failure [19]. Our data indicate that co-administration of bosentan with LCZ 696 in MCT-injected rats is more efficient on TPVR, pulmonary vascular remodeling and cardiac fibrosis than bosentan or LCZ 696 used alone. As frequently observed in several patients treated with LCZ 696 [25], the highest dose of LCZ 696 tested in our MCT-injected rats was associated with a significant reduction in mBP. We found that LCZ 696 at a dose of 30mg/kg/day in association with bosentan (100mg/kg/day) did not impact the mBP neither in MCT-injected rats nor in SuHx rats, and thus represents the best efficacy-safety profile. Since no SuHx rats were treated with bosentan or LCZ 696 alone, we cannot exclude that these monotherapies would have shown a more pronounced positive effects in SuHx rats as compared to the dual therapy. Consistent with previous studies [26, 27], bosentan was only mildly effective as monotherapy at 100mg/kg/day in MCT rats. Indeed, bosentan treatment decreased TPVR by improving cardiac output without changing mPAP. We also noted that co-administration of bosentan with LCZ 696 has better efficacy in MCT-injected rats than in SuHx rats. These differences could be partly explained by the fact that the SuHx rat model tends to progress more slowly than the MCT rat model, together with a more compromised RV function. Because the MCT rat model of PH is characterized by a high mortality in young rats that started at 21 days after injection and also for ethical concerns, treatments were daily administered at D7 for 2 weeks, excluding any effect of survivor bias. Therefore, these differences could also be due to the fact that MCT rats were treated 1-week post-MCT injection just before the increase in pulmonary pressures, whereas SuHx rats were treated when severe PH was already present. However, these differences could also be due to differences in pathophysiology involved in these two animal models or in the age when the treatment period is starting (1 week-post MCT injection *versus* 5 weeks-post SU5416 injection). In contrast to our results obtained in adult rats with the combined use of a neprilysin inhibitor and an angiotensin receptor blocker, mice deficient in neprilysin from birth are more prone to remodel their pulmonary vessels in response to chronic hypoxia compared to their littermate controls [28]. While some of this difference could be explained by interspecies differences, or by the effects of knockout *versus* pharmacological inhibition, the pleiotropic functions of ANP and other natriuretic peptides in the heart and in the vascular system that are independent of blood pressure regulation could also explain this discrepancy [29].

Consistent with our findings showing that combination therapy of LCZ 696 and bosentan attenuates right ventricular hypertrophy, pulmonary vascular remodeling and decreases inflammatory cell infiltration in lungs of MCT or SuHx rats, higher levels of ANP and cGMP were found in plasma of rats treated with the dual therapy. Elevation of circulating ANP in chronically hypoxic-rats treated with LCZ 696 [30-33] and in SuHx-rats treated with LCZ 696 was already reported [6], but no data were available in SuHx rats co-treated with bosentan or in MCT rats. Herein, our findings also indicated that combination therapy of LCZ 696 and bosentan increased circulating cGMP levels. cGMP is a central and critical second messenger generated in response to nitric oxide or natriuretic peptides [34, 35] and regulates contractility and accumulation of PA-SMCs by modulating the activity of cGMP dependent kinases, phosphodiesterase and ion channels [20]. Consistent with our findings, Clements and co-workers have also reported that LC Z696 alone increased cGMP levels in SuHx rats [6], suggesting that treatment efficacy is at least partly related to cGMP and ANP signaling. The demonstration that LCZ 696 has anti-proliferative effect that is more pronounced in presence of bosentan or sildenafil on human PA-SMCs derived from patients with idiopathic PAH strongly support that LCZ 696 could be considered as a potentially promising add-on therapy for PAH. Consistent with this notion, it has been reported that patients taking the neprilysin inhibitor racecadotril exhibit a rapid increase in plasma cGMP levels within the 6 hours following oral administration, a phenomenon associated with a decrease in PVR [36]. Unfortunately, the long-term impact of racecadotril was not assessed in this recent clinical trial. However, LCZ 696 treatment was already reported to reduced pulmonary pressures in two cases of PH patients due to left heart disease [37] and reverses PH in end-stage heart failure patients waiting transplantation [38]. Therefore, these results taken with our observations should encourage evaluation of LCZ 696 or oether angiotensin receptor-neprilysin inhibitors in human PAH.

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

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Conflicts of interest:

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 3 years, M.C.C reports nonfinancial support from Actelion, Bayer, Boehringer Ingelheim, and Novartis outside of the submitted work. M.H. and L.S. report grants, personal fees, and nonfinancial support from Actelion, Bayer, GlaxoSmithKline, and MSD, outside of the submitted work. M.H. is a member of the Scientific Advisory Board of Morphogen-IX.

Authors' contributions:

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

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Figure Legend:

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

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

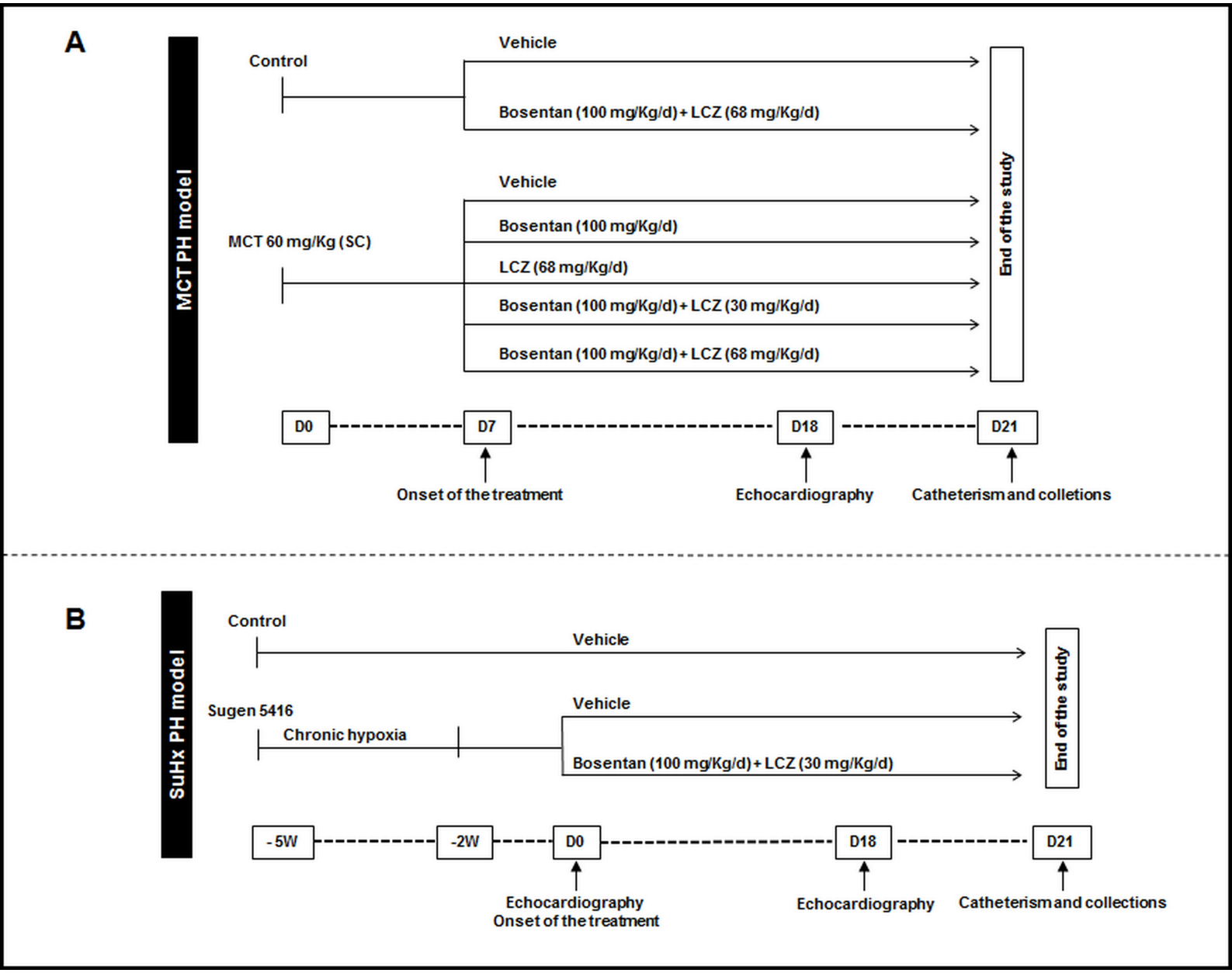
Figure 3: Chronic treatments with LCZ 696 and bosentan attenuate pulmonary vascular remodeling in MCT-injected rats. (A) Representative images of H&E and α-smooth muscle actin (α-SMA) immunostaining, and quantification of the percentage of wall thickness and of muscularized distal pulmonary arteries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

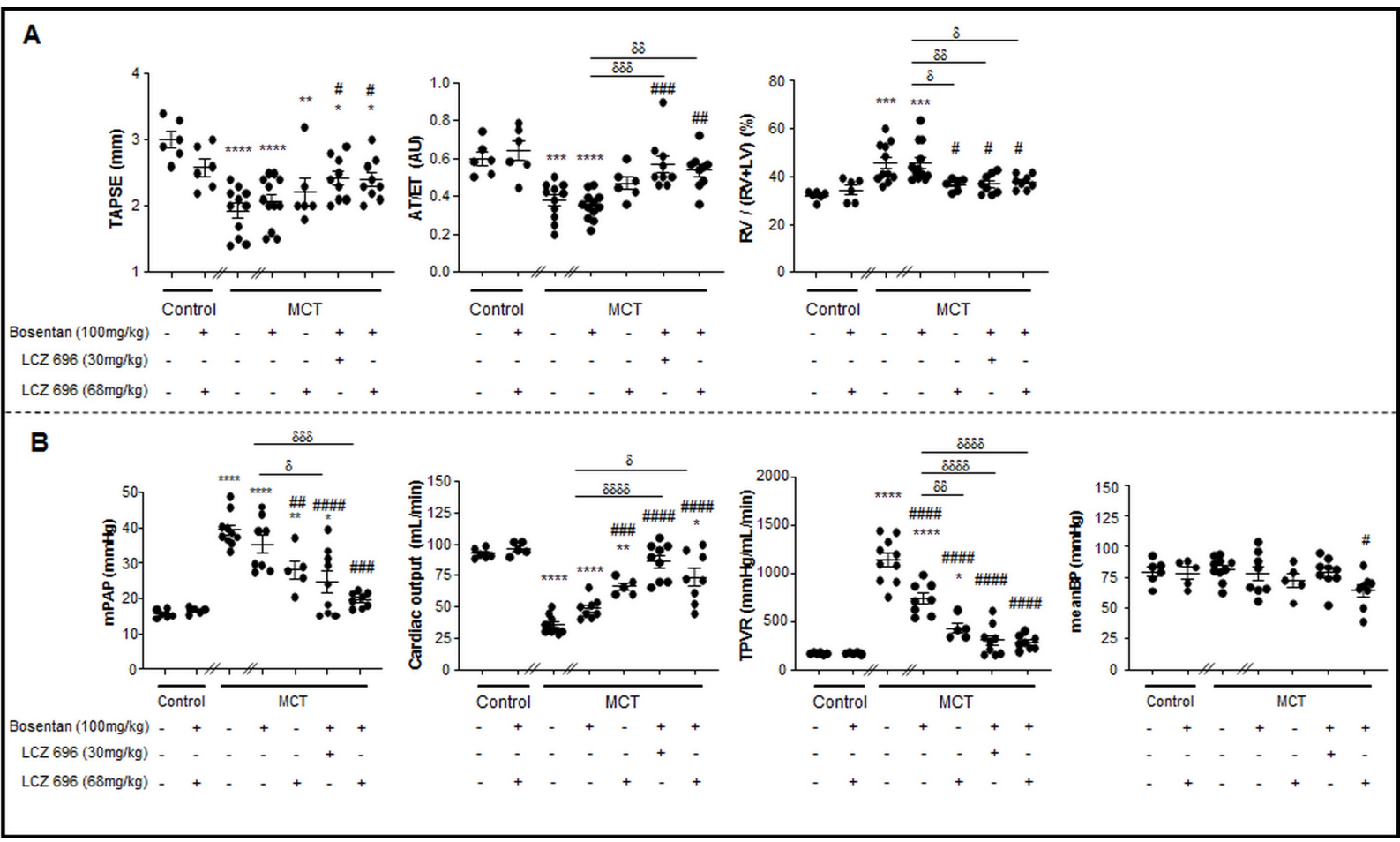
Figure 4: Chronic treatments with LCZ 696 and bosentan attenuate remodeling of the right ventricular wall in MCT-injected rats. Upper left panel: Values of Fulton index/body weight (n=6-10, biological replicates). Upper right and bottom panels: Representative images and quantifications of picrosirius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

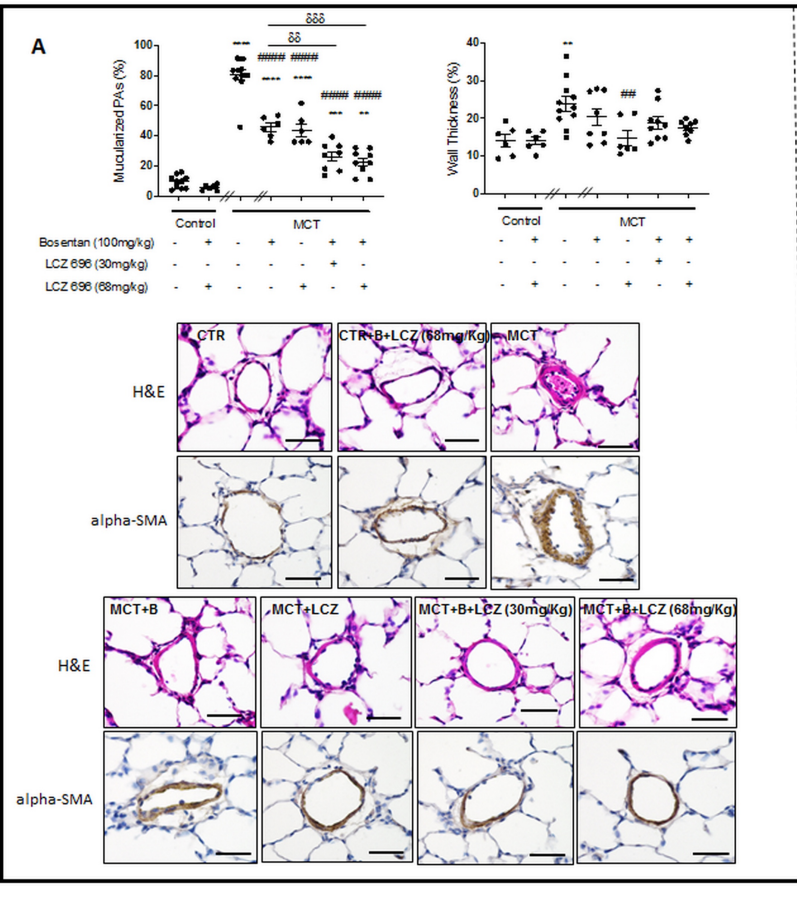
Figure 5: Chronic treatments with LCZ 696 (30mg/kg/d) and Bosentan (100mg/kg) partially reverse PH in SuHx rats. (A) TAPSE, AT/ET ratio, RV/(RV/LV) distance ratio (n=6-12, biological replicates). **(B)** mPAP, CO, TPVR, mBP (n=4-7, biological replicates). Values are means±SD. Comparisons were made using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

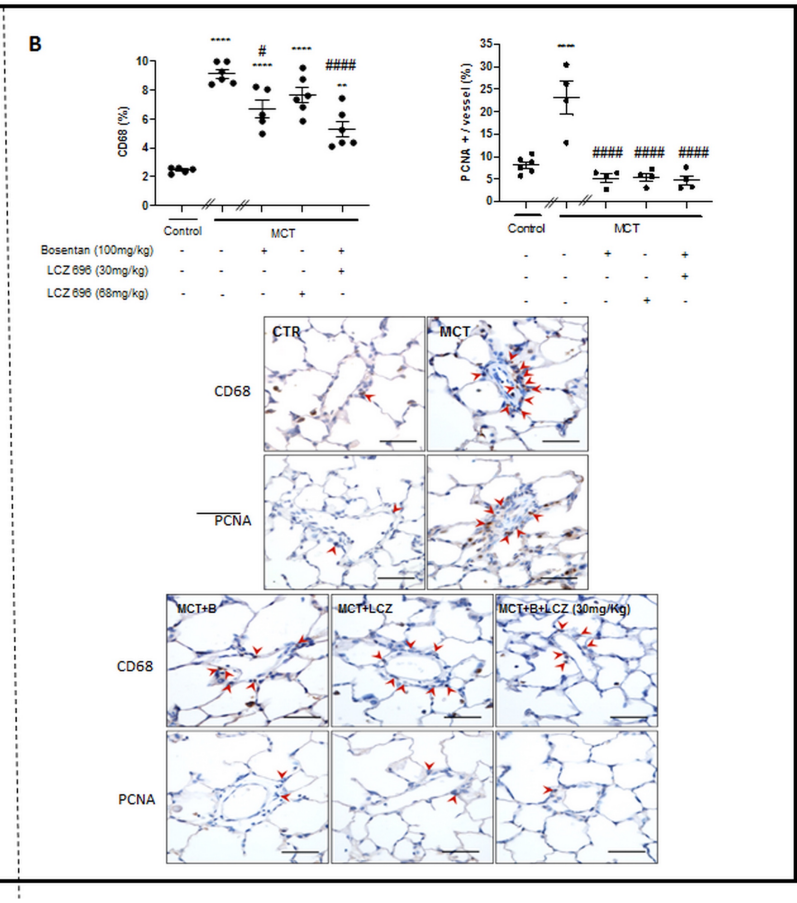
Figure 6: Chronic treatments with LCZ 696 (30mg/kg/d) and Bosentan (100mg/kg) attenuate remodeling of the right ventricular wall in the SuHx rats. (A) Representative images of H&E and α-smooth muscle actin (α-SMA) immunostaining, and quantification of the percentage of wall thickness and of muscularized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

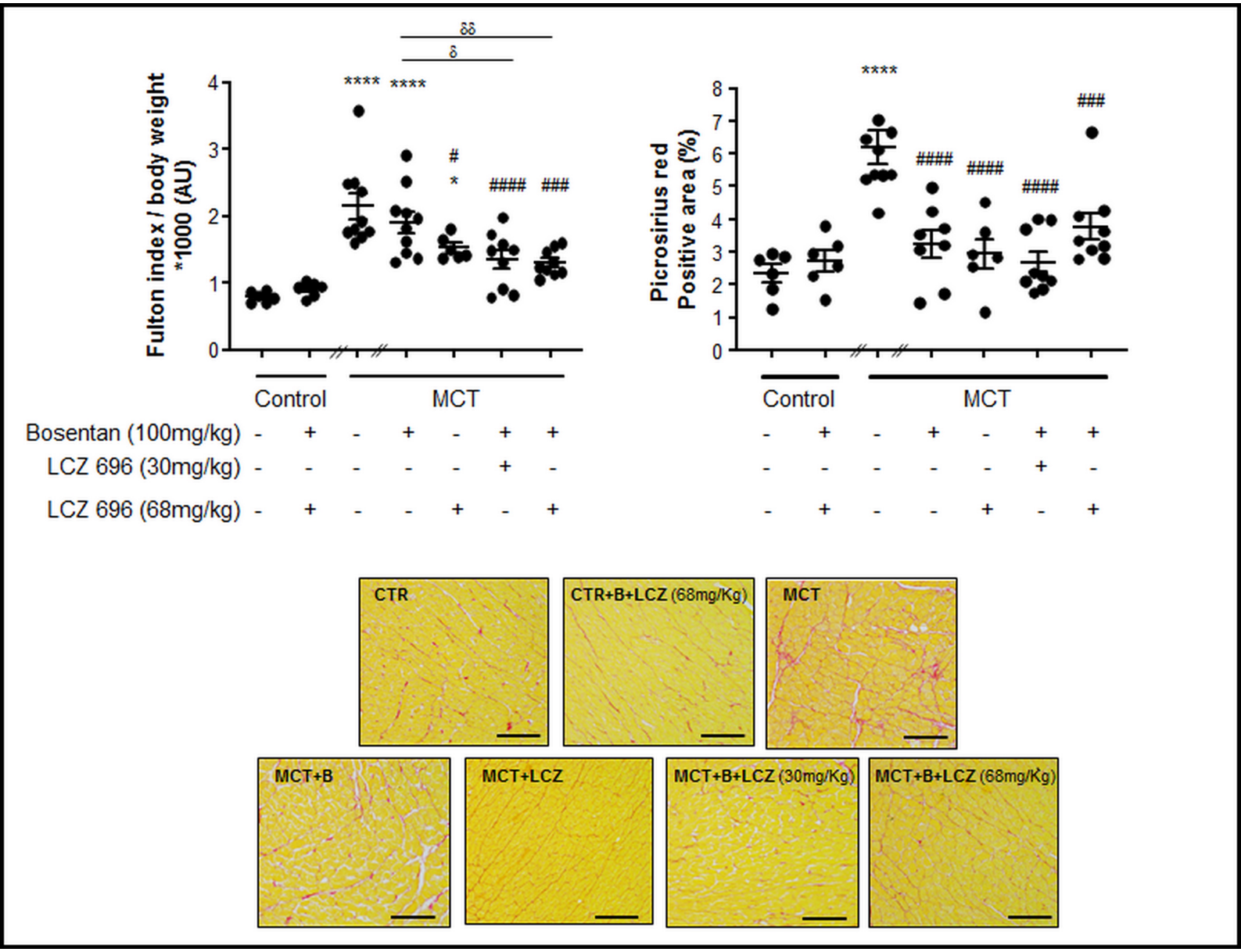
Figure 7: Effects of LCZ 696 alone or in presence of bosentan on circulating levels of ANP and cGMP in MCT- and SuHx-rats and on the pro-proliferative phenotype of cultured PA-SMCs derived from idiopathic PAH patients. Quantification of plasma levels of cGMP **(A)** and **(B)** ANP (n=7-10, biological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


A
MCT PH model
Control
Vehicle
Bosentan (100 mg/Kg/d) + LCZ (68 mg/Kg/d)
MCT 60 mg/Kg (SC)
Vehicle
Bosentan (100 mg/Kg/d)
LCZ (68 mg/Kg/d)
Bosentan (100 mg/Kg/d) + LCZ (30 mg/Kg/d)
Bosentan (100 mg/Kg/d) + LCZ (68 mg/Kg/d)
End of the study
D0
D7
D18
D21
Onset of the treatment
Echocardiography
Catheterism and colletions
B
SuHx PH model
Control
Vehicle
Sugen 5416
Chronic hypoxia
Vehicle
Bosentan (100 mg/Kg/d) + LCZ (30 mg/Kg/d)
End of the study
- 5W
-2W
D0
D18
D21
Echocardiography
Onset of the treatment
Echocardiography
Catheterism and collections

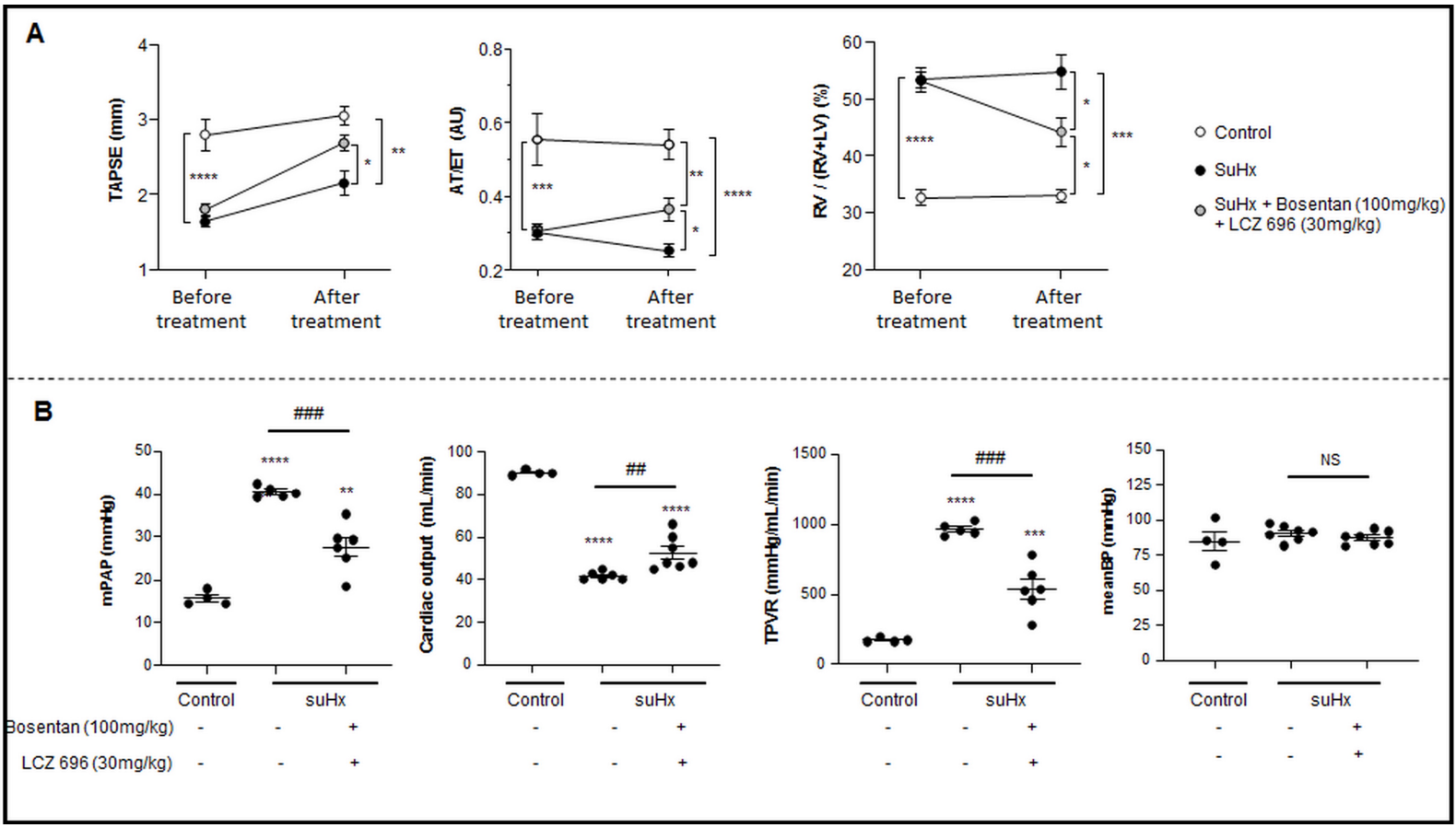

A
TAPSE (mm)
AT/ET (AU)
RV / (RV+LV) (%)
Control
MCT
Bosentan (100mg/kg)
LCZ 696 (30mg/kg)
LCZ 696 (68mg/kg)
B
mPAP (mmHg)
Cardiac output (mL/min)
TPVR (mmHg/mL/min)
meanBP (mmHg)
Control
MCT
Bosentan (100mg/kg)
LCZ 696 (30mg/kg)
LCZ 696 (68mg/kg)

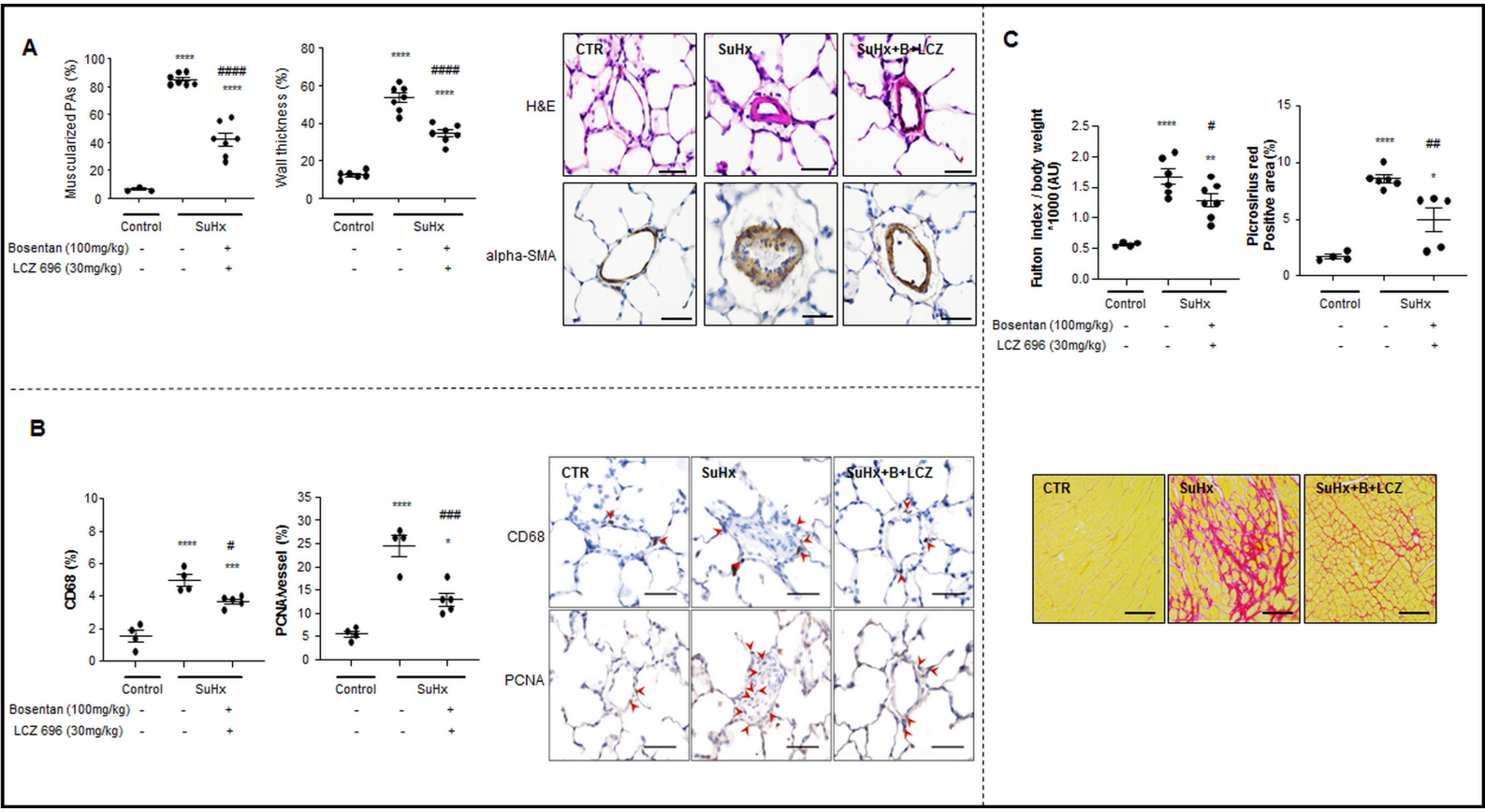

A
Muscularized PAs (%)
Wall Thickness (%)
Control
MCT
Bosentan (100mg/kg)
LCZ 696 (30mg/kg)
LCZ 696 (68mg/kg)
CTR
CTR+B+LCZ (68mg/Kg)
MCT
H&E
alpha-SMA
MCT+B
MCT+LCZ
MCT+B+LCZ (30mg/Kg)
MCT+B+LCZ (68mg/Kg)

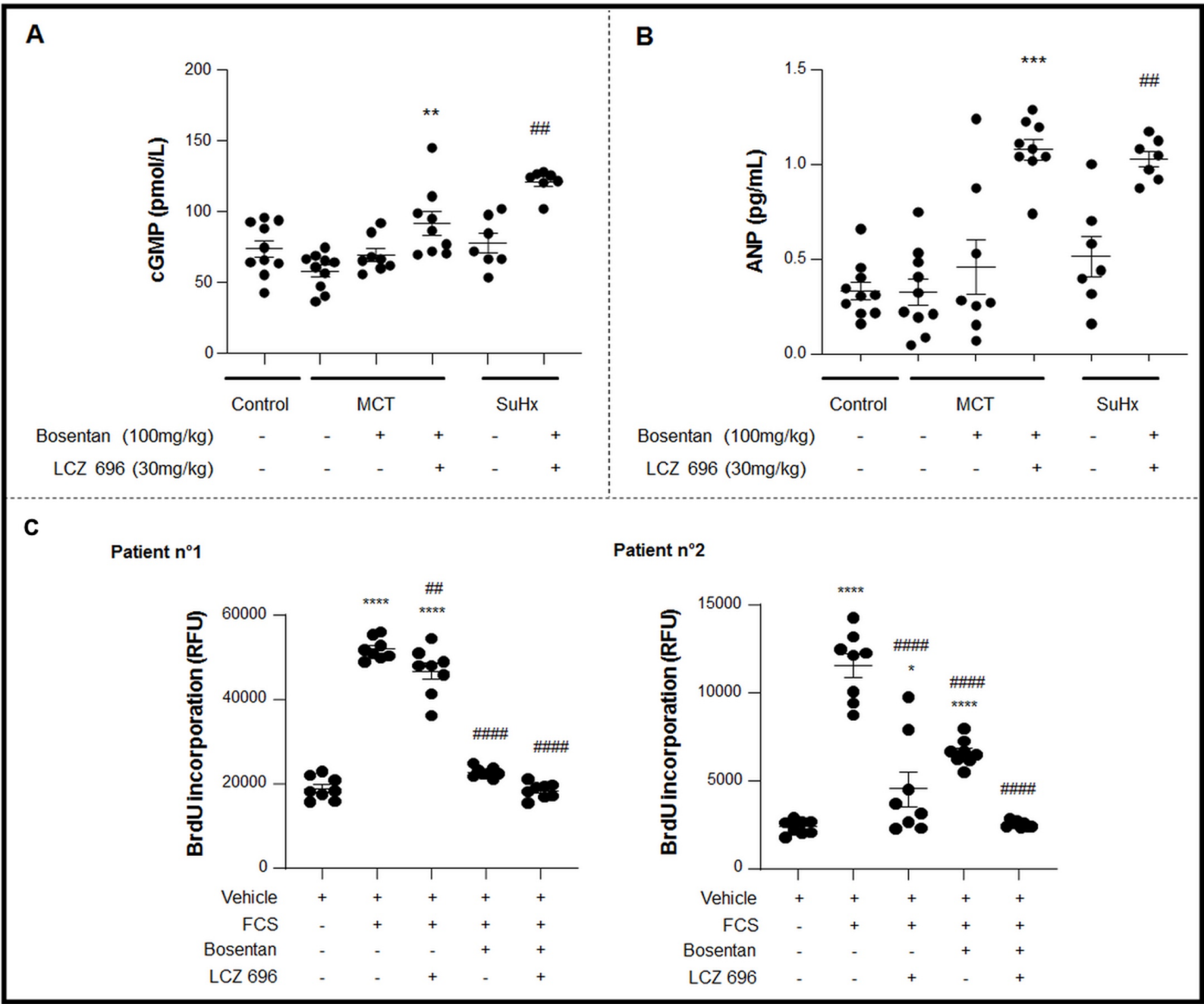

B
CD68 (%)
PCNA + / vessel (%)
Control
MCT
Bosentan (100mg/kg)
LCZ 696 (30mg/kg)
LCZ 696 (68mg/kg)
CTR
MCT
CD68
PCNA
MCT+B
MCT+LCZ
MCT+B+LCZ (30mg/Kg)


Fulton index / body weight
*1000 (AU)
0
1
2
3
4
δδ
δ
****
****
#
*
####
###
Control
MCT
Bosentan (100mg/kg) - + - + - + +
LCZ 696 (30mg/kg) - - - - - + -
LCZ 696 (68mg/kg) - + - - + - +
Picrosirius red
Positive area (%)
0
1
2
3
4
5
6
7
8
****
####
####
####
###
Control
MCT
- + - + - + +
- - - - - + -
- + - - + - +

CTR
CTR+B+LCZ (68mg/Kg)
MCT
MCT+B
MCT+LCZ
MCT+B+LCZ (30mg/Kg)
MCT+B+LCZ (68mg/Kg)


A
TAPSE (mm)
AT/ET (AU)
RV / (RV+LV) (%)
Before treatment
After treatment
Control
SuHx
SuHx + Bosentan (100mg/kg) + LCZ 696 (30mg/kg)
B
mPAP (mmHg)
Cardiac output (mL/min)
TPVR (mmHg/mL/min)
meanBP (mmHg)
Control
suHx
NS
Bosentan (100mg/kg)
LCZ 696 (30mg/kg)


A
Muscularized PAs (%)
Wall thickness (%)
Control
SuHx
Bosentan (100mg/kg)
LCZ 696 (30mg/kg)
H&E
alpha-SMA
CTR
SuHx
SuHx+B+LCZ
B
CD68 (%)
PCNA/vessel (%)
CD68
PCNA
C
Fulton Index /body weight *1000 (AU)
Picrosirius red Positive area (%)


A
cGMP (pmol/L)
200
150
100
50
0
**
##
Control
MCT
SuHx
Bosentan (100mg/kg) - - + + - +
LCZ 696 (30mg/kg) - - - + - +
B
ANP (pg/mL)
1.5
1.0
0.5
0.0
***
##
Control
MCT
SuHx
Bosentan (100mg/kg) - - + + - +
LCZ 696 (30mg/kg) - - - + - +
C
Patient n°1
BrdU incorporation (RFU)
60000
40000
20000
0
****
##
****
####
####
Vehicle + + + + +
FCS - + + + +
Bosentan - - - + +
LCZ 696 - - + - +
Patient n°2
BrdU incorporation (RFU)
15000
10000
5000
0
****
####
*
####
****
####
Vehicle + + + + +
FCS - + + + +
Bosentan - - - + +
LCZ 696 - - + - +