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Angiotensin Converting Enzyme 2 and Angiotensin (1-7) axis in Pulmonary Arterial Hypertension

Julio Sandoval[1] M.D., Leonardo Del Valle-Mondragón[2] M.Sc., Felipe Masso[3] Ph.D., Nayeli Zayas M.D., Tomás Pulido[1] M.D., Ricardo Teijeiro M.D[1]., Hector Gonzalez-Pacheco M.D[1]., Rossana Olmedo-Ocampo[1] M.D., Carlos Sisniega[1] M.D., Araceli Paez-Arenas[3] MSc, Gustavo Pastelin-Hernandez[2] M.D., Jose Gomez-Arroyo M.D. Ph.D. [1,4], and Norbert F. Voelkel[5] M.D.

1) Cardiopulmonary Department, 2) Pharmacology Department, 3) Physiology and Molecular Biology Department of the "Ignacio Chávez" National Institute of Cardiology Mexico City, Mexico; 4)Division of Pulmonary and Critical Care Medicine, University of Cincinnati, Cincinnati, USA; 5) Department of Pulmonary Medicine, Amsterdam University Medical Centers, Amsterdam, The Netherlands.

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Corresponding author:

Julio Sandoval MD

Cardiopulmonary and Immunology Departments, Ignacio Chávez National Institute of Cardiology of Mexico

Juan Badiano # 1, Colonia Sección XVI Tlalpan, 14080, México D.F. México.

Phone number: + 52 55 55732911 ext. 1279. Fax number: + 52 55 55131410

e-mail address: sandovalzarate@prodigy.net.mx

Summary of take home messages

This study demonstrates that in patients with PAH of different etiologies there are alterations of the Angiotensin Converting Enzyme 2-Angiotensin (1-7)-MAS axis the counter regulatory axis of the

Renin-Angiotensin-Aldosterone System. Analysis of blood samples also demonstrates the presence of antibodies directed against Angiotensin Converting Enzyme 2.

Abstract

Background: In animal models of pulmonary arterial hypertension (PAH), angiotensin converting enzyme type 2 (ACE2) and Angiotensin 1-7 [Ang-(1-7)] have been shown to have vasodilatory, anti-proliferative, anti-fibrotic and anti-hypertrophic properties. However, the status and role of the ACE2-Ang-(1-7) axis in human PAH is incompletely understood.

Methods: We studied 85 patients with a diagnosis of PAH of distinct etiologies. Fifty-five healthy blood donors paired for age and sex served as controls. Blood samples were obtained from the pulmonary artery in patients with PAH during right heart catheterization. Peripheral blood was obtained for both groups. Ang-(1-7) and angiotensin II (AngII) were measured by zone capillary electrophoresis. Aldosterone, Angiotensin-(1-9), Angiotensin A, (Ang-A) and ACE2 were measured by ELISA, and ACE2 activity was determined enzymatically.

Results: Of the 85 patients, 47 had idiopathic PAH, 25 had PAH-associated with congenital heart disease, and 13 had PAH-associated with collagen vascular disease. Compared to controls, patients with PAH had a higher concentration of AngII [(1.03(IQR 0.72-1.88)vs.0.19(IQR 0.10-0.37)pmoles/ml;$p<0.001$)] and of aldosterone [(88.7(58.7-132)vs.12.9(9.55-19.9)ng/dL;$p<0.001$)]. Conversely, PAH patients had a lower concentration of Ang-(1-7) than controls [(0.69(0.474-0.91)vs.4.07(2.82-6.73)pmoles/ml;$p<0.001$)], and a lower concentration of Ang-(1-9), and Ang-A. Similarly, the ACE2 concentration was higher than in controls [(8.7(5.35-13.2)vs.4.53(1.47-14.3)ng/ml;$p=0.011$)], whereas the ACE2 activity was significantly reduced [(1.88(1.08-2.81)vs.5.97(3.1-17.8)nmoles/ml;$p<0.001$)]. No significant differences were found among the three

different etiologic forms of PAH.

Conclusions: The AngII-ACE2-Ang- (1-7) axis appears to be altered in human PAH and we propose that this imbalance, in favour of AngII, plays a role in the pathogenesis of the severe PAH. Further mechanistic studies are warranted.

Key words: Pulmonary arterial hypertension, Angiotensin II, Angiotensin converting enzyme 2, Angiotensin-(1-7), Angiotensin-(1-9), Angiotensin A-Alamandine

Introduction

Pulmonary arterial hypertension (PAH) encompasses a group of diseases characterized by a broad spectrum of pulmonary vascular changes leading to elevated pulmonary artery pressures, right heart failure (RHF), and untimely death (1-2). Despite advances in its treatment, RHF and a low cardiac output state are almost inevitable outcomes in the natural history of PAH (3-5). It has been demonstrated that the sympathetic nervous system (SNS) as well as the renin-angiotensin-aldosterone system (RAAS) are hyper-activated in PAH and may negatively impact survival (4, 5). A mechanistic role of sympathetic overdrive and hyperactive RAAS in the development of PAH has also been postulated (6-9).

The angiotensin converting enzyme type 2 (ACE2) converts Angiotensin II (AngII) into Angiotensin 1-7 [Ang-(1-7)], perhaps to counterbalance the deleterious vascular effects of AngII (10-12). *In vitro* studies *and* animal models of PAH have shown that Ang-(1-7) has anti-proliferative, anti-fibrotic, and anti-hypertrophic properties, in addition to its vasodilatory effects (13,14). Based on this knowledge recent attempts to favourably modify the ACE 2-Ang-(1-7) axis have been undertaken (15) despite incomplete knowledge of this system in human PAH. Here, we sought to further characterize the Ang II–ACE2–Ang-(1-7) axis in PAH patients and hypothesized that, compared to healthy controls, patients with PAH will have higher levels of AngII, lower expression of ACE2 and therefore lower levels of Ang-(1-7). We also explored the serum concentration of other novel protective angiotensins such as Angiotensin-1-9 [(Ang-(1-9)], and Angiotensin A-Alamandine (Ang-A) in this population.

Material and methods

Study population. We studied eighty-five patients with PAH of diverse etiologies [(median age 34 (IQR25.5-48yrs.); 85% female)] recruited from and followed by the pulmonary hypertension clinic of the cardiopulmonary department of the National Institute of Cardiology of Mexico. The diagnosis of

PAH was established in accordance with current guidelines for diagnosis and treatment of PAH (1). All patients underwent a thorough diagnostic workup including right heart catheterization (RHC).The diagnosis of PAH was established by exclusion of secondary causes of pulmonary hypertension and demonstration of a mean pulmonary artery pressure (mPAP) ≥25 mmHg at rest, a pulmonary capillary wedge pressure (PCWP) ≤15 mmHg, and a pulmonary vascular resistance (PVR) > 3UW by RHC (1). Fifty-five carefully selected healthy blood-donors, free from cardiovascular disease, from our institutional blood transfusion biobank, paired for age and sex were used as controls. During the diagnostic RHC of PAH patients a blood sample was obtained from the pulmonary artery and from a peripheral (cubital or vena cava) vein for enzyme and peptide measurements. Only peripheral blood was available for controls. Blood samples were immediately processed. The investigation and ethics committees of the Ignacio Chávez National Heart Institute approved the study, and each participant (patients and controls) gave an informed consent.

Methods for the measurements of Angiotensin II and Angiotensin (1-7), Ang-(1-9), Ang-A, Aldosterone levels as well as ACE2 concentration and ACE2 activity (16-21) are described in the On-line Supplemental material.

Statistical analysis

We compared Ang II, Aldosterone, Ang-(1-7), ACE2 levels, ACE2 activity, Ang-(1-9) and Ang-A concentration measurements between PAH patients and controls. For patients, we also compared baseline demographic characteristics, laboratory, echocardiography, hemodynamic, and enzyme and peptide findings among the three etiologic subgroups of PAH. Finally, we compared enzymatic measurements between pulmonary and peripheral vein samples within every single patient.

All data were verified for normal distribution by Shapiro-Wilks test. All categorical data were summarized as frequencies and percentages. Continuous variables were reported as medians and 25th and 75th percentiles (interquartile ranges, IQRs). Statistical differences between groups were assessed, either using the chi-square or Fisher's exact test in the case of categorical variables. For continuous variables, we used the Kruskal-Wallis or Mann-Whitney U tests, as appropriate. We

performed the Kruskal-Wallis test with Dunns non-parametric pairwise post hoc test with Bonferroni corrections to assess group differences. The Wilcoxon signed-rank test was used to compare paired samples. A p-value<0.05 was considered significant. Data were analyzed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp. Armonk, N.Y. USA).

Results

The clinical, laboratory, echocardiography, and hemodynamic characteristics of the 85 studied PAH patients are summarized in **Table 1**. All patients had severe PAH, as evidenced by a median pulmonary artery pressure (mPAP) of 56mmHg (IQR 43.5-70). All PAH patients had a normal pulmonary capillary wedge pressure (PCWP), and the median cardiac index was 2.76 (IQR2.31-4.20) L/min/m^2. Thirty-seven of the 85 patients (43.5%) were in World Health Organization (WHO) functional class III and IV and had a baseline 6-min walk distance (6MWD) of 316 (IQR242-389) meters. Forty-seven patients were diagnosed with idiopathic PAH (IPAH), 13 patients had PAH associated with collagen vascular disease (PAH-CTD) and 25 had PAH associated with congenital heart disease (PAH-CHD). Differences in demographics and clinical variables amongst subtypes of PAH are shown in **Table 1**. Hemodynamically all 3 groups were similar except for a higher right atrial pressure (RAP) and PCWP in PAH-CHD patients, the pulmonary artery pressures were lower in the PAH-CTD group. Prior to the baseline RHC, 52 patients (61%) had been treated with specific PAH drugs including sildenafil, endothelin-receptor antagonists (ERA) and prostanoids either as monotherapy (n=32) or as a combination therapy (n=20). Twenty nine patients were treated with sildenafil alone, 7 were receiving sildenafil plus an ERA, 6 were receiving sildenafil plus calcium channel-blockers, five were treated with sildenafil plus treprostinil, 3 were on calcium channel blockers (CCB) alone, and 2 patients were receiving sildenafil plus an ERA and a CCB. Twenty-four patients (28.2%) were taking spironolactone, and eleven (12.9%) had received either ACE-inhibitors or angiotensin-receptors antagonists. All these medications were discontinued between 8 to 24 hours prior to the RHC.

Peptide concentrations and enzyme activity differences between PAH and controls

Serum concentrations of Ang II, aldosterone, Ang-(1-7), Ang-(1-9), Ang-A, ACE 2 levels, as well as ACE2 activity from peripheral vein blood samples were available in 69 PAH patients and the 55 control subjects (**Table 2** and **Figure 1)**. Compared to controls, patients with PAH had a higher concentration of AngII and aldosterone, but a lower concentration of Ang-(1-7). Thus, the resulting AngII/Ang-(1-7) ratio was significantly higher in PAH patients. Ang-(1-9), and Ang-A concentration was also lower in patients. Unexpectedly PAH patients had a higher serum concentration of ACE2, while the ACE2 activity was significantly lower than that of controls. In order to explain the diminished activity of ACE2, we searched for serum antibodies directed against ACE2 using a modification of the method described by Takahashi et al (22) (On-line Supplemental material), we demonstrated that anti-ACE2 antibodies not only occur in the three etiological groups of PAH patients but the antibody levels are also higher than in healthy controls (**Table 2**).

Peptide concentrations and enzyme activity differences between PAH subgroups

Serum levels – this time obtained from the pulmonary artery during RHC – of Ang II, Ang-(1-7), Ang-(1-9), Ang-A, ACE2 concentration as well as ACE2 activity were remarkably similar among the 3 different subgroups of PAH (**Table 3**). However, the concentration of anti-ACE2 antibodies was higher in PAH-CTD patients ($p=0.015$) and the anti-ACE2 antibodies correlated negatively with the ACE2 activity only in the IPAH patients **(Figure 2**)

As anticipated, for the entire study cohort of PAH patients, the concentration of AngII correlated positively with aldosterone ($r=0.331;p=0.002$), ACE2 concentration ($r=0.607;p<0.001$), and ACE2 activity ($r=0.377;p<0.001$) and negatively with the concentration of Ang-(1-7) ($r= -0.389;p<0.001$). As expected, a higher concentration of ACE2 translated into a similarly higher ACE2 activity ($r=0.799;p< 0.001$).

The levels of Ang-(1-7) correlated negatively with the aldosterone concentration ($r= -0.256;p=0.020$). Lastly, the Ang-(1-9) concentration correlated positively with the Ang II ($r=0.277;p=0.011$) and with

the ACE2 concentration (r=0.227;p=0.039). Ang A did not correlate with any of the other peptide or enzymatic concentrations.

Modifications of enzymes/peptides as a function of passage through the lung.

The transpulmonary gradient of enzymes and peptides, as defined by the difference in the concentration between pulmonary artery and systemic (arterial) samples, could be determined in 57 of the PAH patients. There were small, but significant, changes in the enzymes and peptides as they passaged through the lung. There was an increase in Ang-(1-7) from 0.72 (0.419-0.929) to 0.736 (0.427-0.963); p<0.001); AngII decreased from 1.186 (0.691-1.909) to 1.158 (0.690-1.928); p= 0.013), and aldosterone from 105.9 (68.7-149.5) to 99.7 (67.18-146.9); p=0.020), the ACE2 concentration decreased from 8.43 (5.09-13.36) to 8.12 (5.00-13.08) p<0.001), and ACE2 activity from 1.79 (1.05-2.48) to 1.76 (1.02-2.42); p<0.001). Of interest, in 23 (40.3%) of the patients, aldosterone concentration increased after its passage through the lung circulation (**Figure E1** in the online supplemental material)

Correlation between peptide concentrations and enzyme activity with hemodynamics

We found a weak but statistically significant positive correlation between aldosterone concentration and the pulmonary vascular resistance index (PVRI) (r=0.320;p=0.013), as well as a negative correlation with the cardiac index (r= -0.284;p=0.010).

We also explored the potential clinical importance of the Ang II–ACE2–Ang-(1-7) axis by analysing its behaviour according to the hemodynamic status of the patients at baseline, as assessed by the cardiac index (**Table 4**). Patients with CI between 2.0 and 2.5 L/min.m^2, had lower values of ACE2 activity and tended to have higher values of aldosterone than patients with a normal CI.

Enzymatic/peptide concentrations in patients with and without ACE2 antibodies.

To evaluate the significance of the presence of ACE2 antibodies in PAH patients we determined the cut-off value of antibodies in the PAH patients using the median value of the lower quartile in this population resulting in a median value of 0.076 O.D. at 490 nm. (AUC=0.937;p< 0.001). As noted in

Table 5, PAH patients with ACE2 antibodies greater than 0.076 O.D. at 490 nm, had a lower ACE2 activity and a higher concentration of aldosterone. No other differences in enzymatic or peptide concentrations were found.

The impact of previous specific-PAH treatments or ACE-inhibitors on the axis

As mentioned, fifty-two of the 85 patients (61%) had been treated during different periods [mean time of 7 months (range 0.5 to 60)] with specific-PAH drugs (either with sildenafil alone or in combination with an endothelin-receptor antagonist or a prostanoid). Except for a higher RAP in the group with previous treatment [(6.5(4-10.2)vs.4(2-6) mmHg; p=0.016)] we did not find any other clinical, hemodynamic, or peptide/enzymatic differences between treatment naïve patients and the group that received drug treatment. We then focused on the 15 treatment naïve patients (7 with IPAH, 5 with PAH-CHD and 3 with PAH-CTD) at baseline and assessed differences in the ACE 2-Ang-(1-7) axis as a response to PAH-specific pharmacotherapy. These patients were treated with sildenafil alone or combination with bosentan. The results are summarized in **Table E1** in the online data supplement**.** Despite having improvements in the functional class and 6-min walk distance after a median period of 7 months of treatment, we did not find any significant change in the peptide concentrations or enzyme activity as measured in the peripheral vein blood.

We also analyzed the peptide/enzymatic concentrations in patients with and without medications known to affect the RAAS system, including 26 patients who were taking spironolactone (n=24) with or without ACE inhibitors and ARAs or both (n=11), and we compared them with 43 PAH patients not taking these medications and also with the 55 control subjects. As shown in **Table E2**, except for Ang-(1-9), all differences in enzymes/peptides are detected between patients (with and without anti RAAS drugs) and control subjects, but not between patients. However, there are Ang-(1-9) differences between patients treated with and without RAAS-drugs. Possibly, there is some benefit of these medications in maintaining protective levels of Ang-(1-9) in PAH patients.

Peptide concentrations and enzyme activity between pulmonary and peripheral blood

We found no difference in the peptide concentrations or enzyme activity between pulmonary artery blood and peripheral vein blood samples, and their values were highly correlated (**Table E3 and Figure E2** in the online data supplement).

Discussion

In this prospective observational study, we demonstrate that, compared with age-and gender-paired healthy controls, patients with PAH have higher levels of Ang II, but most importantly, have lower levels of Ang-(1-7), Ang-(1-9), and Ang-A. In addition, PAH patients have increased concentrations of ACE2, while its enzymatic activity is decreased compared to healthy controls. We suggest that the reduced activity is in part explained by autoantibodies against ACE2, and that the lower ACE2 activity may explain the lower Ang-(1-7) concentrations in PAH patients.

We speculate that increased circulating levels of ACE2 may reflect a compensatory mechanism to alter the balance of the renin angiotensin system in favour of the ACE2-Ang-(1–7)-MAS receptor axis and to promote the anti-fibrotic and anti-inflammatory actions of the Ang (1-7), as well as to attenuate the Ang II-AT1 receptor pathway.

Antibodies directed against ACE2 in the serum of idiopathic PAH patients may thus explain the diminished activity of ACE2, **(Figure 2).** Anti-ACE2 antibodies have previously been described in PAH associated with connective tissue disease (CTD) (22). While in CTD the existence of different types of autoantibodies is not a surprise, we do not have an explanation for the occurrence of anti-ACE2 antibodies in IPAH. Yet our finding supplements the previous description of autoantibodies in IPAH (23) and further supports the hypothesis of autoimmunity in the pathobiology of IPAH (24).

The resultant imbalance in favour of Ang II-Aldosterone over ACE2-Ang-(1-7) may be an important factor in the pathobiology of PAH. The potential clinical relevance of this finding may be represented by the fact that PAH patients with lower than normal values of CI had lower values of ACE2 activity and higher values of aldosterone than patients with normal CI (**Table 4**).

The RAAS in PAH

The importance of the renin-angiotensin-aldosterone system as a contributor to the pathophysiology of different forms of pulmonary hypertension including idiopathic PAH has been established (8,9). It has been shown that certain polymorphisms of ACE and the Angiotensin II receptor 1 (AT1) are associated with disease progression in patients with idiopathic PAH, suggesting that the RAAS is mechanistically involved (25). High expression of ACE in the endothelium of small pulmonary arteries in the lung tissue from patients with PAH has also been demonstrated (26).

de Man and co-workers (8) reported that both systemic and pulmonary RAAS activity is increased in patients with IPAH and that these changes are associated with pulmonary vascular remodelling; they demonstrated that in patients with IPAH increased plasma levels of renin, Angiotensin I (AngI), and AngII were closely associated with disease progression and prognosis. They also showed increased ACE activity in isolated pulmonary microvascular endothelial cells, as well as enhanced AngII production after AngI stimulation (8). AngII also caused increased proliferation of pulmonary artery smooth muscle cells, mediated via enhanced AT1 receptor signalling. Finally, they showed that inhibiting RAAS with Losartan may have therapeutic benefits (8). However, although de Man and co-workers did not explore the counter -regulatory ACE2-Ang-(1-7) system, their study demonstrated that the RAAS is indeed activated in patients with IPAH and they provide a rationale for treatment strategies that modify the neuro-hormonal overdrive in these patients (8,9,27).

More recently described components of RAAS might also play a role in the pathobiology of PAH. The angiotensin converting enzyme type 2 (ACE2), which converts Angiotensin II (AngII) to Angiotensin 1-7 [Ang-(1-7)] is part of a counter -regulatory axis of the RAAS that can affect vasoconstriction, cell proliferation, fibrosis, and inflammation (11-14). In preclinical studies, ACE2 gene transfer prevented the increase in RV systolic pressure and RV hypertrophy in the monocrotaline rat model and improved RV function in the pulmonary artery banding model (28-30). Thus, the activation of pulmonary ACE2 likely modifies the pathogenesis of PAH and may serve as a novel therapeutic target in PAH (10, 31,32).

The ACE2–Ang-(1-7) axis in humans with PAH

Previous investigations of the ACE2-Ang-(1-7) axis in PAH patients have been limited. Dai and co-workers (33) reported decreased levels of serum Ang-(1–7) in patients with pulmonary arterial hypertension due to congenital heart disease, and they report a significant negative correlation between the Ang-(1–7) levels and the mean pulmonary artery pressure. We could not confirm this result in our study. In the study by Dai et al, Ang-(1–7) levels appeared significantly diminished only in CHD patients with severe PAH and the authors did not measure Ang II, ACE2 levels, or other components of the axis (33). Likewise, in a recent proof-of-concept, open-label study on the effect of a soluble recombinant human ACE2 (rhACE2), Hemnes et al (15) assessed the Ang II/Ang-(1-7) ratio in 11 patients with idiopathic PAH in order to provide a rationale for the rhACE2 treatment. They showed that the AngII/Ang-(1-7) ratio was increased in PAH, but the authors, did not assess other components of the RAAS system.

We considered that ACE2 was also affecting other components of the angiotensin system such as Ang-(1-9) or Ang-A, in addition to Ang-(1-7), and measured these peptides in the blood samples. Ang-(1-9), another component of the counter-balancing system, is generated from Ang I by ACE2, or can be cleaved by ACE to form Ang (1-7) (34,35). While little is known about its effects in pulmonary hypertension, preclinical studies (36) have demonstrated that Ang-(1-9) reduced RVSP and RV hypertrophy and attenuated endothelial damage and medial hypertrophy of pulmonary arterioles as well as pulmonary fibrosis induced by MCT. Ang-(1-9) inhibited the infiltration of inflammatory cells, reduced the pro-inflammatory cytokines and attenuated expression of apoptosis-related proteins. All these pulmonary vascular disease-modifying effects of Ang-(1-9) are being signaled through the AT2 receptor.

The concentration of Angiotensin-(1-9) in our PAH patients was lower than that in control subjects (**Table 2**); this is consistent with the lower ACE2 activity. As mentioned, Angiotensin (1-9) can be cleaved by ACE to form Ang (1-7) (34-36), and thus it is not possible to establish here whether the low levels of Ang (1-7) in our patients are a consequence of a low concentration of Ang (1-9).

Whatever the mechanism, our results indicate that, in a similar way to the ACE2/Ang-(1-7)/Mas receptor, the ACE2/Ang-(1-9)/AT2R axis, another counter regulatory arm of RAS to ACE/Ang II/AT1 R, is also dysfunctional in PAH (Figure 3).

The concentration of Angiotensin A was also diminished in our PAH patients as compared to normal controls. (**Table 2**). Its role in the setting of PAH remains uncertain. Angiotensin A may elicit either direct vasoconstrictive and pro-proliferative actions via the angiotensin II type I receptor (AT1) (37), or it can be further metabolized to Alamandine (hydrolyzed by ACE2 from Ang A), triggering opposing effects via the MrgD receptor. Alamandine can be synthesized from Ang (1-7) by decarboxylation of aspartate to form alanine. Accordingly, Alamandine, a central molecule of this counter-regulatory cascade, can be generated both from Ang A as well as from angiotensin 1–7 (37). As these peptides, Ang (1-7) and Alamandine, have similar amino acid sequence and structure their effects are highly likely to be similar. Their respective receptors [Mas receptor (Ang (1-7)) and MrgD receptor (Alamandine)] are expressed on endothelial cells and they may play important roles in vascular physiology (37).

Taken together, our results suggest that in patients with PAH, several components of the counter-balancing axis of RAAS [Ang-(1-7), Ang-(1-9), and Ang A-Alamandine] are affected and likely impaired. A schematic representation of our findings is depicted in **Figure 3**. The diminished ACE2 activity in this protective mediator cascade is likely of central importance, giving support to current studies targeting the downstream ACE2/Ang (1–7)/Mas receptor pathway. It is interesting that the components of the ACE2–Ang-(1-7), axis, including Ang-(1-9), and Ang-A are remarkably similar among the three different subgroups of PAH despite clinical and hemodynamic differences between them, a finding which may be explained by a possible shared endothelial cell pathobiology. It is also of interest that the peptide/enzymatic activity values were not altered in the patients following targeted therapy for PAH– we had initially postulated that the drug treatment would likely normalize these peptide values. The finding of an increased level of Ang-(1-9) in patients taking drugs that modify the RAAS (**Table E2** supplemental material) is interesting and deserves further investigation.

Implications for treatment

Therapeutic modification of the neuro-hormonal axis in patients with severe PAH appear to be possible and the search for additional therapies for this catastrophic group of diseases is warranted. There is evidence for the beneficial effect of modifying conventional components of the RAAS (8-10, 27). Most importantly, however, there is now evidence for the potential benefit of modifying the ACE2-Ang-(1-7) axis in humans with PAH. Hemnes, et al (15) found that a single infusion of rhACE2, a purified intravenous formulation of soluble recombinant human ACE 2 (GSK2586881) was well-tolerated and was associated with improved pulmonary hemodynamics and reduced marker levels of oxidant and inflammatory stress. The results of our large cohort study strengthen the rationale for such a therapeutic approach by confirming that in human PAH the ACE2-Ang-(1-7) axis is indeed abnormally expressed. Our data also suggest that increasing the level of ACE2 may be ineffective when the ACE2 is not functional. An effective strategy might be to assure ACE2 functionality, to increase the levels of Ang-(1-7) and to assure the proper functioning of its MAS receptor. Removal of the ACE2 antibodies, for example by means of plasmapheresis, or perhaps by recombinant antibodies that neutralize the action of ACE2 antibodies, may optimize the therapeutic benefits of ACE2 agonists/activators.

Another important finding of our study was the confirmation of a disproportionate increase of aldosterone in PAH patients and that high aldosterone levels inversely correlated with the CI (**Figure E3** Supplemental material). Preclinical and clinical studies have demonstrated that AngII and aldosterone regulate cellular signalling processes that increase cell proliferation, migration, and vascular hypertrophy as well as initiate cardiovascular fibrosis and interrupt repair processes (38). Indeed, animal models of PAH have demonstrated the beneficial effects of mineralocorticoid receptor antagonism with spironolactone or eplerenone (31, 32). In the clinical setting, the addition of spironolactone to targeted treatment with ambrisentan in the ARIES studies (31, 39) has suggested some clinical benefit as well. The precise role of these drugs remains to be studied in randomized clinical trials.

Study limitations

First, our study does not have validation cohort, therefore, our results must be confirmed in another study within a different population and with a larger sample size. 2) Despite our analysis did no show significant differences in patients with and without PAH-treatment, we cannot exclude completely other PAH-drug influences in our results, as this intervention in our study was not a prospectively designed intervention. 3) Our patients were diagnosed in a relatively late stage of the disease and some of the etiology groups (PAH-CTD) were relatively small, accordingly, further information on early stage and etiology remain to be elucidated. 4) Finally, in the 3 different groups of patients we studied, inflammation likely plays a role. Accordingly, biomarkers of inflammation should be assessed/monitored in future studies.

Conclusions

In patients with idiopathic, CTD and CHD-associated PAH there are significant abnormalities in the AT II–ACE2– Ang-(1-7) axis characterized by elevated levels of Ang II and aldosterone. Decreased Ang-(1-7), Ang-(1-9) and Angiotensin A levels are perhaps explained by decreased ACE2 activity. Although serum levels of ACE2 are elevated, its activity is diminished, likely at least in part due to autoantibodies against ACE2. We also found elevated concentration of aldosterone. Elevated blood levels of aldosterone have been reported in patients with left heart failure (40), and it has been proposed that in pulmonary hypertension extra-adrenal lung vascular endothelial cells produce aldosterone (38) which may stimulate vascular smooth muscle cell growth in a paracrine fashion (41). As shown in **Figure E1**, aldosterone concentration increased after its passage through the lung in a significant proportion of our patients, but not in all patients. These findings are an illustration of the endothelial cell phenotypical alterations, which characterize the "sick lung circulation" of PAH patients (42).

Our results not only show reduced ACE2 activity, but also illustrate how Angiotensin II may preferentially signal via the angiotensin 1 and/ or 4 receptor, shifting the balance towards

vasoconstriction, cell proliferation, inflammation and fibrosis, and thus contributing, together with aldosterone (38), to the pathobiology of PAH.

In a different and likely highly relevant setting it is also of interest that ACE2 is the cellular receptor for corona viruses (43). Variable expression of ACE2, for example due to gender-associated polymorphisms may influence the pulmonary inflammatory response and possibly play a role in the development of pulmonary vascular diseases, as ACE2 has been shown to modify angiogenesis via inhibition of VEGFR2 signalling (44).

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Figure legends

Figure 1. **Peptide and enzymes differences in patients versus controls.** Peripheral serum concentration of Angiotensin II (Ang II); Angiotensin-(1-7) (Ang-1-7); Angiotensin-(1-9), Angiotensin A, Angiotensin Converting Enzyme type 2 (ACE2) concentration (ACE2 co); ACE2 activity (ACE2 Ac); Aldosterone, and ACE2 antibodies in PAH patients and control subjects. The ratio AngII/Ang-(1-7) is also shown. All differences are $p < 0.001$.

Figure 2. **Correlation between the amount of ACE2 antibodies and ACE2 activity in the pulmonary artery blood sample of idiopathic PAH patients.** Elevated concentration of antibodies against ACE2 is associated with a decreased ACE2 activity ($r = - 0.368$; $p < 0.032$) in the pulmonary arterial blood sample of idiopathic PAH patients.

Figure 3. Schematic representation of the RAAS and its counterbalance in pulmonary arterial hypertension according to our findings. In patients with PAH, several components of the counter-balancing axis of RAAS [Ang-(1-7), Ang-(1-9), and Ang A-Alamandine] are affected and likely impaired. Although serum levels of ACE2 are elevated, its activity is diminished (*), perhaps in part due to autoantibodies against ACE2. Renin, Angiotensin I, ACE, and peptide receptors were not measured. Abbreviations: ACE2: Angiotensin converting enzyme type 2. APA: aminopeptidase A; APM: aminopeptidase M; AT1: angiotensin type-1 receptor; AT2: angiotensin type-2 receptor; AT4: angiotensin type-4 receptor; Mas: Mas receptor; MrgD: Mas-related G protein coupled receptor; MLDAD: mononuclear leukocyte-derived aspartate decarboxylase. Modified from Guignabert C, et al. (45).

Table 1. Demographic, clinical, functional, laboratory, echocardiography and hemodynamic characteristics of PAH patients

Variable	All PAH patients (n = 85)	IPAH (n=47)	PAH-CHD (n=25)	PAH-CTD (n=13)	p value (*)
Age, years median, (IQR)	34 (25.5-48)	33 (25-50)	34 (23-44)	47 (30.5-55)	0.057
Female, n (%)	72 (84.7)	42 (89.4)	18 (72)	12 (92.3)	0.106
BMI, median, (IQR)	23.8 (20.9-27.0)	23.5 (20.9-27.4)	23.5 (20.5-25.8)	26.0 (23.6-30)	0.134
Dyspnea, n (%)	67 (78.8)	37 (78.7)	17 (68)	13 (100)	0.073
Syncope, n (%)	27 (31.8)	22 (46.8)	2 (8)	3 (23.1)	**0.003**
Edema, n (%)	18 (21.2)	5 (10.6)	6 (24)	7 (53.8)	**0.003**
Angina, n (%)	11 (12.9)	9 (19.1)	2 (8)	0 (0)	0.130
WHO Functional Class ≥ III, n (%)	37 (43.5)	23 (49)	6 (24)	8 (61.5)	**0.046**
6MWT, meters median, (IQR)	316.5 (242-389)	352 (242.7-390.2)	360 (270.5-448)	250 (209-331.5)	0.082
NT-Pro BNP, pg/mL median, (IQR),	958.9 (335.2-2101.0)	780.0 (223.0-1859.0)	839.5 (139.4-1885)	1741.0 (949.5-2771)	0.202
TAPSE, mm, median, (IQR)	17 (12-20)	16.5 (12-20)	18 (14.2-21)	14 (12.2-17.3)	0.102
Hemoglobin, g/dL median, (IQR)	15 (13.6-16.7)	14.8 (13.8-16.6)	15.9 (13.5-18.3)	14.0 (12.1-15.5)	**0.038**[a]
Hematocrit % median, (IQR)	44.6 (41.7-49.8)	44.3 (41.9-48.2)	48.0 (42.5-54.8)	43.2 (37.8-47.8)	0.136
RBC, 10^6/ml median, (IQR)	5.07 (4.65-5.51)	4.95 (4.64-5.34)	5.31 (4.77-6.09)	5.03 (434-5.70)	0.238

BUN, mg/mL median, (IQR)	14.7 (11.4-19.6)	14.5 (11.5-21.1)	15.8 (11.1-19.7)	15.2 (11.7-19.7)	0.861
Creatinine, mg/mL median, (IQR),	0.75 (0.65-0.93)	0.75 (0.66-0.94)	0.75 (0.66-0.97)	0.65 (0.53-0.90)	0.187
Uric acid, mg/mL median, (IQR)	6.2 (4.9-7.6)	6.05 (4.9-7.69)	6.2 (5.1-7.59)	6.2 (5.1-7	0.837
LVEF, (%) median, (IQR)	61.9 (57.0-68.7)	62.0 (57.0-70.0)	60.9 (55.0-68.9)	61.5 (60.0-65.0)	0.668
RAP, mmHg median, (IQR)	5.0 (3.0-9.0)	4.0 (2.8-8.0)	8.0(6.0-10.7)	4.0(2.0-7.0)	**0.008**[b]
PAP systolic, mmHg median, (IQR)	91 (70-107)	86 (70-110)	99 (78-112.5)	72 (49.5-88)	**0.025**[a]
PAP diastolic, mmHg median, (IQR)	37 (28-46.5)	39 (27-50)	40 (29.5-53.5)	28 (23.5-35)	**0.033**[c]
mPAP, mmHg median, (IQR)	56 (43.5-70)	58 (46-74)	60 (44-71.5)	48 (36-54)	**0.033**[a]
PCWP, mmHg median, (IQR)	6.0 (3.0-9.0)	5.0 (3.0-8.2)	9.0 (6.5-11.5)	3.0 (1.0-6.5)	**0.001**[a,b]
CI, L/min/m^2 median, (IQR)	2.76 (2.31-4.2)	2.96 (2.32-4.2)	2.62 (1.98-3.70)	2.7 (2.32-4.03)	0.566
SaO_2%, median, (IQR)	91 (85.5-94)	92 (88-94)	87 (79-94)	90 (83-96)	0.291

Abbreviations: PAH: pulmonary arterial hypertension; IPAH: idiopathic PAH; PAH-CHD: PAH associated to congenital heart disease; PAH-CTD: PAH associated to collagen tissue disease; BMI: body mass index; WHO: World Health Organization; 6MWT: six-minute walk test; TAPSE: tricuspid annulus plane systolic excursion; RBC: red blood cells; BUN: blood urea nitrogen; LVEF: left ventricular ejection fraction; RAP: right atrial pressure; mPAP: mean pulmonary artery pressure; PCWP: pulmonary capillary wedge pressure; CI: cardiac index; SaO_2: arterial oxygen saturation. SD: standard deviation. (*) Kruskal–Wallis; a: differences between PAH-CHD vs. PAH-CTD; b: differences between IPAH vs. PAH-CHD; c: differences between IPAH vs. PAH-CHD by Dunns non-parametric pairwise post hoc test.

Table 2. Peripheral blood serum level measurements of Ang II, Aldosterone, Ang-(1-7), Ang-(1-9), Angiotensin A, ACE2 concentration, ACE2 activity, and ACE2 antibodies in PAH patients and control subjects.

Variable	Control (n = 55)	PAH patients (n = 69)	p value (*)
Age, years, median, (IQR)	30 (27-38)	35(27-49.5)	0.063
Female, n (%)	42 (76.4)	58 (84.1)	0.280
BMI, median (IQR)	25.9 (23.2-28.9)	23.8 (20.9-27)	0.026
Ang II, pmoles/L, median, (IQR)	0.199 (0.105-0.378)	1.03 (0.721-1.88)	**<0.001**
Aldosterone, ng/dL, median, (IQR)	12.9 (9.55-19.96)	88.7 (58.7-132.3)	**<0.001**
Ang-(1-7), pmoles/L, median, (IQR)	4.07 (2.82-6.73)	0.69 (0.474-0.914)	**<0.001**
Ang II / Ang-(1-7) ratio, median, (IQR)	0.04 (0.03-0.06)	1.58 (0.81-3.54)	**<0.001**
Ang-(1-9), pg/mL, median (IQR)	34.4 (30.31-45.98)	24.1 (14.40-34.00)	**<0.001**
Ang-(1-9)/Ang-(1-7) ratio (pg/mL), median, (IQR)	0.0093 (0.0059-0.160)	0.0344 (0.021-0.068)	**<0.001**
Angiotensin A, pg/mL, median (IQR),	120.4 (80.49-224.7)	18.93 (11.28-37.03)	**<0.001**
Angiotensin A/Ang-(1-7) ratio, (pg/mL), median, (IQR)	0.0315 (0.015-0.064)	0.0319 (0.013-0.071)	0.664
ACE2 concentration, ng/mL, median, (IQR)	4.53 (1.47-14.35)	8.70 (5.35-13.23)	**0.011**

ACE2 activity, mM, median, (IQR)	5.97 (3.11-17.81)	1.88 (1.08-2.81)	**<0.001**
ACE2 antibodies, O.D. at 490 nm., median, (IQR)	0.023 (0.005-0.04)	0.15 (0.10-0.22)	**<0.001**

Abbreviations: Ang II: angiotensin II; Ang-(1-7): angiotensin1-7; Ang-(1-9): Angiotensin-(1-9); ACE2: angiotensin converting enzyme 2. (*) Mann–Whitney U tests.

Table 3. Pulmonary artery blood serum measurements of Ang II, Aldosterone, Ang-(1-7), Ang-(1-9), Angiotensin A, ACE2 concentration, ACE2 activity, and ACE2 antibodies among different PAH etiologies

Variable	All PAH patients (n = 85)	IPAH (n=47)	PAH-CHD (n=25)	PAH-CTD (n=13)	p value (*)
Ang II, pmoles/L, median, (IQR)	1.20 (0.85-1.83)	1.13 (0.78-1.64)	1.33 (0.93-1.90)	1.28 (0.74-1.97)	0.333
Aldosterone, ng/dL, median, (IQR)	85.8 (52.9-129.6)	74.5 (47.3-119.1)	92.08 (63.2-177.3)	87.0 (76.3-132.4)	0.144
Ang-(1-7),pmoles/L, median, (IQR)	0.66 (0.43-0.88)	0.61 (0.43-0.92)	0.71 (0.40-0.85)	0.73 (0.58-0.81)	0.915
Ang II / Ang-(1-7) ratio, median, (IQR)	1.83 (0.90-3.68)	1.70 (0.72-3.3)	1.99 (1.25-4.41)	2.24 (0.96-4.16)	0.420
Ang-(1-9), pg/mL median (IQR)	23.58 (13.99-32.24)	21.42 (13.65-28.71)	23.94 (13.6-28.7)	28.57 (16.4-37.8)	0.719
Ang-(1-9)/Ang-(1-7) ratio (pg/mL), median, (IQR)	0.0344 (0.021-0.068)	.0313 (.0178-.0692)	.0376 (.0213-.0763)	.045 (.0216-.0627)	0.885
Angiotensin A, pg/mL, median (IQR),	20.46 (11.37-41.15)	24.9 (11.42-51.66)	14.43 (10.79-42.48)	18.15 (11.3-23)	0.532
Ang-A/Ang-(1-7) ratio (pg/mL), median, (IQR)	0.0319 (0.013-0.071)	.0389 (.0129-0.1266)	.0359 (.0136-.0660)	.0232 (.0155-.0419)	0.763
ACE2, ng/mL median, (IQR),	8.74 (6.6-13.19)	8.47 (6.15-14.74)	8.74 (7.02-13.06)	10.15 (5.29-10.8)	0.918

ACE2 activity, mM median, (IQR)	2.07 (1.27-2.81)	2.11 (1.30-2.76)	2.0 (1.24-2.51)	1.94 (0.82-3.61)	0.579
ACE2 antibodies, O.D. at 490 nm., median, (IQR)	0.16 (0.10-0.24)	0.15 (0.08-0.24)	0.12 (0.10-0.21)	0.21 (0.17-0.37)	**0.015**[a,c]

Abbreviations: As in Table 1 and 2. (*) Kruskal–Wallis; a: differences between PAH-CHD vs. PAH-CTD; c: differences between IPAH vs. PAH-CHD by Dunns non-parametric pairwise post hoc test.

Table 4. Clinical, functional, hemodynamic, echocardiography, and pulmonary artery peptide concentration characteristics of PAH patients according to the value of cardiac index.

	All patients (n=84)	CI > 2.5 L/min/m^2 (n=51)	CI 2.0-2.5 L/min/m^2 (n=21)	CI < 2.0 L/min/m^2 (n=12)	p value (*)
Age, years Median, (IQR)	33.5 (25.2-47)	31 (25-45)	39 (29.5-53)	39.5 (23.5-46.7)	0.311
Female, n (%)	72 (85.7)	44 (86.2)	19 (90.4)	9 (75)	0.466
BMI, Median, (IQR)	23.8 (20.9-27)	23.8 (20.9-27.3)	23.2 (21.4-26.7)	23.9 (20.2-28.8)	0.928
Dyspnea, n (%)	67 (79.8)	40 (78.4)	17 (81)	10 (83.3)	0.919
Syncope, n (%)	27 (32.1%)	14 (27.5)	8 (38.1)	5 (41.7)	0.508
Edema, n (%)	18 (21.4)	9 (17.6)	6 (28.6)	3 (25)	0.560
WHO Functional Class $\geq$ III, n (%)	37 (44)	22 (43)	9 (42.8)	6 (50)	0.904
6MWT, meters, median (IQR)	311 (242-390)	341 (284-386)	305 (219-416)	267 (203.5-336)	0.230
NT-ProBNP, pg/mL median, (IQR),	953 (320.5-2240)	808 (291-1840)	968 (465-4613)	462 (208-1932)	0.418
TAPSE, mm median, (IQR)	17 (12-20)	17 (14-20)	13 (11-19.5)	16.5 (11.7-20)	0.217

LVEF (%) Median (IQR)	61.9 (57-68.8)	63.5 (57 (65.5)	61 (57-67.5)	60 (55-66)	0.450
Hemoglobin, g/dL median, (IQR),	14.9 (13.6-16.8)	15 (13.6-16.6)	14.2 (13-16.7)	15.7 (13.6-16.8)	0.807
Hematocrit % median (IQR)	44.5 (41.7-49.9)	44.7 (41.7-50.5)	43.2 (40.6-47.8)	45.2 (42.1-53.8)	0.421
Creatinine, mg/mL median, (IQR)	0.75 (0.65-0.93)	0.73 (0.64-0.86)	0.73 (0.64-0.99)	0.96 (0.80-1.05)	**0.007*, [a]**
Uric acid, mg/mL mean ± SD	6.2 (4.9-7.6)	5.86 (4.8-7.24)	6.79 (5.12-7.96)	8.1 (5.4-9.15)	0.089
Heart rate, beats/min Median (IQR)	78 (71-89)	78 (70-89)	79 (70.5-93)	75 (72.7-79.5)	0.578
RAP, mmHg median (IQR)	5 (3-9)	4 (3-7)	5 (4-10.5)	9 (3.2-13.5)	0.071
Systolic PAP, mmHg median (IQR)	88.5 (70-107)	86 (70-103)	92 (70-104.5)	97.5 (71-119.7)	0.633
Diastolic PAP, mmHg median (IQR)	36 (28-45.7)	35 (27-46)	40 (27-46.5)	39.5 (30-50.5)	0.765
mPAP, mmHg median (IQR)	56 (44.2-70)	56 (44-67)	55 (44-71.5)	58 (43.5-76)	0.915
PCWP, mmHg median (IQR)	6 (3-9)	6 (3-9)	6 (3-9)	7.5 (2-12)	0.668
CI, L/min/m^2 median, (IQR)	2.76 (2.31-4.18)	3.81 (2.95-4.47)	2.34 (2.12-2.46)	1.9 (1.8-1.94)	< 0.001*
SaO2 %, median, (IQR)	91 (86-94)	92 (86-95)	90 (88.5-92)	86.5 (82-92.2)	0.172
Ang II, pmoles/L median, (IQR)	1.203 (0.84-1.83)	1.24 (0.89-1.87)	1.15 (0.61-1.83)	1.04 (0.62-1.55)	0.451
Aldosterone, ng/dL median, (IQR)	81 (58.6-129.8)	73.3 (54.4-116)	131.6 (62-183)	98 (63-147.4)	0.087
Ang-(1-7), pmoles/L median, (IQR)	0.668 (0.436-0.887)	0.650 (0.49-0.86)	0.63 (0.33-1.09)	0.78 (0.57-1.07)	0.644
Ang II/Ang-(1-7) ratio, median (IQR)	1.59 (0.809-3.55)	1.75 (0.84-3.66)	2.07 (0.50-4.66)	1.10 (0.51-2.15)	0.373

Ang-(1-9),pg/mL median (IQR)	23.5 (13.9-32.4)	23.5 (13.9-29.7)	26.3 (18.3-40.6)	18.5 (12.22-37.1)	0.244
Angiotensin A, pg/mL median (IQR),	20.19 (11.3-41.3)	23.2 (11.1-37.6)	19.9 (13.1-37.1)	13.1 (9.35-69.1)	0.933
ACE2 concentration, ng/mL median, (IQR)	9.04 (6.59-13.28)	9.4 (6.9-13.5)	7.84 (3.91-10.88)	8.65 (5.3-15.1)	0.244
ACE2 activity, mM/mL median, (IQR)	2.07 (1.28-2.83)	2.36 (1.63-3.15)	1.65 (1.03-2.01)	2.19 (1.02-3.15)	**0.028*, b**
ACE2 antibodies, O.D. at 490 nm., median, (IQR)	0.157 (0.103-0.2429	0.176 (0.110-0.248)	0.161 (0.085-0.225)	0.120 (0.067-0.241)	0.549

Abbreviations: As in Table 1 and 2. (*) Kruskal–Wallis; a: differences with CI < 2.0 L/min/m^2; b: differences between CI 2.0-2.5 and CI > 2.5 L/min/m^2 by Dunns non-parametric pairwise post hoc test.

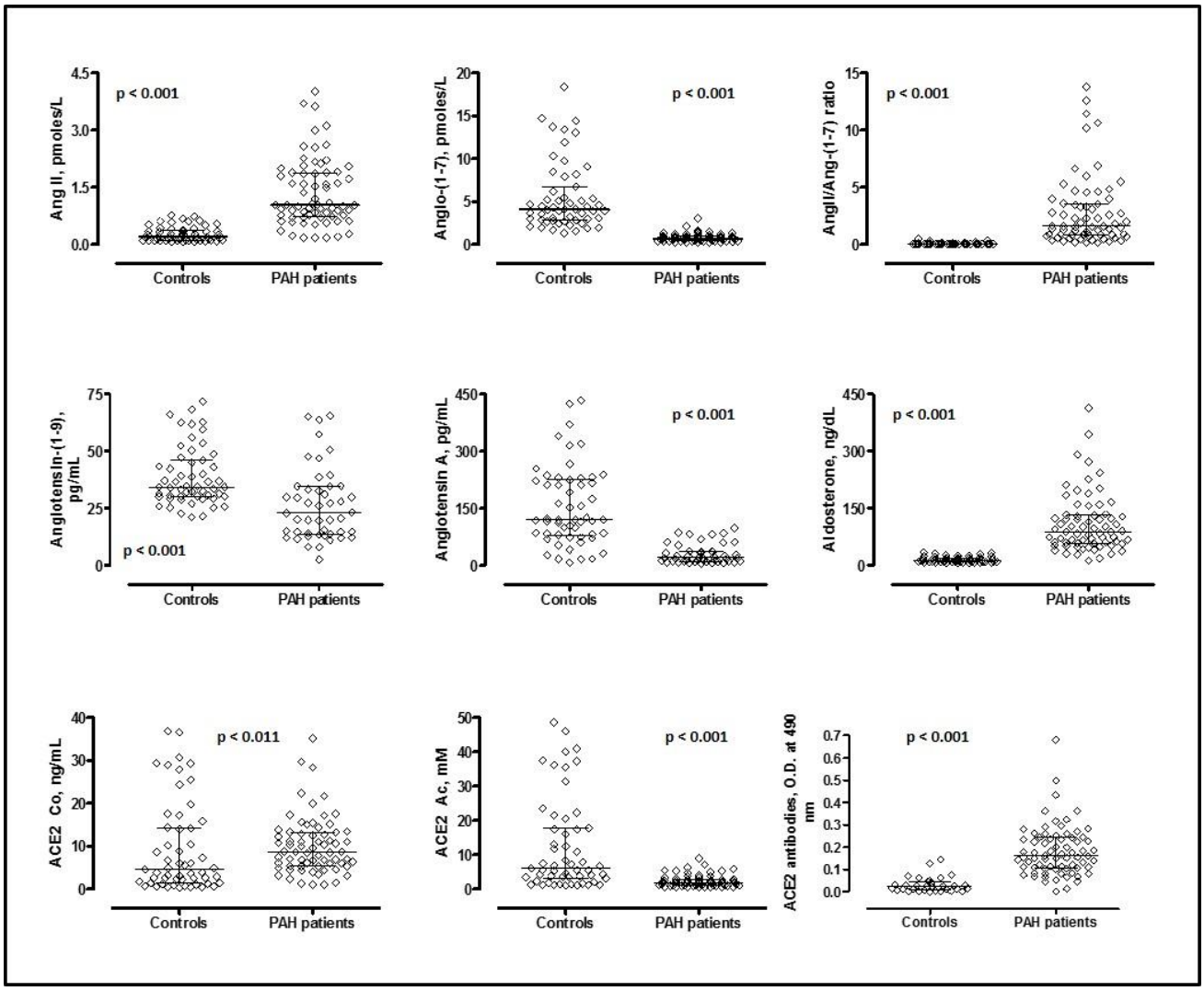
Table 5. Clinical, functional, hemodynamic, echocardiography, and pulmonary artery peptide concentration characteristics of PAH patients according to the presence of ACE2 antibodies

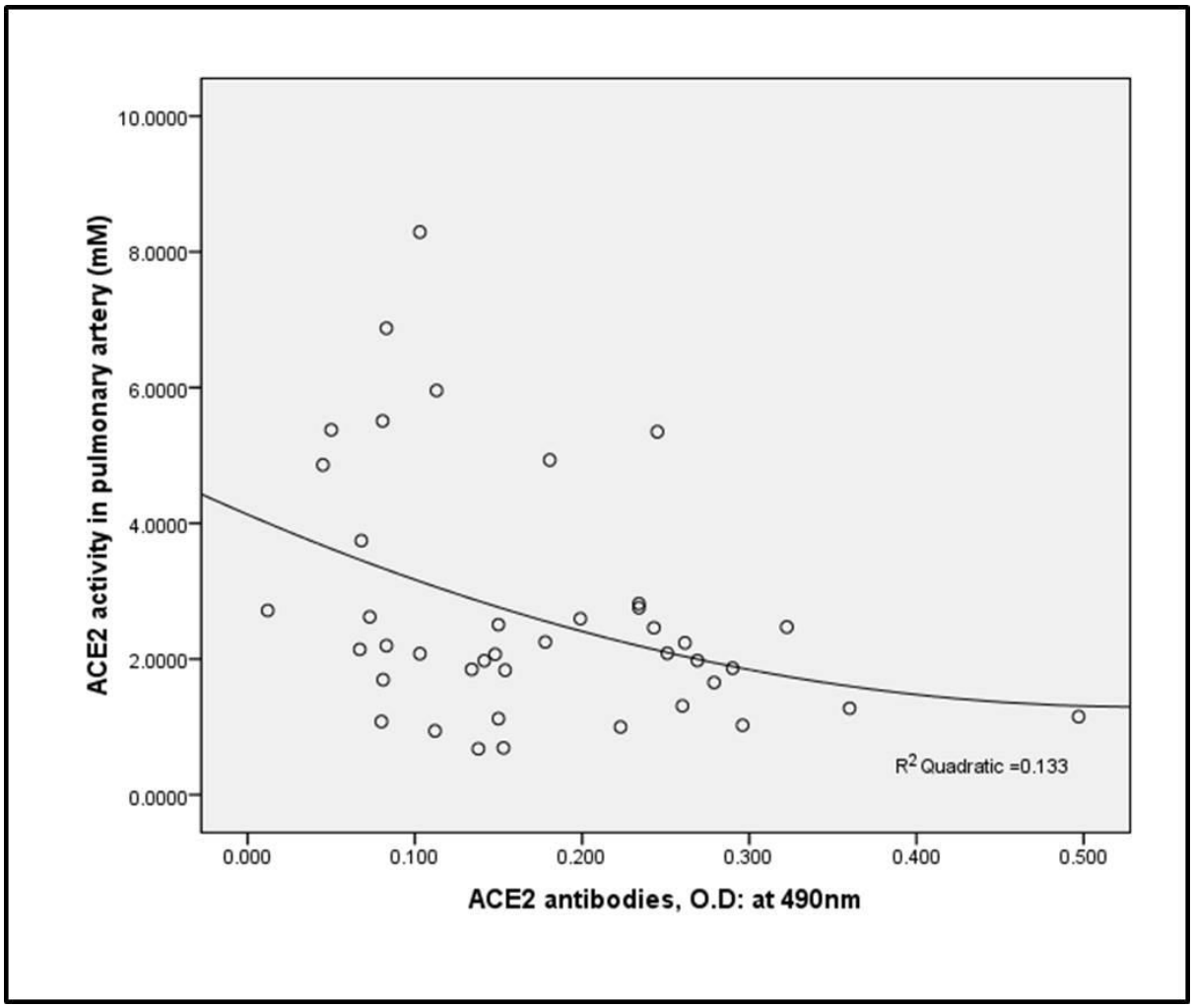
	ACE2 antibodies ≤ 0.0705 O.D. at 490 nm. n = 9 (12.3%)	ACE2 antibodies > 0.0706 O.D. at 490 nm. n = 64 (87.7%)	p value (*)
Age, years median, (IQR)	38 (31.5-44)	31 (25-48.5)	0.356
Female, n (%)	8 (88.9)	53 (82.8)	0.645
BMI, median, (IQR)	20.76 (20.42-23.01)	23.93 (21.1-27.1)	**0.039**
Dyspnea, n (%)	8 (88.9)	50 (78.1)	0.454
Syncope, n (%)	4 (44.4)	16 (25)	0.221
Edema, n (%)	3 (33.3)	12 (18.8)	0.311
WHO Functional Class ≥ III, n (%)	5 (55.6)	24 (37.5)	0.300
6MWT, meters median (IQR)	370 (272-400)	309 (239-385)	0.286
NT-Pro BNP, pg/mL median, (IQR),	2179 (348.5-6238.7)	965 (403.5-2268)	0.525
TAPSE, mm, mean ± SD	19 (12-23)	16.5 (12-19)	0.360
Hemoglobin, g/dL median, (IQR),	14.6 (12.8-16.9)	15.2 (13.9-16.6)	0.603
Hematocrit %, median (IQR)	44.3 (40.3-54.2)	45.5 (42.1-49.8)	0.980
Creatinine, mg/mL median, (IQR),	0.75 (0.64-0.89)	0.75 (0.66-0.96)	0.663

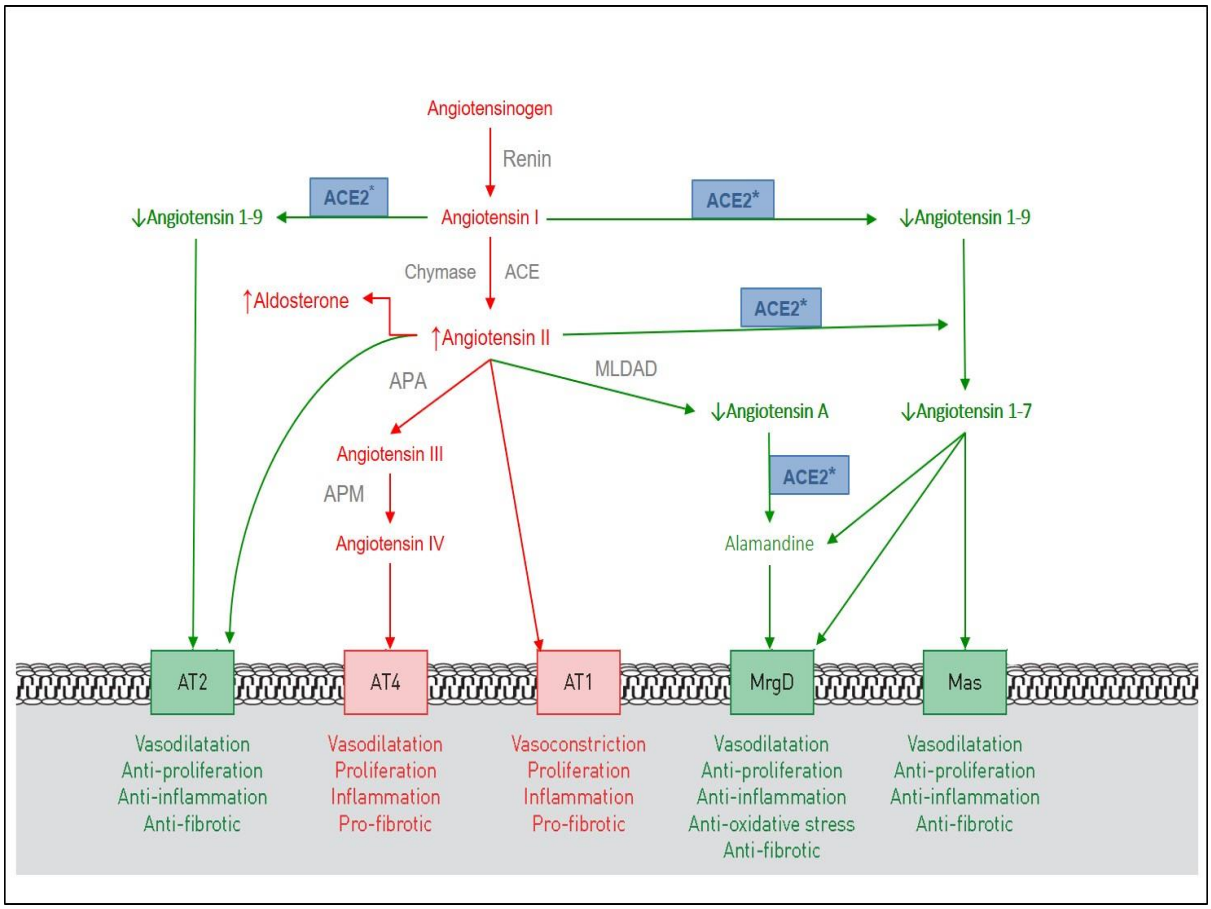
Uric acid, mg/mL mean ± SD,	5.35 (4.6-9.8)	6.7 (5.1-7.64)	0.615
LVEF %, Median (IQR)	56 (55-60)	61.9 (57-69.2)	**0.035**
RAP, mmHg median (IQR)	6 (4-14.5)	5 (4-9)	0.199
mPAP, mmHg mean ± SD	70 (52-77)	56 (46-71.5)	0.220
PCWP, mmHg median (IQR)	6 (3-14)	7 (3-9)	0.573
CI, L/min/m^2 median, (IQR)	2.47 (1.92-2.9)	2.95 (2.31-4.22)	0.074
SaO2 %, median, (IQR)	0.85 (0.82-0.93)	0.92 (0.86-0.95)	0.290
Ang II, pmoles/L median, (IQR),	1.240 (0.924-1.951)	1.186 (0.819-1.730)	0.638
Aldosterone, ng/dL median, (IQR)	58.93 (47.71-80.56)	90.37 (59.35-140.74)	**0.030**
Ang-(1-7), pmoles/L median, (IQR),	0.704 (0.402-1.11)	0.623 (0.426-0.877)	0.687
Ang II/Ang-(1-7) ratio, median (IQR)	2.05 (0.714-3.40)	1.62 (0.832-4.45)	0.695
Ang-(1-9), pg/mL median (IQR)	26.13 (13.53-39.93)	23.51 (13.34-31.08)	0.627
Ang-(1-9)/Ang-(1-7) ratio (pg/mL), median, (IQR)	.0314 (.0183-.0612)	.392 (.0198-.0803)	0.558
Angiotensin A, pg/mL median (IQR),	36.23 (13.59-78.07)	22.81 (11.46-41.31)	0.196
Ang-A/Ang-(1-7) ratio (pg/mL), median, (IQR)	.0445 (.0372-.1260)	.0403 (.0138-.0940)	0.671
ACE2concentrationng/mL, median, (IQR)	10.14 (7.21-16.53)	9.56 (6.79-13.54)	0.580

ACE2 activity, mM/mL median, (IQR)	3.11 (2.07-4.30)	2.07 (1.28-2.72)	**0.050**

Abbreviations: As in Table 1 and 2. (*) Mann–Whitney U tests


Ang II, pmoles/L
p < 0.001
Controls
PAH patients
Anglo-(1-7), pmoles/L
p < 0.001
AngII/Ang-(1-7) ratio
p < 0.001
Angiotensin-(1-9), pg/mL
p < 0.001
Angiotensin A, pg/mL
p < 0.001
Aldosterone, ng/dL
p < 0.001
ACE2 Co, ng/mL
p < 0.011
ACE2 Ac, mM
p < 0.001
ACE2 antibodies, O.D. at 490 nm
p < 0.001


ACE2 activity in pulmonary artery (mM)
10.0000
8.0000
6.0000
4.0000
2.0000
0.0000
0.000
0.100
0.200
0.300
0.400
0.500
ACE2 antibodies, O.D: at 490nm
R2 Quadratic =0.133


Angiotensinogen
Renin
↓Angiotensin 1-9
ACE2*
Angiotensin I
ACE2*
↓Angiotensin 1-9
Chymase
ACE
↑Aldosterone
↑Angiotensin II
ACE2*
APA
MLDAD
↓Angiotensin A
↓Angiotensin 1-7
Angiotensin III
APM
ACE2*
Angiotensin IV
Alamandine
AT2
AT4
AT1
MrgD
Mas
Vasodilatation
Anti-proliferation
Anti-inflammation
Anti-fibrotic
Vasodilatation
Proliferation
Inflammation
Pro-fibrotic
Vasoconstriction
Proliferation
Inflammation
Pro-fibrotic
Vasodilatation
Anti-proliferation
Anti-inflammation
Anti-oxidative stress
Anti-fibrotic
Vasodilatation
Anti-proliferation
Anti-inflammation
Anti-fibrotic

Angiotensin converting enzyme 2 and angiotensin (1-7) axis in pulmonary arterial hypertension

Julio Sandoval[1] M.D., Leonardo Del Valle-Mondragón[2] M.Sc., Felipe Masso[3] Ph.D., Nayeli Zayas M.D., Tomás Pulido[1] M.D., Ricardo Teijeiro M.D[1]., Hector Gonzalez-Pacheco M.D[1]., Rossana Olmedo-Ocampo[1] M.D., Carlos Sisniega[1] M.D., Araceli Paez-Arenas[3] MSc, Gustavo Pastelin-Hernandez[2] M.D., Jose Gomez-Arroyo M.D. Ph.D.[1,4,], and Norbert F. Voelkel[5] M.D.

Online Data Supplement

Measurement of antibodies directed against ACE2.

We used a modification of the method described by Takahashiet al (Arthritis Research &Therapy 2010; 12: R85). Briefly, Anti-ACE-2 antibodies ELISA test. Recombinant Human Angiotensin-Converting Enzyme 2/ACE-2 produced by transfected human cells, purity greater than 95% was purchased from novo protein (Summit, NJ USA). 96 well ELISA microplates (NuncMaxiSorp ™ Thermo Fisher Scientific, Waltham, MA USA) were coated with a solution of purified recombinant ACE-2 at 5 µg/ml in bicarbonate buffer (0.05M pH 9.6) 50 µl per well (corresponding to 250 ng/well). Plates were incubated overnight at 4^0C, the plates were washed trice with 200 µl of PBS containing 0.1% Tween 20 (Sigma-Aldrich St Louis Mo. USA). Plates were blocked with 200 µl of PBS Tween 20 containing 1% pig gelatin (Sigma-Aldrich St Louis Mo. USA) and incubated for 1 hour at room temperature with orbital agitation (120 rpm). Then, the plates were washed as before and 100 µl of human serum of control or HAP individuals were incubated for 3 hours at room temperature as before, blank wells received only 100 µl of PBS Tween 20. After three washes, 100 µl of horseradish peroxidase-conjugated rabbit anti-human IgG (Sigma-Aldrich St Louis Mo. USA) diluted 1:1000 in PBS 0.1% Tween 20 was added to each well and incubated for 45 minutes. After incubation, the plates were washed again and 100 µl of a mixture of enzyme substrate containing H_2O_2 and o-Phenylenediamine (Sigma-Aldrich St Louis Mo. USA) was added and incubated for 45 minutes in the dark. Absorbance was measured at 490 nm using a Biotek EL311 reader (Winooski,

USA). Plates were corrected with the wells containing PBS Tween 20 only. All assays were performed by triplicate. Data were plotted as the average of O.D. from 3 wells for each individual. This assay was previously optimized by selecting ACE-2 coating concentration, antibody dilution, time of incubation and time of color development reaction. Results are expressed as O.D at 400 ηm.

Table E1. Supplemental material. Changes in clinical, echocardiography, and laboratory variables and in peripheral blood measurements of Ang II, Ang- (1-7), ACE2 concentration, and ACE2 activity in naïve PAH patients after specific treatment (n=15)

Variable	Baseline	After PAH treatment	p value
Dyspnea, n (%)	9 (60)	7 (46.7)	0.715
Angina, n (%)	2 (13.8)	0 (0)	0.483
Syncope, n (%)	7 (46.7)	1 (6.7)	**0.035**
Edema, n (%)	4 (26.7)	4 (26.7)	1.000
WHO Functional Class ≥ III, n (%)	10 (66.7)	2 (13.3)	**0.008**
6MWT, meters, median, (IQR)	276 (240-400)	356 (276-453)	0.174
NT-Pro BNP, pg/mL, median, (IQR)	2778 (396-7402)	1328 (236-3750)	0.529
TAPSE, mm, median, (IQR)	17 (14-21)	17.4 (12-21)	0.967
RVSP, mmHg, median, (IQR)	77 (60-102)	94 (72-109)	0.340
LVEF (%), median, (IQR)	64 (57-70)	64 (60-70)	0.838
Uric acid, mg/mL, median, (IQR),	6.5 (5.6-7.8)	6.8 (4.4-8.1)	0.799
Creatinine, mg/mL, median, (IQR)	0.67 (0.60-0.75)	0.79 (0.68-0.83)	0.185
SaO2 (%), median, (IQR)	92 (88-95)	92 (89.5-94)	0.785
Ang II, pmoles/L, median, (IQR)	0.901 (0.601-2.051)	0.827 (0.679-1.184)	0.622
Aldosterone, ng/dL, median, (IQR)	70.21 (57.2-133.2)	93.2 (64.9-112)	0.758
Ang-(1-7), pmoles/L, median, (IQR)	0.789 (0.534-1.152)	0.913 (0.710-1.226)	0.424
ACE2 concentration, ng/mL, median, (IQR)	7.032 (5.223-10.394)	7.191 (5.845-10.035)	0.712
ACE2 activity, mM/mL, median, (IQR)	23.030 (11.333-25.254)	22.233 (11.312-30.263)	0.792

Abbreviations: PAH: pulmonary arterial hypertension; WHO: World Health Organization; 6MWT: six-minute walk test; TAPSE: tricuspid annulus plane systolic excursion; RVSP : Right ventricular systolic pressure; LVEF: left ventricular ejection fraction; Ang II: angiotensin II; Ang-(1-7): angiotensin1-7; ACE2: angiotensin converting enzyme 2.

Table E2. Supplemental material. Comparison of measurements of Ang II, Aldosterone, Ang (1-7), Ang-(1-9), Angiotensin A, ACE2 concentration, and ACE2 activity in peripheral blood among control subjects and PAH patients with and without anti-RAAS drugs.

	Control subjects (n = 55)	PAH patients without modifying drugs of the RAAS drugs (n=43)	PAH patients with modifying drugs of the RAAS (n=26)	p value *
Ang II, pmoles/L median, (IQR)	0.199 (0.105-0.378)	0.956 (0.617-1.88)	1.295 (0.818-1.886)	< 0.001*,[a]
Ang-(1-7), pmoles/L median, (IQR)	4.070 (2.825-6.738)	0.685 (0.527-0.985)	0.750 (0.407-0.877)	< 0.001*,[a]
Ang II / Ang-(1-7) ratio, median, (IQR)	0.040 (0.034-0.062)	1.519 (0.724-3.270)	2.065 (0.877-4.632)	< 0.001*,[a]
Aldosterone, ng/dL median, (IQR)	12.92 (9.55-19.96)	95.69 (57.29-160.37)	85.98 (64.93-120.97)	< 0.001*,[a]
Ang-(1-9),pg/m/L median (IQR)	34.42 (30.31-45.98)	19.76 (13.05-29.30)	30.57 (22.85-36.07)	< 0.001*,[b]
Angiotensin A, pg/mL median (IQR),	120.45 (80.49-224.72)	18.22 (10.92-37.7)	19.46 (12.55-37.37)	< 0.001*,[a]

ACE2 concentration, ng/mL, median, (IQR)	4.539 (1.47-14.35)	8.459 (5.223-13.221)	9.618 (6.018-13.390)	0.035*,a
ACE2 activity, mM median, (IQR)	5.977 (3.110-17.814)	1.887 (1.082-2.578)	1.917 (1.181-3.469)	< 0.001*,a
ACE2 antibodies, O.D. at 490 nm., median, (IQR)	0.023 (0.005-0.043)	0.160 (0.106-0.242)	0.157 (0.157-0.240)	< 0.001*,a

Abbreviations: RAAS: Renin-angiotensin-aldosterone system; Ang II: angiotensin II; Ang-(1-7): angiotensin1-7; Ang-(1-9): Angiotensin-(1-9); ACE2: angiotensin-converting enzyme 2. (*) Kruskal–Wallis; a: differences between control subjects and patients with and without modifying drugs of the RAAS, but no difference between patients; b: differences between control subjects and patients without modifying drugs of the RAAS and between patients with and without drugs, and no difference between control subjects and patients with modifying drugs of the RAAS by Dunns non-parametric pairwise post hoc test.

Table E3. Supplemental material. Comparison of measurements of Ang II, Aldosterone, Ang (1-7), Ang-(1-9), Angiotensin A, ACE2 concentration, and ACE2 activity between pulmonary and peripheral vein blood samples in patients with PAH.

Variable	Pulmonary artery sample (n=85)	Peripheral vein sample (n=69)	p value
Ang II, median, (IQR) (pmoles/L)	1.2 (0.85-1.8)	1.03 (0.7-1.9)	0.585
Aldosterone, median, (IQR) (ng/dL)	85.8 (58.9-123.3)	89.7 (58.9-133.7)	0.701
Ang-(1-7), median, (IQR) (pmoles/L)	0.68 (0.45-0.89)	0.7 (0.50-0.9)	0.710
Ang-(1-9), median (IQR) (pg/mL)	23.58 (13.99-32.24)	23.71 (14.20-32.28)	0.864
Angiotensin A, median (IQR), (pg/mL)	20.46 (11.37-41.15)	20.53 (11.41-41.3)	0.875
ACE2, median, (IQR) (ng/mL)	8.7 (6.7-13.1)	8.7 (5.4-13.2)	0.403
ACE2 activity, median, (IQR) (mM)	2.0 (1.3-2.8)	1.9 (1.1-2.8)	0.221

Abbreviations: Ang II: angiotensin II; Ang-(1-7): angiotensin1-7; Ang-(1-9): Angiotensin-(1-9); ACE2: angiotensin-converting enzyme 2;

Figure E1. Supplemental material. Transpulmonary gradient of aldosterone (n=57). **(A):** In 23 (40.3%) PAH patients, aldosterone concentration increased from 98.2 (74.2-156.5) to 99.8 (74.6-156.6) ng/dL (p‹0.001); In the remaining 34 (59.6%) patients **(B)**, aldosterone decreased from 106.1 (61.79-147.2) to 101.3 (60.6-142.4) ng/dL (p‹0.001).

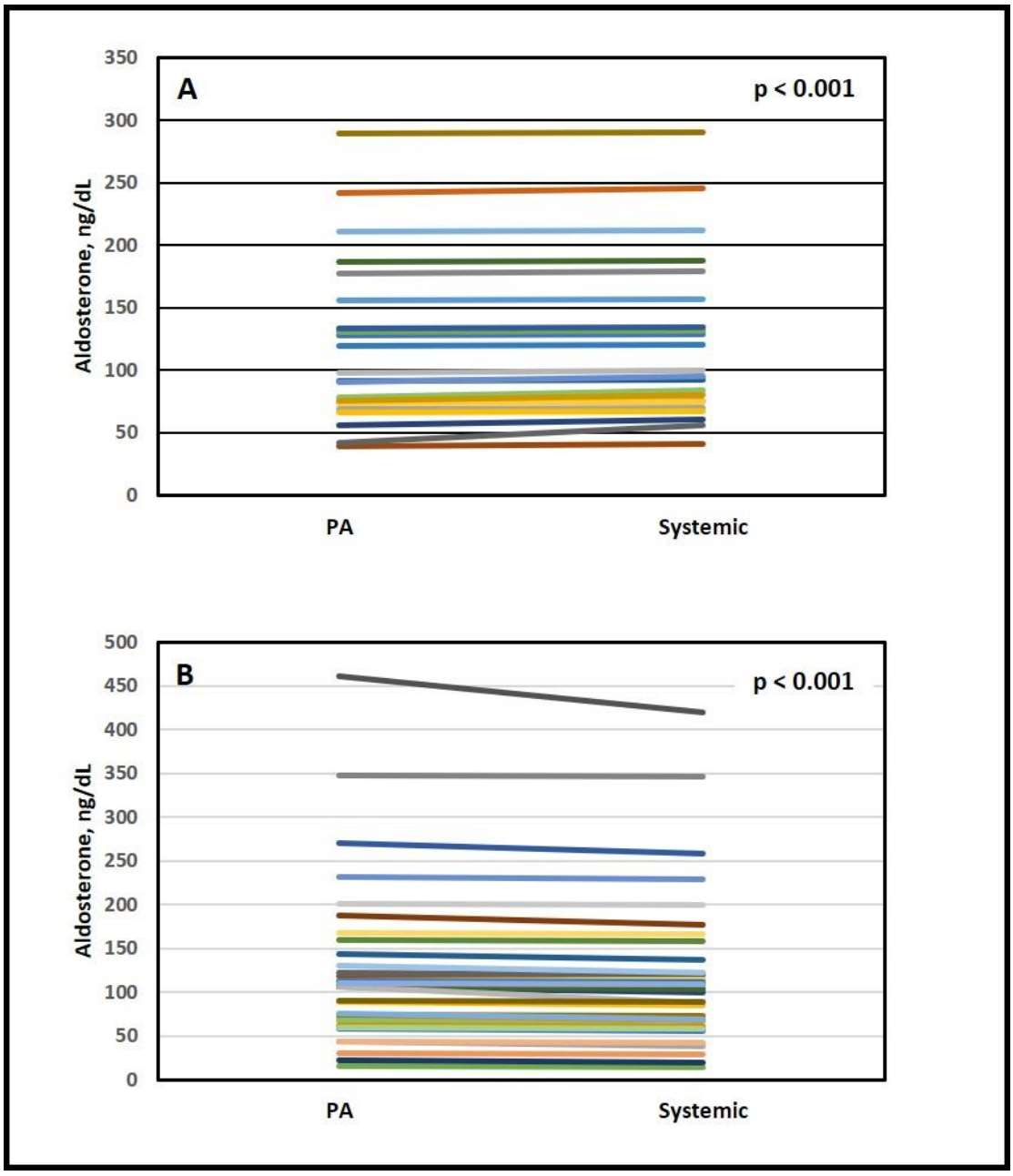
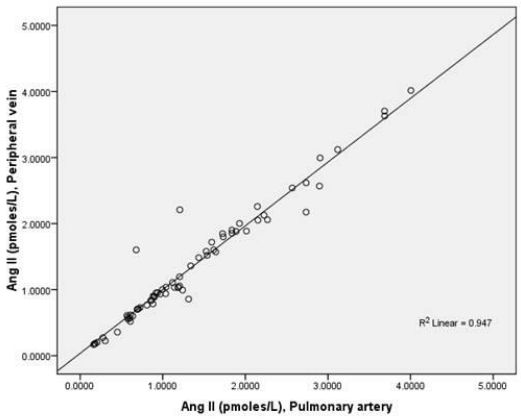
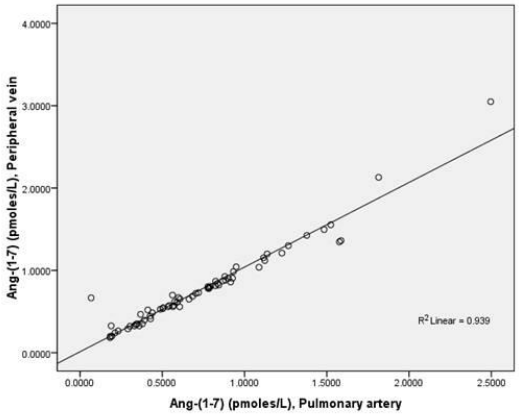
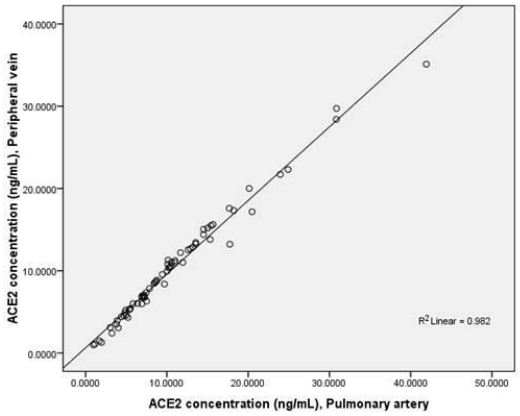
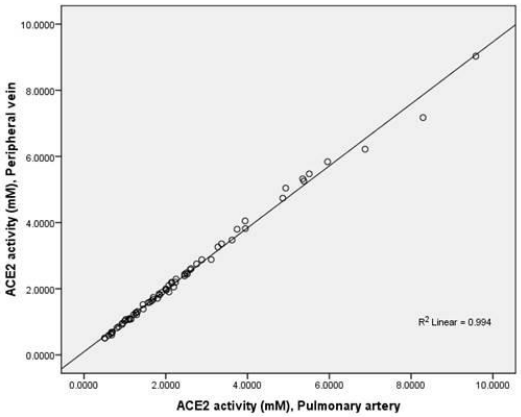


Figure E2. Supplemental material. Correlation between the concentration of enzymes/peptides in peripheral blood and pulmonary artery samples. All correlations are highly significant ($p \leq 0.001$).
Abbreviations: Ang II: angiotensin II; Ang-(1-7): angiotensin1-7; Ang-(1-9): Angiotensin-(1-9); ACE2: angiotensin-converting enzyme 2;


Ang II (pmoles/L), Peripheral vein
Ang II (pmoles/L), Pulmonary artery
R² Linear = 0.947


Ang-(1-7) (pmoles/L), Peripheral vein
Ang-(1-7) (pmoles/L), Pulmonary artery
R² Linear = 0.939


ACE2 concentration (ng/mL), Peripheral vein
ACE2 concentration (ng/mL), Pulmonary artery
R² Linear = 0.982


ACE2 activity (mM), Peripheral vein
ACE2 activity (mM), Pulmonary artery
R² Linear = 0.994

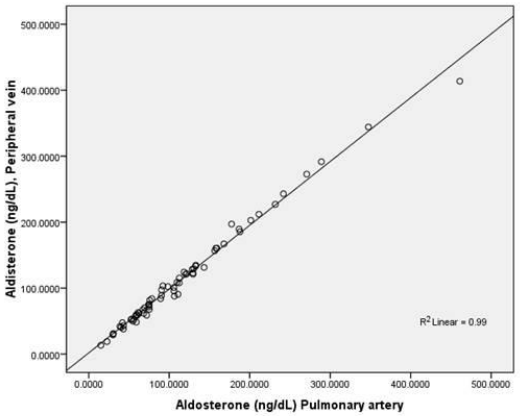

Aldisterone (ng/dL), Peripheral vein
Aldosterone (ng/dL) Pulmonary artery
R² Linear = 0.99

Figure E3 Supplemental material. Correlation between cardiac index (CI) and aldosterone (yellow dots), and between CI and NT-ProBNP (blue dots) in patients with PAH. Only the correlation CI-Aldosterone was significant.