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

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Online Data Supplement

Angiotensin II and Angiotensin (1-7) measurements

Angiotensin II and angiotensin (1–7) levels were measured by capillary zone electrophoresis (CZE) as described by Tenorio et al (16). Briefly, serum samples were homogenized (1:3 w/v) in 25 mM boric acid, pH 9.0. Proteins were removed by the addition of ice-cold methanol (2:1 v/v).The supernatant was further exposed to ice-cold 20% trichloro-acetic acid and the mix was centrifuged for 15 min at 16,000×*g*/10 °C and filtered through 0.22 μm nitrocellulose filters. The samples were analyzed in a CZE system (P/ACE MDQ Capillary Electrophoresis System, Beckman Coulter, Inc., Fullerton, CA, USA). Capillary electrophoretic separation was achieved using a 60 cm (50 cm to the detector) ×75 μmi.d. fused-silica capillary maintained at 25 °C in a cartridge with a 100 μm×800 μm detection window. Prior to any analysis, the capillary was pre-conditioned by flushing 1.0 M of NaOH for 60 min, deionized water for 30 min, and finally the background electrolyte (100 mM boric acid + 3 mM tartaric acid + 10 fM gold chloride III, pH 9.0). The applied voltage was set at 20 kV. The samples were injected using 0.5psi/10 s of hydrodynamic pressure. Photodiode

array detection was set at 200 nm and data acquisition was achieved with a ThinkCentre (IBM Corporation, White Plains, NY, USA) workstation. To determine angiotensin II or angiotensin-(1-7) concentrations, a standard curve was performed. Results are expressed in pmoles/mL.

Aldosterone, ACE2 concentration, ACE2 activity, Ang-(1-9) concentration, and Ang-A concentration measurements

Aldosterone was quantified in the patients' serum, by using a commercial Kit (Aldosterone ELISA Kit (Cat. ADI-900-173, Enzo Life Sciences, Lausen, Switzerland). The sera were processed according to the evaluation protocol established by the manufacturer (17). The quantification was performed spectrophotometrically at 405 nm (Evolution 220, Thermo Scientific, Palo Alto, CA, USA). The concentrations were determined by using a standard curve of aldosterone from 0 to 1000 pg / mL. ACE2 concentration was determined by using a commercial kit (ACE2 human ELISA Kit, AdipoGen International, Palo Alto, IL, USA / Cat. AG-45A-0022EK-KIO1, 96 wells) (18). ACE2 activity was determined by using a commercial kit (ACE2-human ELISA Kit, Sensolyte, AnasPecInc., Seraing, Belgium / Cat. 72086, 96 wells) (19). Angiotensin-(1-9) concentration was determined by using a commercial kit (Angiotensin-(Ang 1-9) ELISA Kit, MYBioSource, San Diego, CA, USA, Cat. MBS2022456) (20) and Angiotensin A-Alamandine concentration was determined by using a commercial kit (Angiotensin A ELISA Kit. Abcam, Cambridge, MA, USA. Cat ab136935) (21); for these measurements, the sera were processed according to the evaluation protocol established by the manufacturers.

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 $\geq$ 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).

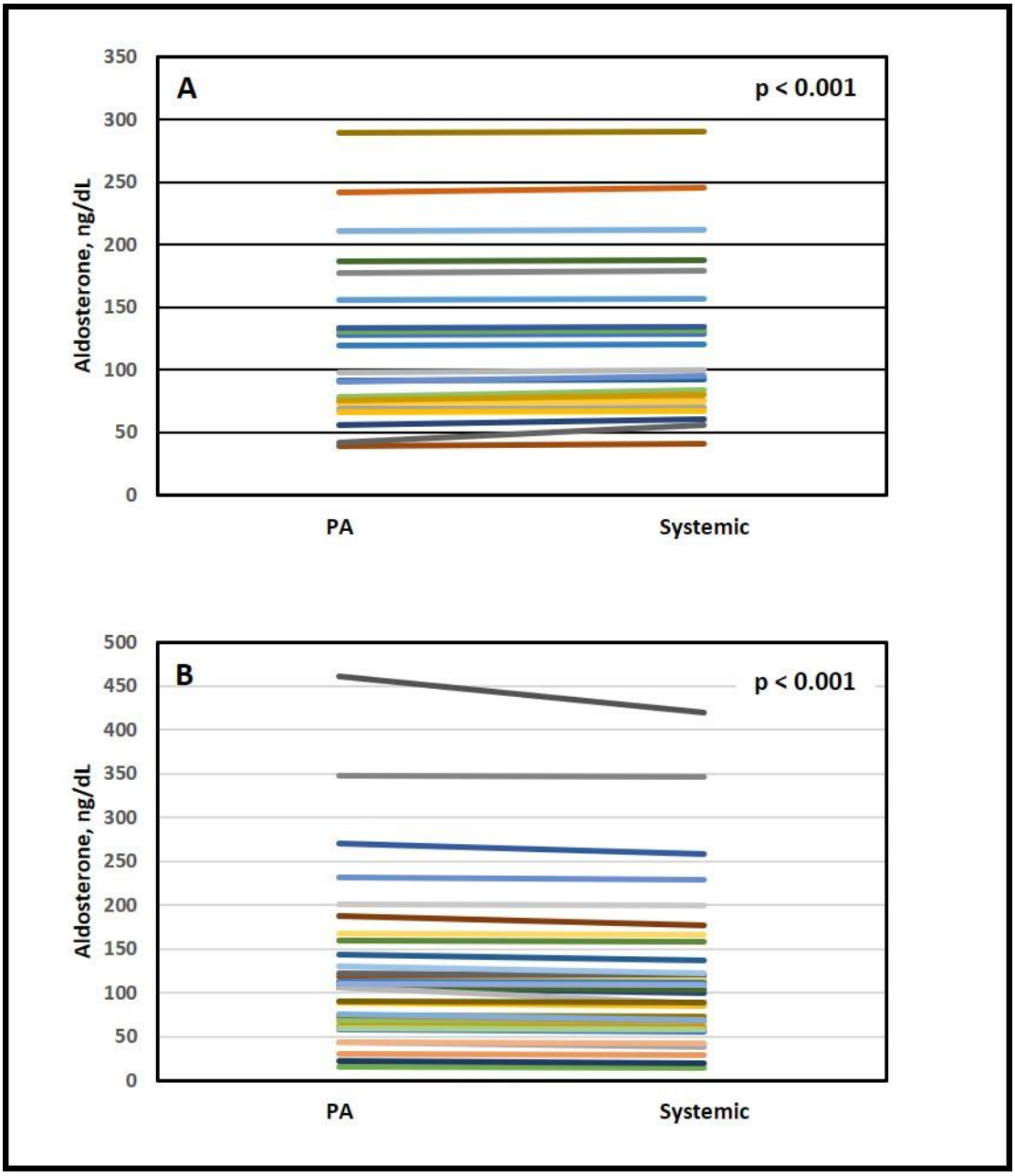

Figure E2. Supplemental material. Correlation between the concentration of enzymes/peptides in peripheral blood and pulmonary artery samples. All correlations are highly significant ($p \leq 0.001$). **Abbreviations:** Ang II: angiotensin II; Ang-(1-7): angiotensin1-7; Ang-(1-9): Angiotensin-(1-9); ACE2: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

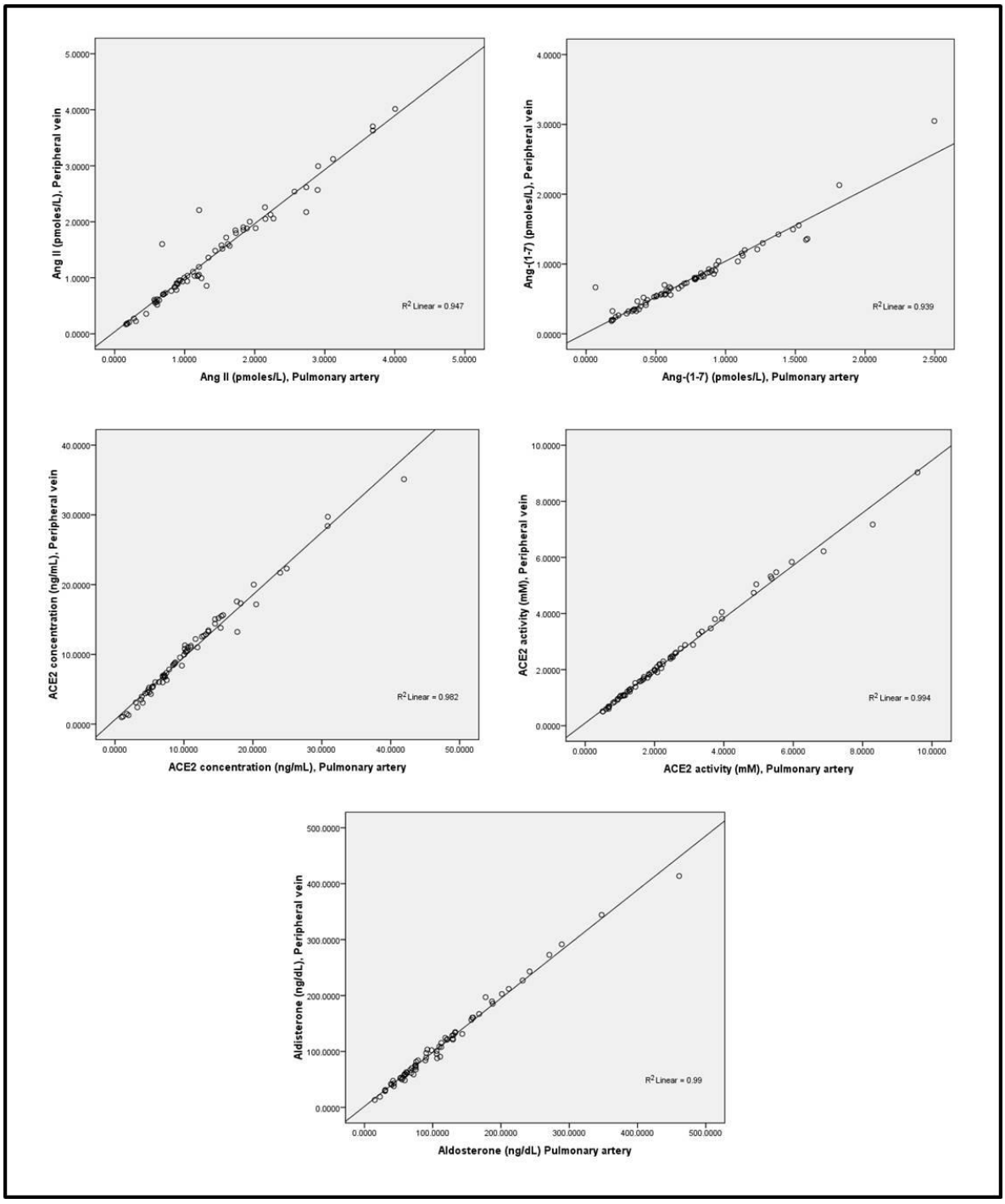

Figure E3 Supplemental material. Correlation between cardiac index (CI) and aldosterone (yellow dots), and between CI and NT-ProBNP (blue dots) in patients with PAH. Only the correlation CI-Aldosterone was significant.