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# Serum and Pulmonary Expression Profiles of the Activin Signaling System in Pulmonary Arterial Hypertension

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**Nonstandard Abbreviations and Acronym:**

<b>6MWD</b>	six-minute walk distance
<b>BMP</b>	bone morphogenetic protein
<b>BNP</b>	B-type natriuretic peptide
<b>CD</b>	clusters of differentiation
<b>CTR</b>	control
<b>FMI</b>	fluorescence mean intensity
<b>FSTL3</b>	follistatin-like 3
<b>GDF</b>	growth and differentiation factor
<b>IL</b>	interleukin
<b>NT-proBNP</b>	N-terminal pro B-type natriuretic peptide
<b>NYHA-FC</b>	New York Heart Association functional class
<b>PAH</b>	pulmonary arterial hypertension
<b>ROC</b>	receiver operating characteristic

## Abstract

**Background:** Activins are novel therapeutic targets in pulmonary arterial hypertension (PAH). We therefore studied whether key members of the activin pathway could be used as PAH biomarkers.

**Methods:** Serum levels of activin A, activin B,  $\alpha$ -subunit of inhibin A and B proteins, and the antagonists follistatin and follistatin-like 3 (FSTL3) were measured in controls and in patients with newly diagnosed idiopathic, heritable, or anorexigen-associated PAH (n=80) at baseline and 3 to 4 months after treatment initiation. The primary outcome was death or lung transplantation. Expression patterns of the inhibin subunits, follistatin, FSTL3, Bambi, Cripto, and the activin receptors type I (ALK), type II (ACTRII), and betaglycan were analyzed in PAH and control lung tissues.

**Results:** Death or lung transplantation occurred in 26 of 80 patients (32.5%) over a median follow-up of 69 (interquartile range, 50-81) months. Both baseline (hazard ratio, 1.001 [95% CI, 1.000-1.001];  $P=0.037$  and 1.263 [95% CI, 1.049-1.520];  $P=0.014$ , respectively) and follow-up (hazard ratio, 1.003 [95% CI, 1.001-1.005];  $P=0.001$  and 1.365 [95% CI, 1.185-1.573];  $P<0.001$ , respectively) serum levels of activin A and FSTL3 were associated with transplant-free survival in a model adjusted for age and sex. Thresholds determined by receiver operating characteristic analyses were 393 pg/mL for activin A and 16.6 ng/mL for FSTL3. When adjusted with New York Heart Association functional class, 6-minute walk distance, and N-terminal pro-B-type natriuretic peptide, the hazard ratios for transplant-free survival for baseline activin A  $<393$  pg/mL and FSTL3  $<16.6$  ng/mL were, respectively, 0.14 (95% CI, 0.03-0.61;  $P=0.009$ ) and 0.17 (95% CI, 0.06-0.45;  $P<0.001$ ), and for follow-up measures, 0.23 (95% CI, 0.07-0.78;  $P=0.019$ ) and 0.27 (95% CI, 0.09-0.78,  $P=0.015$ ), respectively. Prognostic values of activin A and FSTL3 were confirmed in an independent external validation cohort. Histological analyses showed a nuclear accumulation of the phosphorylated form of Smad2/3, higher immunoreactivities for ACTRIIB, ALK2, ALK4, ALK5, ALK7, Cripto, and FSTL3 in vascular endothelial and smooth muscle layers, and lower immunostaining for inhibin- $\alpha$  and follistatin.

**Conclusions:** These findings offer new insights into the activin signaling system in PAH and show that activin A and FSTL3 are prognostic biomarkers for PAH.

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Pulmonary arterial hypertension (PAH) refers to a rare and severe cardiopulmonary disorder in which occlusive remodeling of the small peripheral lung vasculature is largely responsible for the rise in pulmonary vascular resistance and mean pulmonary artery pressure, resulting in right heart failure and ultimately death without effective intervention.<sup>1,2</sup> Patient survival of advanced PAH remains poor with a 5-year survival rate  $\approx 60\%$ ,<sup>3–5</sup> and lung transplantation remains a suitable option for eligible patients who are refractory to the currently approved PAH medications and continue to have progressive clinical deterioration.<sup>6</sup>

Growing preclinical and clinical evidence indicates a detrimental role played by an enhanced Smad2/3-mediated transcriptional response, often accompanied by impaired bone morphogenetic protein (BMP)–Smad1/5/8 signal transduction, in PAH.<sup>7–16</sup> Although the exact mechanisms behind these alterations are unknown, activin A, the dimer of inhibin- $\beta$ A subunit encoded by *INHBA*, is suspected of having a role in both the Smad2/3-pathway over activation and the progression of obstructive vascular remodeling in PAH.<sup>13–16</sup> The function of activin A (inhibin- $\beta$ A–inhibin- $\beta$ A) and activin B (inhibin- $\beta$ B–inhibin- $\beta$ B) through activin receptors (ie, ACTRIIB, ACTRIIA combined with ALK4, ALK2, ALK7) are indeed pleiotropic, and constrained by 2 distinct classes of endogenous inhibitors, inhibins (ie, inhibin A and B) and follistatins (ie, follistatin and follistatin-like 3 [FSTL3]). To date, there are 2 reports of elevated circulating levels of activin A and follistatin in PAH,<sup>11,13</sup> but these studies are limited by the relatively small numbers of patients, a lack of independent validation cohorts, and absence of follow-up.

Therefore, the main objective of this translational study was to analyze the prognostic effect of serum levels of activin ligands and their inhibitors at baseline and during follow-up in a French multicenter prospective cohort of patients with idiopathic, heritable, or anorexigen-associated PAH and to determine whether the PAH pulmonary vascular lesions were associated with differential expression patterns of the activin signaling members

## Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

### Cohort Data Collection

#### *Discovery Cohort*

This study was conducted in accordance with the Declaration of Helsinki and informed consent was obtained for each patient before their enrollment. The study was approved by the ethics committee CPP Ile-de-France – VII on March 12, 2010 (approval number 10-003).

This is an ancillary study from the EFORT (Evaluation of Prognostic Factors and Treatment Goals in PAH) cohort (NCT 01185730). The EFORT study is a prospective study to assess prognostic factors at both baseline and follow-up in a French multicenter prospective cohort of incident (ie, newly diagnosed) patients with PAH. From January 2011 and December 2013, all incident patients entered in the French Registry with a diagnosis of idiopathic, heritable, or anorexigen-induced PAH were enrolled in the EFORT study. Inclusion criteria were age  $\geq 18$  years and PAH diagnosis confirmed by right heart catheterization showing a mean pulmonary artery pressure  $\geq 25$  mm Hg at rest, a pulmonary artery wedge pressure  $\leq 15$  mm Hg, and

pulmonary vascular resistance  $\geq 3$  Wood units. Participants with other conditions associated with PAH (connective tissue disease, portal hypertension, HIV infection, schistosomiasis, and congenital heart disease with the exception of atrial septal defect) or other causes of pulmonary hypertension (chronic lung disease, left heart disease, chronic thromboembolic pulmonary disease, and unclear or multifactorial mechanisms, or both) were excluded. Patients were also excluded if they were adults under guardianship, incarcerated persons, in emergency situations, refused or were unable to give informed consent, and uninsured participants. Assessments were performed at baseline (ie, time of PAH diagnosis) just before initiation of any PAH treatment and 3 to 4 months after treatment initiation (first followup visit). Occurrence of death or transplantation was assessed until December 2020. Serial assessments included New York Heart Association functional class (NYHA-FC), non-encouraged 6-minute walk distance (6MWD), right heart catheterization, and biomarkers BNP (B-type natriuretic peptide) or NT-proBNP (N-terminal pro-B-type natriuretic peptide). Eighty patients with available serum samples at both baseline and first follow-up visit for measurements of circulating biomarkers constituted our study population (Table 1).

**Table 1.** Baseline Characteristics for Patients With Pulmonary Arterial Hypertension in the EFORT Cohort (Table view)

Characteristics	Incident patients from EFORT cohort (n=80)
Female, n (%)	57 (71)
Age, y	51±19
Pulmonary arterial hypertension pathogenesis, n (%)	
Idiopathic	53 (66)
Heritable	16 (20)
Drug-induced pulmonary arterial hypertension	11 (14)
New York Heart Association functional class, n (%)	
I–II	28 (35)
III	45 (56)
IV	7 (9)
6-min walk distance, m	343±143
Hemodynamics	
Right atrial pressure, mm Hg	9±5
Mean pulmonary artery pressure, mm Hg	51±11
Pulmonary artery wedge pressure, mm Hg	9±3
Cardiac output, L/min	4.5±1.2
Cardiac index, L·min <sup>-1</sup> ·m <sup>-2</sup>	2.5±0.6
Pulmonary vascular resistance, Wood units	10±4
Mixed venous oxygen saturation, %	63±8
Brain natriuretic peptide, ng/L (n=72)	158 (54–312)
N-terminal pro-B-type natriuretic peptide, ng/L (n=4)	2349 (106–4977)

EFORT indicates Evaluation of Prognostic Factors and Treatment Goals in PAH.

### ***Validation Cohort***

A London cohort of 129 patients with PAH constituted our validation cohort collected as part of the Imperial College Prospective Study of Patients with Pulmonary Vascular Disease cohort (UK Research Ethics Committee approval EC Reference 17/LO/0563). A collection of serum samples was available in 125 patients at the time of PAH diagnosis and in 37 patients during follow-up.

### ***Controls***

Peripheral blood from healthy blood donors was provided by the French Blood Establishment (EFS, Paris, France).

### **Ella Microfluidic Platform and ELISA**

Blood analyses were performed centrally in the Université Paris-Saclay/Inserm UMR\_S 999 research laboratory (Le Plessis-Robinson, France). Activin A (DAC00B, Biotechne, Boston, MA), activin B (AL-150, AnshLabs, Webster, TX), FSTL3 (AL-152, AnshLabs, Webster, TX), and inhibin- $\alpha$  subunit (AL-A34, AnshLabs, Webster, TX) were measured using specific ELISA kits according to the manufacturer's instructions. CXCL9 (chemokine ligand 9), IL1- $\alpha$  (interleukin 1- $\alpha$ ), IL-6 (interleukin 6), and follistatin were measured using the SimplePlex Ella microfluidic platform (Protein Simple, San Jose, CA) according to the manufacturer's instructions.

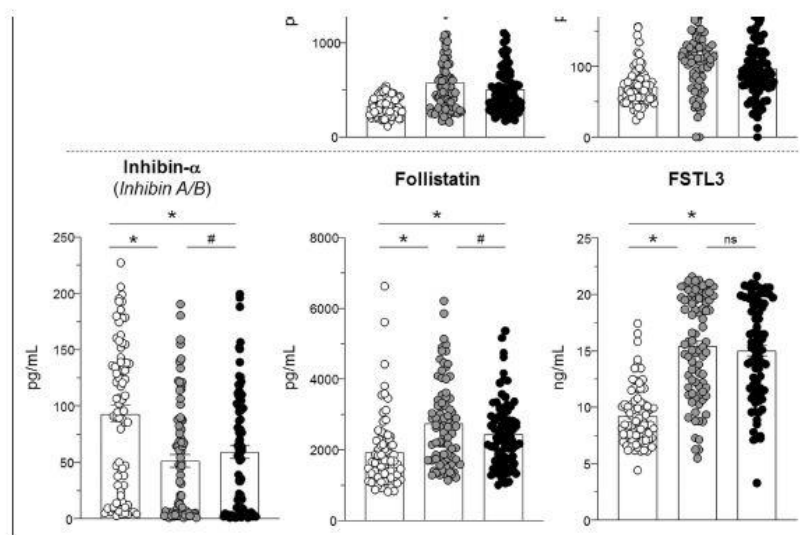
### **Immunofluorescence and Confocal Microscopy**

Lung studies were performed on pulmonary samples obtained at the time of lung transplantation in patients with PAH and at the time of lobectomy or pneumonectomy performed for localized lung cancer in controls (Table S1). Preoperative echocardiography was performed in controls to rule out pulmonary hypertension. The lung specimens from the controls were collected at a distance from the tumor foci. The absence of tumoral infiltration was retrospectively established in all tissue sections by histopathological analysis. This study was approved by the local ethics committee (CPP EST-III n°18.06.06, Le Kremlin-Bicêtre, France). All patients gave informed consent before the study. Immunofluorescence staining of  $\alpha$ -smooth muscle actin, ACTRIIA, ACTRIIB, ALK2, ALK4, ALK5, ALK7, BMP, and activin membrane-bound inhibitor, betaglycan (TGF $\beta$ RIII), CD31, Cripto, connective tissue growth factor, follistatin, follistatin-like 3 (FTSL3 or FLRG), inhibin- $\alpha$ , inhibin- $\beta$ A, inhibin- $\beta$ B, PAI-1 (plasminogen activator inhibitor-1; serpine), phospho-Smad2/3, and von Willebrand factor were performed in human lung paraffin sections (Table S2). In brief, at least 3 random lung sections (5- $\mu$ m-thick) were deparaffinized and incubated in retrieval buffer. The sections were then saturated with blocking buffer and incubated overnight with specific antibodies, followed by corresponding secondary fluorescently labeled antibodies (Thermo Fisher Scientific). Nuclei were labeled using 4',6-diamidino-2-phenylindole (Thermo Fisher Scientific). Slides were mounted using ProLong Gold antifade reagent (Thermo Fisher Scientific). The images were captured using an LSM700 confocal microscope (Zeiss) with ZEN software.

## Statistical Analyses

Clinical information was collected from the web-based French Pulmonary Hypertension Registry (PAHTool; Inovultus Ltd. Santa Maria da Feira, Portugal). Statistical analysis was performed using SPSS Statistics version 26 (SPSS Inc., Chicago, IL). Continuous variables are expressed as the means±SD or medians (interquartile range, 25%–75%) according to the data distribution.

After treatment with PAH therapies, a decline in activin B ( $97.7\pm 4.7$  versus  $121.9\pm 6.7$  pg/mL,  $P<0.05$ ) and follistatin ( $2460\pm 105$  versus  $2750\pm 131$  pg/mL,  $P<0.05$ ) was seen, whereas inhibin- $\alpha$  increased ( $59.6\pm 5.9$  versus  $51.6\pm 5.5$  ng/mL,  $P<0.05$ ). No significant difference was observed in activin A and FSTL3 levels between patients with PAH at diagnosis and at first follow-up ( $583.7\pm 46.5$  versus  $515.2\pm 33.7$  pg/mL and  $15.4.0\pm 0.5$  versus  $15.0\pm 0.5$  pg/mL, respectively). Pearson correlation identified a significant positive correlation between serum circulating activin A and FTSL3 (Figure S1).



**Figure 1.** Levels of activin A, activin B, the  $\alpha$ -subunit of inhibin A and B proteins, and the antagonists FST and FSTL3 in the serum of patients with PAH at diagnosis, first treatment follow-up, and in the serum of healthy persons. \* $P<0.05$  versus healthy subjects; # $P<0.05$  versus PAH at diagnosis. FST indicates follistatin; FSTL3, FST like-3; ns, nonsignificant; and PAH, pulmonary arterial hypertension.

## Associations Between Serum Levels of Activin A, Activin B, Follistatin, FSTL3, Inhibin A/B, and Outcomes in PAH

Patients were followed for long-term outcomes over a median of 69 (interquartile range, 50–81) months, during which time 21 patients died and 5 patients underwent lung transplantation. The prognostic value of serum levels of activin ligands and their inhibitors were analyzed at baseline and at first follow-up (landmark analysis), performed after a median (IQR) of 4.6 (3.9–7.3) months. First-line PAH therapy was started after baseline assessments and included calcium channel blockers ( $n=5$ , 6%), oral monotherapy ( $n=34$ , 43%), oral dual combination therapy ( $n=33$ , 41%), and triple combination therapy ( $n=8$ , 10%). At baseline, median values of activin A, follistatin, and FSTL3 were significantly elevated in non-survivors versus survivors, whereas serum levels of activin B and inhibin- $\alpha$  were similar in the 2 groups. Similarly, median values of activin A, follistatin, and FSTL3 were also higher at first follow-up in non-survivors (Table S3). Univariable Cox proportional hazard ratios for biomarkers of



activin pathway at baseline and follow-up are presented in Table 2. Serum levels of activin A and FSTL3 were associated with transplant-free survival at both baseline and follow-up. Follistatin was associated with transplant-free survival at follow-up only. The relationship between elevated serum levels of activin A or FSTL3 and transplant-free survival persisted in a model adjusted for age and sex at baseline and follow-up.

**Table 2.** Identification of Cytokines Associated With Transplant-Free Survival by Univariable Analysis at Diagnosis and 3 to 4 Months After Treatment Initiation (Table view)

Cytokines	Hazard ratios	95% CI	P value
At diagnosis			
Activin A, per SD	1.562*	1.261–1.935*	<0.001*
Activin A, per SD (adjusted for age and sex)	1.319*	1.018–1.709*	0.037*
Activin B, per SD	0.979	0.630–1.519	0.923
Follistatin, per SD	1.388	0.975–1.977	0.069
FSTL3, per SD	4.534*	2.223–9.245*	<0.001*
FSTL3, per SD (adjusted for age and sex)	3.209*	1.269–8.112*	0.014*
Inhibin- $\alpha$ subunit, per SD	1.041	0.667–1.625	0.861
At first follow-up			
Activin A, per SD	3.053*	2.003–4.654*	<0.001*
Activin A, per SD (adjusted for age and sex)	2.616*	1.516–4.512*	0.001*
Activin B, per SD	0.860	0.567–1.302	0.476
Follistatin, per SD	2.049*	1.469–2.857*	<0.001*
Follistatin, per SD (adjusted for age and sex)	1.613*	1.105–2.354*	0.013*
FSTL3, per SD	3.238*	1.869–5.608*	<0.001*
FSTL3, per SD (adjusted for age and sex)	2.685*	1.401–5.146*	0.003*
Inhibin- $\alpha$ subunit, per SD	0.854	0.560–1.302	0.463

Biological values are expressed as continuous variables. FSTL3 indicates follistatin-like 3.

\*Significant result.

Time-dependent ROC curve analyses were performed to determine the best threshold of transplant-free survival at baseline, corresponding to 393 pg/mL for activin A and 16.6 ng/mL for FSTL3. The ROC curves from which the thresholds were identified are presented in Figure S2. Comparison of clinical, hemodynamic, and biological values between patients with high and low levels of activin A are detailed in Table 3. Circulating levels of key proinflammatory cytokines known to be associated with PAH<sup>18,19</sup> were also compared among patients with high and low levels of activin A. Patients with PAH who have high serum levels of activin A had higher right atrial pressure, lower 6MWD, higher values of BNP, IL-6,  $\beta$ -NGF ( $\beta$ -nerve growth factor), and CXCL9 (Table 3).

In univariable analysis, the hazard ratios of activin A and FSTL3 expressed as dichotomous variables (according to thresholds previously determined) at diagnosis were 9.505 (2.233–40.462;  $P=0.002$ ) and 7.747 (2.888–20.787;  $P<0.001$ ), respectively. At first follow-up, the hazard ratios of activin A and FSTL3 were 6.835 (2.342–19.948;  $P<0.001$ ) and 6.932 (2.596–18.509;  $P<0.001$ ), respectively. Kaplan-Meier survival curves according to thresholds of activin A and FSTL3 are presented in Figure 2. The prognostic value of activin A (model A) and FSTL3 (model B) remained significant when adjusted for other noninvasive parameters currently used for PAH risk assessment (NYHA-FC, 6MWD, and NT-proBNP; Table 4).

**Table 3.** Comparison of Clinical, Hemodynamic, and Biological Parameters Between Patients With High and Low Levels of Circulating Activin A at Baseline (Table view)

Parameter	High level of activin A (n=44)	Low level of activin A (n=36)	P value
Sex, female	27 (61)*	30 (83)*	0.03*
Age, y	60±16*	40±16*	<0.01*
Event (death or lung transplantation), n (%)	22 (50)*	4 (11)*	<0.01*

Parameter	High level of activin A (n=44)	Low level of activin A (n=36)	P value
New York Heart Association, n (%)			0.59
I-II	14 (32)	14 (39)	
III	25 (57)	20 (56)	
IV	5 (11)	2 (5)	
6-min walk distance, m	309±134*	383±144*	0.02*
Right atrial pressure, mm Hg	10±5*	7±4*	<0.01*
Mean pulmonary artery pressure, mm Hg	52±10	49±13	0.24
Pulmonary artery wedge pressure, mm Hg	9±3	8±3	0.35
Cardiac output, L/min	4.4±1.2	4.5±1.2	0.56
Cardiac index, L·min <sup>-1</sup> ·m <sup>-2</sup>	2.4±0.6	2.6±0.7	0.19
Pulmonary vascular resistance, Wood units	11±4	10±5	0.47
Mixed venous oxygen saturation, %	62±9	64±7	0.45
Brain natriuretic peptide, ng/L	202 (83–558)*	71 (38–245)*	0.01*
Activin B, pg/mL	122 (104–171)	107 (71–131)	0.04
Follistatin, pg/mL	2876 (2193–4026)*	1957 (1412–3028)*	<0.01*
Follistatin-like 3, ng/mL	19 (15–20)*	12 (10–15)*	<0.01*
Inhibin- $\alpha$ subunit, pg/mL	34 (4–87)	47 (8–77)	0.33
$\beta$ -Nerve growth factor, pg/mL	4.2 (3.5–5.2)*	3.4 (2.5–3.8)*	0.01*
Chemokine (C-X-C motif) ligand, pg/mL	787 (554–1175)*	449 (349–679)*	<0.01*
Interleukin-1 $\alpha$ , pg/mL	0.15 (0.04–0.29)	0.14 (0.09–0.25)	0.83
Interleukin -6, pg/mL	5.1 (3.5–7.8)*	2.7 (1.7–4.0)*	<0.01*

Threshold was determined by receiver operating characteristic analysis.

\*Significant result.

These findings were validated in an independent external PAH cohort that comprised 125 UK patients (69% female, mean age 59±17 years, 91% idiopathic PAH, and 9% heritable PAH). Characteristics of the validation cohort are detailed in the Table S4. After a median follow-up of 59±30 months, 56 patients had died. A collection of serum samples was available in 125 patients at the time of PAH diagnosis and in 37 patients during follow-up. Kaplan-Meier survival analysis in this cohort according to the status of activin A and FSTL3 confirmed the results previously observed in the French cohort (Figure S3).

**Table 4.** Multivariable Cox Regression Analysis Including 3 Noninvasive Low-Risk Variables and Activin A (Model A) or Follistatin-Like 3 (Model B) Assessed at Diagnosis and Follow-Up in the Discovery EFORT Cohort (Table view)

Variable	Model A			Model B		
	Hazard ratios	95% CI	<i>P</i> value	Hazard ratios	95% CI	<i>P</i> value
<b>At diagnosis</b>						
New York Heart Association functional class I-II	1.38	0.58–3.30	0.471	1.23	0.50–2.99	0.653
6-min walk distance >440 m	1.15	0.26–5.19	0.853	0.99	0.26–3.75	0.993
Brain natriuretic peptide <50 or N-terminal pro-B-type natriuretic peptide <300 ng/L	0.27	0.05–1.59	0.147	0.30	0.06–1.49	0.140
Activin A < threshold	0.14*	0.03–0.61*	0.009*			
Follistatin-like 3 < threshold				0.17*	0.06–0.45*	<0.001*
<b>At first follow-up</b>						
New York Heart Association functional class I-II	0.50	0.21–1.15	0.103	0.68	0.29–1.57	0.362
6-min walk distance >440 m	1.06	0.28–4.04	0.932	0.55	0.16–1.87	0.336
Brain natriuretic peptide <50 or N-terminal pro-B-type natriuretic peptide <300 ng/L	0.38	0.12–1.27	0.116	0.47	0.14–1.57	0.218
Activin A < threshold	0.23*	0.07–0.78*	0.019*			
Follistatin-like 3 < threshold				0.27*	0.09–0.78*	0.015*

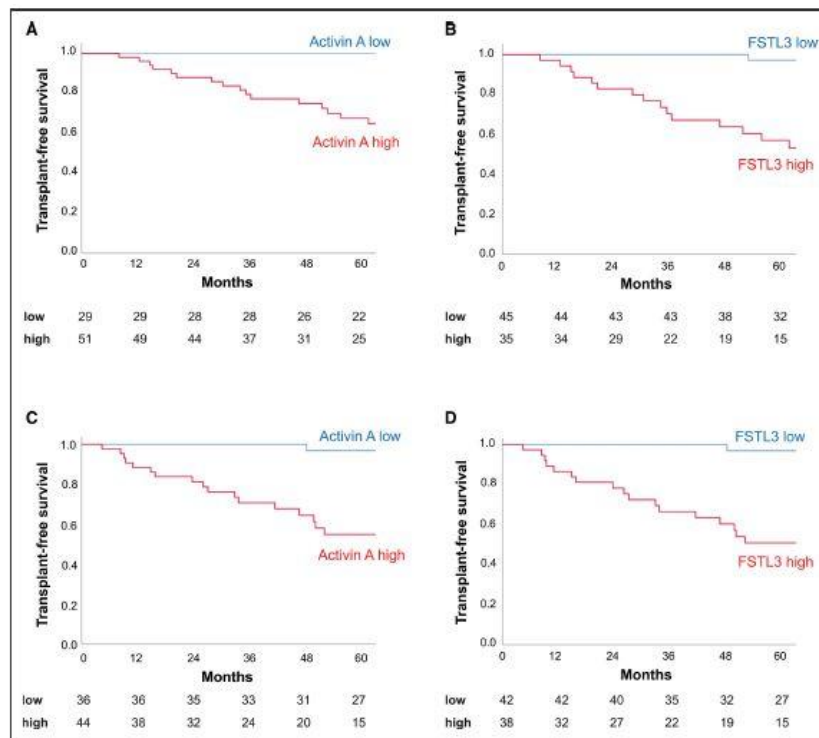
Thresholds determined by time-dependent receiver operating characteristic curves: 393 pg/mL for activin A and 16.6 ng/mL for FSTL3. EFORT indicates Evaluation of Prognostic Factors and Treatment Goals in PAH.

\*Significant result.

### **Pulmonary Vascular Expression of the Activin Subunits (Inhibin- $\beta$ A and Inhibin- $\beta$ B) in PAH**

To determine whether pulmonary vascular remodeling is associated with changes in the expression of activin ligands, confocal microscopic analyses and triple labeling of either inhibin $\alpha$ ,  $\beta$ A, or  $\beta$ B subunits with von Willebrand factor and  $\alpha$ -smooth muscle actin were used to investigate the activin expression patterns in lung specimens from 4 patients with PAH and 4 controls (Table S1). We found strong staining for  $\beta$ A- and  $\beta$ B-subunits (Figure 3A, Figure S4A and S4B, and Table S5) and a weak staining of  $\alpha$ -subunit in paraffin-embedded lungs of patients with PAH compared with controls (Figure 3B, Figure S4C, and Table S5). More specifically, we found increased inhibin-  $\beta$ A immunogenicity within the endothelium of distal pulmonary arteries from patients with PAH compared with weak staining of the endothelium of control specimens (fluorescence mean intensity [FMI]:  $12.7 \pm 1.1$  versus  $8.3 \pm 0.7$ , respectively,  $P < 0.01$ ; Figure 3A). Similar differential staining was observed for inhibin- $\beta$ B (FMI:  $43.4 \pm 19.3$  versus  $19.3 \pm 3.6$ , respectively,  $P = 0.006$ ), but we also noted a strong signal for inhibin- $\beta$ B within the smooth muscle layer in PAH-remodeled vessels relative to the smooth muscle of control specimens (FMI:  $19.2 \pm 3.6$  versus  $8.0 \pm 0.7$ , respectively,  $P = 0.023$ ; Figure 3A). In addition, alveolar macrophages displayed strong immunogenicity for the 2 activin subunits inhibin- $\beta$ A and inhibin- $\beta$ B (Figure 3A). These results support that both activin subunits inhibin- $\beta$ A and inhibin- $\beta$ B are overexpressed in the remodeled pulmonary arterial walls, with inhibin- $\beta$ A expressed mainly by the

dysfunctional pulmonary endothelium and inhibin- $\beta$ B by both the vascular endothelial and smooth muscle layers.

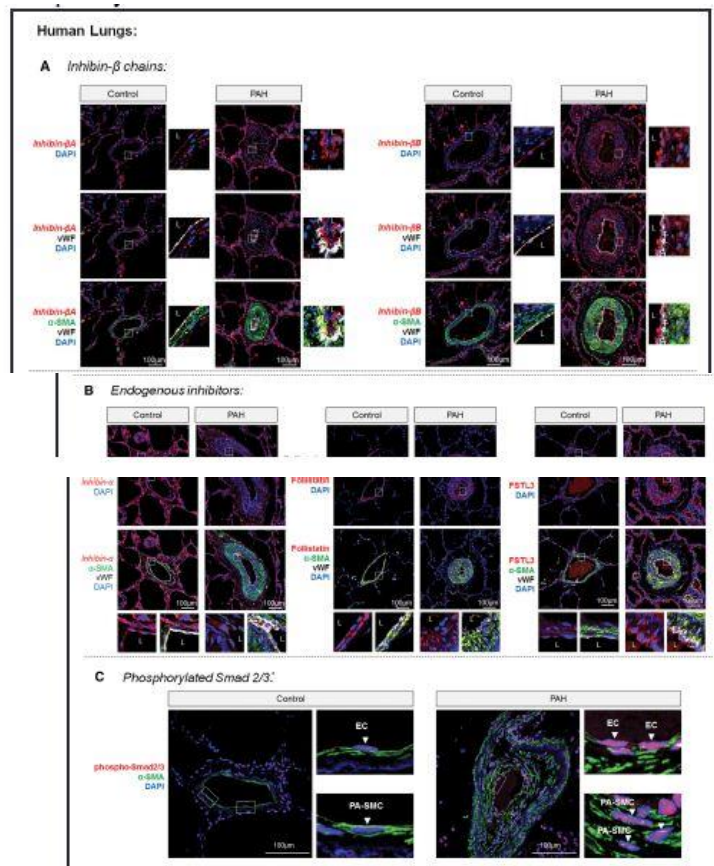


**Figure 2.** Kaplan-Meier survival curves according to levels of activin A and FSTL3 at the diagnosis (A, B) and at the follow-up (C, D). Thresholds determined by receiver operating characteristic curves: 440 pg/mL for activin A and 16.2 ng/mL for FSTL3. Log rank  $P < 0.001$  for both analyses. FSTL3 indicates follistatin-like 3.

## Pulmonary Vascular Expression of Activin Receptors and Smad2/3 Phosphorylation in PAH

Activin binding to ACTRIIA or ACTRIIB is followed by recruitment, phosphorylation, and activation of the type I receptors to initiate signaling through intracellular Smad2/3 proteins. To study the expression of activin type-I, and type-II receptors on pulmonary vascular cells, subsequent studies were performed using confocal microscopic analyses and triple labeling of either ACTRIIA, ACTRIIB, ALK2, ALK4, ALK5, and ALK7, with CD31 or von Willebrand factor and  $\alpha$ -smooth muscle actin (Table S2 and Figure S5) and lung specimens from 4 patients with PAH and 4 controls. In control samples, low expression of ACTRIIA and ACTRIIB, ALK2, and ALK7 was observed in pulmonary vessels. Furthermore, ALK4 and ALK5 were expressed by the endothelial and smooth muscle layers in control pulmonary vessels (Table S5 and Figure S5). In contrast, explanted PAH lung specimens showed strong staining for activin type-I and type-II receptors. More specifically, we found that ACTRIIB, ALK4, and ALK5 were highly expressed in the endothelium and smooth muscle of muscularized pulmonary arterioles in PAH and that ALK2 and ALK7 were highly expressed in the endothelium of muscularized pulmonary arterioles (FMI:  $68.4 \pm 14.4$  versus  $19.8 \pm 2.9$ ,  $P = 0.016$ ; FMI:  $42.4 \pm 6.4$  versus  $1.7 \pm 0.7$ ,  $P = 0.0007$ , respectively; Figure S5 and Table S5). To assess the nuclear abundance of the phosphorylated form of Smad2/3 (phospho-Smad2/3) in pulmonary vascular cells, phospho-Smad2/3 immunofluorescent staining was next performed in human PAH and control lungs. Consistent with our previous findings, our confocal

microscopic analyses demonstrated a nuclear accumulation of phospho-Smad2/3 within PAH-remodeled arterial walls as reflected by a strong nuclear staining for phospho-Smad2/3 in both the vascular endothelial and smooth muscle layer relative to controls (FMI:  $101.8 \pm 6.4$  versus  $16.7 \pm 3.9$ ,  $P < 0.0001$ ; FMI:  $66.5 \pm 6.0$  versus  $21.2 \pm 1.3$ ,  $P = 0.0003$ , respectively; [Figure 3C](#) and [Table S5](#)).



**Figure 3.** Expression patterns of key members of the activin signaling in lungs of controls and patients with PAH. Representative photomicrographs of distal pulmonary arteries showing overexpression of inhibin-βA and inhibin-βB (red color; **A**), downregulation of the α-subunit of inhibin A and B proteins with a concomitant overexpression of the two antagonists follistatin and follistatin like-3 (FSTL3; red color; **B**), and nuclear accumulation of phospho-Smad2/3 (red color; **C**). Pulmonary artery smooth muscle cells (PA-SMCs) and endothelial cells (ECs) were visualized with an anti-smooth muscle α-actin (α-SMA) antibody (green color) and an anti-von Willebrand Factor (vWF) antibody (white color), respectively. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole; blue color). L indicates lumen; and PAH, pulmonary arterial hypertension.

To validate these observations, subsequent studies analyzed the expression patterns of the 2 known target genes downstream of Smad2/3, named connective tissue growth factor and PAI-1. Consistent with an over-activation of Smad2/3 in PAH, we found an increase in the staining for connective tissue growth factor and PAI-1 in the lungs of patients with PAH relative to controls ([Figure S6](#) and [Table S5](#)).

In conclusion, these findings obtained in explanted lungs from patients with PAH revealed increased expression of activin type-I, and type-II receptors in vascular cells of remodeled pulmonary arterioles in PAH.

## Pulmonary Vascular Expression of Activin Regulators in PAH-Remodeled Pulmonary Vessels

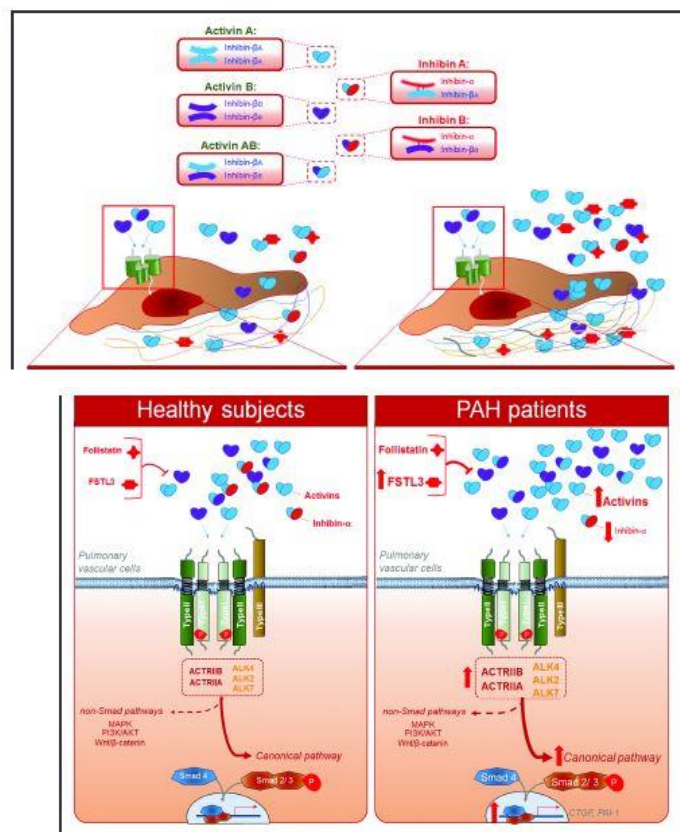
Negative regulators of activin signals include the 2 activin-binding and neutralization proteins, follistatin and FSTL3,<sup>20,21</sup> and the cell membrane antagonistic coreceptors, betaglycan (TGF $\beta$ RIII)<sup>22</sup> and Cripto or BMP and activin membrane-bound inhibitor.<sup>23,24</sup>

To determine whether pulmonary vascular remodeling is associated with changes in follistatin or FSTL3 expression, immunofluorescent staining was next performed in human PAH and control lungs (Figure 3B and Table S5). Even if our confocal microscopic analyses revealed a small decrease in follistatin in the pulmonary endothelium and smooth muscle in PAH compared with control lungs (FMI: 8.2 $\pm$ 0.5 versus 23.3 $\pm$ 4.7,  $P=0.0193$ ; FMI: 30.6 $\pm$ 9.4 versus 89.2 $\pm$ 3.5,  $P<0.0011$ , respectively), a substantial increase in FSTL3 proteins was observed in PAH compared with controls (FMI: 69.4 $\pm$ 2.8 versus 10.4 $\pm$ 1.4,  $P<0.0001$ ; FMI: 51.0 $\pm$ 6.3 versus 16.4 $\pm$ 0.7,  $P=0.0016$ , respectively). Although no difference was observed in the level of betaglycan, a low staining for the pseudo-receptor BMP and activin membrane-bound inhibitor that sequesters activin was also noted in both the vascular endothelial and smooth muscle layer in PAH relative to controls (FMI: 16.0 $\pm$ 1.5 versus 25.6 $\pm$ 3.0,  $P=0.0272$ ; FMI: 6.5 $\pm$ 1.6 versus 26.0 $\pm$ 3.6,  $P=0.0027$ , respectively; Figure 3B and Table S5). In addition, our confocal microscopic also revealed a strong expression of Cripto that blocks the recruitment of type-I receptors in vascular endothelial cells in PAH (FMI: 75.9 $\pm$ 5.5 versus 16.9 $\pm$ 3.7,  $P<0.0001$ , respectively; Figure S5 and Table S5).

Taken altogether, these findings suggest that activin–Smad2/3 signaling is overactive in PAH and accompanied by alterations in the abundance of several activin antagonistic regulators not only in the blood, but also within remodeled pulmonary arterial walls. Figure 4 shows a summary of our results.

## Discussion

A better understanding of dysregulated biological pathways involved in the exaggerated accumulation of vascular cells in the pulmonary arterial wall is needed to develop innovative PAH therapies. In this study, we present the first comprehensive analysis of protein expression profiling of the activin signaling system in the serum and explanted lungs of patients with PAH. We provide evidence that activin–Smad2/3 signaling is overactive in human PAH and that elevated levels of activin A and FSTL3 levels in serum predict death or lung transplantation in a discovery cohort of patients with PAH. This finding was confirmed in an independent external PAH cohort, suggesting that both activin A and FSTL3 could serve as prognostic biomarkers in PAH. We also provide evidence showing that the activin signaling pathway is overactive in lungs of patients with PAH.



**Figure 4.** The activin signaling pathway is overactive in PAH. The activins are members of the transforming growth factor- $\beta$  superfamily, which are composed of disulfide linked dimers of  $\beta$ -subunits (inhibin- $\beta$ A and inhibin- $\beta$ B) that can also dimerize with an  $\alpha$ -subunit to form inhibin A (inhibin- $\alpha$ -inhibin- $\beta$ A) or inhibin B (inhibin- $\alpha$ -inhibin- $\beta$ B). Consequently, 3 forms of activin have been described, activin A, activin B, and activin AB. Propeptides (pro- $\beta$ A and - $\beta$ B) interact extracellularly with several heparan sulfate proteoglycans through their pro-regions, thereby protecting them from proteolysis and regulating their localization within tissues. Activins signal through complexes formed with 2 type II (ACTRIIB >ACTRIIA) and 2 type I (ALK4 >ALK2 >ALK7) serine/threonine kinase receptors to activate the canonical Smad2/3 pathway and other noncanonical pathways, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling, the mitogen-activated protein kinases (MAPK) pathway including extracellular signal-regulated kinase 1/2 (ERK 1/2), p38, and c-Jun N-terminal kinases and the Wnt/ $\beta$ -catenin signaling pathway. Follistatin and follistatin like-3 (FSTL3) are 2 endogenous neutralizing proteins that neutralize activins and myostatin (also known as GDF8). Our data indicate that PAH pulmonary vascular cells overexpress the 2 activin subunits inhibin- $\beta$ A and inhibin- $\beta$ B, and the activin type I (ALK2, ALK4, ALK5, and ALK7) and type II receptors (ACTRIIA and ACTRIIB). In addition, we found downregulation of the  $\alpha$ -subunit of inhibin A and B proteins. No significant change was observed in the expression of the type III receptor betaglycan.

Recently completed randomized controlled trials have shown that, trapping activin A, activin B, GDF-11, and GDF-8 with sotatercept, a fusion protein composed of the extracellular domain of the human ActRIIA fused to the Fc domain of human IgG1 represents a promising treatment option in PAH. The phase 2 randomized controlled trial PULSAR (A Study of Sotatercept for the Treatment of Pulmonary Arterial Hypertension [PAH]) met its primary end point of a reduction in pulmonary vascular resistance, supporting the concept that targeting the activin pathway has the potential to serve as an add-on therapy in PAH.<sup>12,15</sup> The phase 3

STELLAR study (A Study of Sotatercept for the Treatment of Pulmonary Arterial Hypertension [MK-7962-003/A011-11]; NCT04576988) also met its primary end point of 6MWD improvement.<sup>16</sup> Herein, our data highlight that activin A, in addition to being a treatment target, can also be regarded as a PAH biomarker.

Our data show that serum levels of activin A and FSTL3 outperformed risk stratification tools in 2 independent cohorts of patient with PAH. Validated risk stratification instruments combine clinical, exercise, biomarker, imaging, and hemodynamic variables to obtain a multidimensional estimate of prognosis.<sup>25–30</sup> The most recent updates of these methods underscore the value of simplified approaches using noninvasive measures to predict survival during follow-up, including 6MWD, NYHA-FC, and BNP or NT-proBNP.<sup>31–33</sup> Of note, these biomarkers are closely linked to right ventricular dysfunction.<sup>32,33</sup> Likewise, our data show that elevated serum levels of activin are associated with higher right atrial pressure and BNP or NT-proBNP and lower 6MWD. Other biomarkers have been individually identified as prognostic factors, such as GDF-15,<sup>34</sup> red blood cell distribution width,<sup>35</sup> uric acid,<sup>36</sup> creatinine,<sup>37</sup> IL-6, IL-8, IL-10,<sup>38</sup>  $\beta$ -NGF,<sup>39</sup> CXCL9,<sup>39</sup> and TRAIL.<sup>39</sup> More recently, -omic studies have markedly enlarged the panel of biomarkers in PAH.<sup>40–42</sup> However, none has been validated to date as a therapeutic target in clinical practice.

Serum activin A levels were identified in our study as a biomarker predictive of the survival of patients with PAH. The prognostic value of serum activin A levels remains strong and independent after adjustment for other noninvasive variables included in risk stratification tools (including 6MWD, NYHA-FC, and BNP or NT-proBNP). Of note, FSTL3, a counter-regulatory protein in this pathway, was also identified as a prognostic factor at both baseline and follow-up. However, a positive correlation between the abundance of activin A and FSTL3 was noted in our PAH serum samples that might be explained in part by a feedback loop upregulating follistatin in response to activin A signals.<sup>43</sup>

This translational study is the first to provide a comprehensive analysis of the expression and prognosis value of key members of the activin/inhibin family in 2 independent cohorts of patients with PAH from the time of diagnosis and 3 to 4 months after initiation of PAH targeted therapy.

However, a future large validation study is still needed to confirm the clinical utility of these 2 biomarkers to predict the response to currently approved PAH-specific therapies. Future studies are also needed to study whether these observations can be extended to other forms of PAH and pulmonary hypertension and to determine whether patients with PAH who have high levels of activin A or FSTL3 respond better than others to drugs that target the activin signaling pathway.

Pulmonary vascular remodeling in PAH was associated with changes in the expression of the different activin receptors and their inhibitory regulators in the serum and lung tissues of patients with PAH. Our data indicate that explanted PAH lung specimens displayed strong staining for the activin type-II receptor ACTRIIB, and for the activin type-I receptors ALK2, ALK4, ALK5, and ALK7, as well. In accordance, there was a significant increase in the staining of phosphorylated Smad2/3 levels in vascular endothelial and smooth muscle cells within remodeled pulmonary arterial walls in PAH lungs. Decreased circulating levels of inhibins in patients with newly diagnosed PAH compared with healthy controls and PAH-treated patients is of interest, because these proteins have the ability to compete with activin and to block its action through an interaction with betaglycan and ACTRII. Some patients with PAH exhibited very low levels of inhibin A and B, but without substantial changes in betaglycan protein levels. The loss of the



inhibin/betaglycan regulatory mechanism could, at least in part, contribute to the increase in activin signaling in PAH.

Because betaglycan is also known to bind tissue growth factor- $\beta$  isoforms and other BMP/GDF ligands, we cannot exclude a role for the betaglycan overexpression in the accumulation of pulmonary artery smooth muscle cells in PAH. In addition, we observed an overexpression of Cripto that is not only known to inhibit activin, but also to facilitate nodal signaling by forming receptor complexes with these ligands that are structurally and functionally similar.<sup>24</sup> Because we used explanted lung tissues from 4 patients with advanced PAH, more work is needed to validate that these alterations in the activin-Smad2/3 signaling are present in the early stage of the disease. Consistent with previous studies,<sup>11,13</sup> we found higher levels of activin A in the serum and lungs of rats with established pulmonary hypertension induced by chronic hypoxia, monocrotaline, and Sugen hypoxia relative to controls, respectively (Figure S7).

The mechanism of action of the activin signaling system in the maintenance of vascular integrity is complex. We have already reported that inhibin- $\beta$ A-specific endothelial activation (*VEcad-INHBA-Tg* mice) exacerbates chronic hypoxia-induced pulmonary hypertension, whereas conditional endothelial knockout (*INHBA-ECKO* mice) prevents its progression in mice.<sup>14</sup> We have previously shown mechanistically that activin A triggers the internalization and subsequent degradation of BMPR-II, a phenomenon that can explain in part why enhanced activin-Smad2/3 signal transduction is often accompanied by the loss or impaired BMP-Smad1/5/8 response.<sup>7,14</sup>

We were also able to demonstrate that primary cultures of pulmonary endothelial cells derived from patients with PAH displayed higher inhibin- $\beta$ A expression and produced more activin A than pulmonary endothelial cells isolated from control lungs.<sup>14</sup> There is still a gap in our knowledge on the underlying mechanisms behind activin over-activation in PAH and dysregulation of different negative regulators of activin signals. Activin A is a proinflammatory and profibrotic mediator, and it has been shown that ACTRIIA-Fc can suppress inflammation in rodent models.<sup>44</sup> We have measured higher circulating levels of IL-6,  $\beta$ -NGF, and CXCL9 in patients with levels of activin A higher than the threshold determined by the ROC analysis in the I cohort. Further studies focusing on the relationships between inflammation and the activin pathway should be prioritized.

In summary, our study provides the first extensive analysis of protein expression profiling of the activin signaling system in human PAH. Although further experiments are required to identify the exact mechanisms underlying the activin overexpression in pulmonary vascular cells, we clearly demonstrated that the activin signaling system is overactive and that its serum level and those of FSTL3 represent 2 prognostic biomarkers that remain strong and independent after adjustment for all other variables included in contemporary risk stratification approaches.

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