









ORIGINAL RESEARCH

Prognostic Significance of Proteomics-Discovered Circulating Inflammatory Biomarkers in Patients With Pulmonary Arterial Hypertension

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BACKGROUND: Pulmonary arterial hypertension (PAH) ultimately leads to right ventricular failure and premature death. The identification of circulating biomarkers with prognostic utility is considered a priority. As chronic inflammation is recognized as key pathogenic driver, we sought to identify inflammation-related circulating proteins that add incremental value to current risk stratification models for long-term survival in patients with PAH.

METHODS AND RESULTS: Plasma levels of 384 inflammatory proteins were measured with the proximity extension assay technology in patients with PAH (n=60) and controls with normal hemodynamics (n=28). Among these, 51 analytes were significantly overexpressed in the plasma of patients with PAH compared with controls. Cox proportional hazard analyses and C-statistics were performed to assess the prognostic value and the incremental prognostic value of differentially expressed proteins. A panel of 6 proteins (CRIM1 [cysteine rich transmembrane bone morphogenetic protein regulator 1], HGF [hepatocyte growth factor], FSTL3 [follistatin-like 3], PLAUR [plasminogen activator, urokinase receptor], CLSTN2 [calsyntenin 2], SPON1 [spondin 1]) were independently associated with death/lung transplantation at the time of PAH diagnosis after adjustment for the 2015 European Society of Cardiology/European Respiratory Society guidelines, the REVEAL (Registry to Evaluate Early and Long-Term PAH Disease Management) 2.0 risk scores, and the refined 4-strata risk assessment. CRIM1, PLAUR, FSTL3, and SPON1 showed incremental prognostic value on top of the predictive models. As determined by Western blot, FSTL3 and SPON1 were significantly upregulated in the right ventricle of patients with PAH and animal models (monocrotaline-injected and pulmonary artery banding-subjected rats).

CONCLUSIONS: In addition to revealing new actors likely involved in cardiopulmonary remodeling in PAH, our screening identified promising circulating biomarkers to improve risk prediction in PAH, which should be externally confirmed.

Key Words: biomarkers ■ inflammation ■ prognosis ■ proteomics ■ pulmonary hypertension

Pulmonary arterial hypertension (PAH) is a complex and multifactorial cardiopulmonary disorder characterized by sustained vasoconstriction and obliterative remodeling of small pulmonary arteries

(PA), leading to progressive elevation of pulmonary vascular resistance, right ventricular failure and premature death.¹ Despite advances in drug therapy that target the pulmonary vasoconstrictive component

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RESEARCH PERSPECTIVE

What Is New?

- Proteomic analysis identified several inflammatory-related proteins independently associated with death or lung transplantation at the time of pulmonary arterial hypertension diagnosis, with 4 (CRIM1 [cysteine rich transmembrane bone morphogenetic protein regulator 1], PLAUR [plasminogen activator, urokinase receptor], FSTL3 [follistatin-like 3], and SPON1 [spondin 1]) showing incremental prognostic value on top of the 2015 European Society of Cardiology/European Respiratory Society guidelines, the REVEAL (Registry to Evaluate Early and Long-Term PAH Disease Management) 2.0 risk scores, and the refined 4-strata risk assessment.
- FSTL3 and SPON1 were significantly upregulated in the right ventricle of patients with pulmonary arterial hypertension and pulmonary arterial hypertension animal models, suggesting that the stressed right ventricle is a source of these circulating proteins.

What Question Should Be Addressed Next?

- Further studies are needed to confirm the predictive value of these proteins and to clarify their function in the pathophysiology of the disease.

Nonstandard Abbreviations and Acronyms

CRIM1	cysteine rich transmembrane bone morphogenetic protein regulator 1
ESC/ERS	European Society of Cardiology/ European Respiratory Society
FSTL3	follistatin-like 3
PAB	pulmonary artery banding
PAH	pulmonary arterial hypertension
PLAUR	plasminogen activator, urokinase receptor
REVEAL	Registry to Evaluate Early and Long-Term PAH Disease Management
SPON1	spondin 1

of the disease, long-term prognosis for patients with PAH is still poor with a survival rate around 40% to 75% at 3 years^{1,2} due, at least in part, to nonspecific symptoms making diagnosis and severity assessment challenging, thus delaying initiation or up-titration of PAH-specific supportive therapies.

The development of clinically useful risk stratification models is an essential step to assess disease severity

and progression, as well as to modulate appropriate proactive interventions. To date, the US REVEAL (Registry to Evaluate Early and Long-Term PAH Disease Management) risk score³ and the European Society of Cardiology/European Respiratory Society (ESC/ERS) guidelines risk assessment table⁴ are most often used to guide treatment decisions for patients with PAH with various causes. These models integrate patient demographics, exercise capacity, hemodynamics, and natriuretic peptides to establish risk scores predicting prognosis. Although these existing tools are of recognized value, they suffer from the invasiveness of right heart catheterization. Moreover, most patients are categorized at intermediate risk, limiting their discriminative properties for a significant proportion of patients. To provide a more nuanced assessment of patients' prognosis, a refined noninvasive 4-strata risk stratification model was recently proposed⁵ and externally validated⁶ to prognosticate patients with PAH, modestly increasing the discrimination of the model. Thus, refinements in risk stratification are mandatory and incorporation of new predictors of disease severity is needed. Because circulating biomarkers allow longitudinal monitoring, the identification of new circulating biomarker that add incremental benefit to already existing clinical predictors is considered a key priority.^{7,8}

Although the exact pathophysiology underlying lung and right ventricular adverse remodeling remains unclear, chronic inflammation and altered immune processes are recognized as key pathogenic drivers of PAH.⁹ Indeed, perivascular inflammatory infiltrates and associated release of inflammatory mediators are prominent features of PAH lungs promoting adventitial fibroblast activation, PA smooth muscle cell proliferation, and endothelial-mesenchymal transition,⁹ which together establish a self-perpetuating inflammatory loop and contribute to the progressive narrowing of the PA lumen. In addition, persistent inflammation is also intimately linked to cardiomyocyte death and fibrosis that adversely affect right ventricular function.¹⁰

In view of the importance of inflammation in PAH initiation and progression, we hypothesized that changes in circulating inflammatory factors levels may provide insights into the prognosis of well-characterized patients with idiopathic or connective tissue disease-associated PAH. Using proximity extension assay, we examined the plasma levels and discriminative properties of 384 immune-related proteins as biomarkers for the assessment of the severity and prognosis of the disease.

METHODS

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

Human Lung and Right Ventricle Tissue Acquisition

All experimental procedures using human tissues or plasma were performed in accordance with the principles of the Declaration of Helsinki, as well as the approval of the Institut Universitaire de Cardiologie et Pneumologie de Québec-University of Laval Ethics Committee (CER-20735 and CER-20773). Written informed consent was obtained from all study subjects.

Tissues were obtained from patients who had previously given signed informed consent. PAH and control tissues were obtained from Respiratory Health Network tissue bank (www.rsr-qc.ca). Healthy lung tissues (controls) were obtained during lung resection for tumors. Lung samples were taken at distance from the tumor and demonstrated normal lung parenchyma. PAH tissues were from lung explants or warm autopsies. PAH diagnosis was previously confirmed by right heart catheterization. The patients' characteristics are described in [Tables S1](#) and [S2](#).

Proteomic Analysis in Human Plasma Samples

Study Population

Sixty patients with idiopathic PAH or PAH associated with connective tissue disease undergoing right heart catheterization at the Institut Universitaire de Cardiologie et Pneumologie de Québec in Canada were enrolled from June 2012 to March 2019. Diagnostic criteria for PAH included mean PA pressure ≥ 25 mmHg with PA wedge pressure ≤ 15 mmHg and pulmonary vascular resistance > 3 Wood units, together with the exclusion of known associated diseases, according to contemporary guidelines.¹¹ More specifically, exclusion criteria included patients with left ventricular ejection fraction $< 40\%$, restrictive (lung fibrosis on computed tomography or total lung capacity $< 80\%$ predicted) or obstructive (forced expiratory volume in 1 s/forced vital capacity $< 70\%$) lung disease, perfusion defects on lung scintigraphy, or active or recent (< 5 years) cancer. Patients were diagnosed and treated according to the guidelines.⁴ Patients with PAH were categorized as compensated right ventricle (RV, defined as cardiac index > 2.2 L/min per m^2) versus decompensated RV (defined as cardiac index ≤ 2.2 L/min per m^2). Similarly, 28 subjects referred for right heart catheterization resulting in normal pulmonary hemodynamics and the absence of cardiac or respiratory diseases were enrolled as a control group.

Blood samples were obtained from each subject in EDTA tubes during right heart catheterization using the central venous line. The blood samples were immediately centrifuged to obtain plasma samples and kept frozen at -80°C until proteomics assay. Baseline

clinical information was obtained from the clinical database (age, sex, PAH type, World Health Organization functional class, exercise capacity, hemodynamics, NT-proBNP [N-terminal fragment of brain natriuretic peptide], estimated glomerular filtration rate, and treatment). Estimated glomerular filtration rate was calculated by Modification of Diet in Renal Disease formula.¹²

Plasma of the cohort was screened using proximity extension, a high-throughput multiplex proteomic immunoassay, for changes in the expression of 384 immune-related proteins, primarily cytokines and chemokines (Olink Explore Inflammation Panel, Olink Proteomics, Uppsala, Sweden). The assay uses epitope-specific binding and hybridization of a set of paired oligonucleotide antibody probes, which is subsequently amplified and detected by next generation sequencing, resulting in log base-2 normalized protein expression values. Technicians from Olink were blinded to patient status. This study complied with Strengthening the Reporting of Observational Studies in Epidemiology guidelines.¹³

Animal Models of PAH and RV Remodeling

All animal protocols were approved by the Institut Universitaire de Cardiologie et Pneumologie de Québec-University of Laval Biosafety and Ethics Committees (#2022-1049 and 2023-1317). RV tissues from monocrotaline-injected and pulmonary artery banding (PAB)-operated rats used in our previous publications were de novo examined.^{14,15} Proteins were extracted from human and rodent RVs and PAs using a 2% Chaps lysis buffer supplemented with a protease inhibitor cocktail (Roche). Protein concentrations were determined by the Bradford method. Equal amounts of proteins (15 μg) were separated by electrophoresis in SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were subsequently blocked in 5% nonfat dry milk in TBS-T buffer for 1 hour. The membranes were incubated with a rabbit polyclonal anti-SPON1 (spondin 1) antibody (#PA5-116176, ThermoFisher Scientific) or a rabbit polyclonal anti-FSTL3 (follistatin-like 3) antibody (#PA1915, Boster Biological Technology) overnight at 4°C . After being rinsed 3 times with TBS-T buffer, membranes were incubated with goat-anti-rabbit antibody conjugated to horseradish peroxidase for 2 hours at room temperature in 5% nonfat dry milk in TBS-T buffer. Immunolabeling was detected using ECL reagents (Perkin-Elmer) with the imaging Chemidoc MP system (Bio-Rad Laboratories). Protein expression was quantified using the Image lab software (Bio-Rad Laboratories) and normalized to Amido black as previously described.^{14,15}

Statistical Analysis

Normally distributed variables, nonnormally distributed variables, and categorical variables were shown as mean±SD, median (interquartile range), and counts and percentages, respectively. Normally distributed variables were compared by the Student's *t* test, and nonnormally distributed variables were compared by the Mann–Whitney *U* test. For more than 2 groups, the 1-way ANOVA for normally distributed variables or the Kruskal–Wallis test for nonnormally distributed variables was performed for comparison. Categorical variables were compared by the chi-square test or the Fisher's exact test. The relationship between circulating protein levels and hemodynamics (pulmonary vascular resistance, cardiac index, and stroke volume), NT-proBNP, and estimated glomerular filtration rate were assessed using Pearson's correlation analysis.

We first compared the expression of the immune-related proteins between controls and patients with PAH and between controls and patients with PAH categorized as decompensated RV (cardiac index ≤ 2.2 L/min per m²). Proteins identified by Olink proteomic extension assay with an adjusted *P* value ≤ 0.01 (using Benjamini–Hochberg correction) and a fold change ≥ 1.4 were considered to be differentially expressed in the plasma of patients with PAH. We then performed univariate and multivariate Cox proportional hazard analyses for death or lung transplantation using differentially expressed proteins. Event-free survival was analyzed from sampling to death or lung transplantation. The cutoff date was September 30, 2021. No patients were lost to follow-up.

The multivariate models were adjusted for age, sex, and the 2015 ESC/ERS risk score (model 1), the REVEAL 2.0 risk score (model 2), and the refined 4-strata noninvasive risk assessment (model 3). The multivariate logistic regression analyses for the discrimination of patients with death or lung transplantation were used for calculation of the combined models. The C-statistics between models were compared by DeLong's test.¹⁶ Calibration was tested using the Hosmer–Lemeshow goodness-of-fit test for the logistic regression model.

As a complementary approach, receiver operating characteristics curves and time-dependent receiver operating characteristics curves of 1 and 3 years with area under the curve were used to assess the performance of the proteins found to be independently associated with long-term outcomes alone or in combination to validated risk stratification models to predict PAH transplant-free survival. The Kaplan–Meier analysis was used to estimate transplant-free survival rate and a log-rank test was used for comparisons between groups using the optimal cutoff point determined by the curve point nearest to the 100% of axis

of the ordinates on receiver operating characteristics curves for the survival curve.¹⁷

A significance level inferior to 5% ($P < 0.05$) was considered statistically significant, unless specifically stated. All statistical analyses were made with Graph Pad Prism version 9.0.0 (GraphPad Software, Inc., San Diego, CA), SPSS version 27 (IBM, Armonk, NY), or R version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Study Subjects

Baseline clinical characteristics of the cohort are shown in [Table 1](#). Patients with PAH were well matched to controls. As expected, patients with PAH had severe hemodynamic impairment, elevated NT-proBNP, and poor exercise capacity, whereas patients with connective tissue disease-PAH were older than patients with idiopathic PAH ([Table S3](#)).

Identification of Inflammatory Biomarkers for RV Function and Long-Term Outcomes

Out of the 384 proteins analyzed, 26 immune-related analytes were significantly overexpressed in plasma of patients with PAH compared with controls (adjusted $P < 0.01$ and fold change ≥ 1.4). Using the same thresholds, 49 proteins were significantly upregulated in patients with PAH with decompensated RV compared with controls, of which 24 were found to overlap ([Figure 1A](#) and [1B](#)). From the correlation and cluster analysis summarized in [Figure 1C](#), it emerges that, among the measured proteins, some of them strongly correlated with each other. The major cluster consisted of MGMT (O-6-methylguanine-DNA methyltransferase), IRAK1 (interleukin 1 receptor associated kinase 1), ATP5IF1 (ATP synthase inhibitory factor subunit 1), HEXIM1 (HEXIM P-TEFb complex subunit 1), LAP3 (leucine aminopeptidase 3), DFFA (DNA fragmentation factor subunit alpha), and GMMP (guanosine monophosphate reductase). Another independent secondary cluster of highly correlated proteins consisted of TGF- α (transforming growth factor alpha), FSTL3, PLAUR [plasminogen activator, urokinase receptor], ESM1 (endothelial cell specific molecule 1), CRIM1 (cysteine rich transmembrane bone morphogenetic protein regulator 1), SPON1, and CXCL17 (C-X-C motif chemokine ligand 17). The correlation between these proteins suggests potential coregulation and connection between them into pathologic processes.

During a median follow-up of 2.5 years (interquartile range, 0.9–4.3 years) after blood sample was drawn, 31 patients with PAH died and 2 underwent lung transplantation. Among the 51 analytes found to differ significantly between PAH and controls, 17 were significantly associated with death or lung transplantation

Table 1. Baseline Clinical Characteristics of Control Patients and Patients With PAH

	Control (n=28)	PAH (n=60)	P value
Female sex, n (%)	18 (64)	39 (65)	0.948
Age, y	61±16	65±14	0.297
World Health Organization functional class, n (%)			
I		0 (0)	
II		10 (17)	
III		41 (68)	
IV		9 (15)	
Hemodynamics			
Mean pulmonary arterial pressure, mmHg	17±3	47±9	<0.001
Mean right atrium pressure, mmHg	5±4	8±4	0.005
Cardiac index, L/min per m ²	3.0±0.6	2.4±0.8	<0.001
Pulmonary vascular resistance, dynes·s·cm ⁻⁵	123±58	832±379	<0.001
Heart rate, bpm	71±13	81±11	0.002
Mixed venous oxygen blood saturation, %	72±8	63±9	<0.001
N-terminal pro-B-type natriuretic peptide, pg/mL*	155 (79–244)	1375 (369–4082)	<0.001
Estimated glomerular filtration rate (Modification of Diet in Renal Disease), mL/min per 1.73m ²	83±28	69±22	0.009
6-minute walking distance, m		306±121	
PAH medication categories, n (%)			
None	28 (100)	37 (62)	
Mono therapy	0 (0)	15 (25)	
Double therapy	0 (0)	7 (12)	
Triple therapy	0 (0)	1 (2)	
Specific PAH therapy at enrollment, n (%)			
Endothelin receptor antagonist	0 (0)	10 (17)	
Phosphodiesterase type 5inhibitor	0 (0)	19 (32)	
Epoprostenol	0 (0)	3 (5)	

Data are expressed by mean±SEM, median (interquartile range)*, or n (%). PAH indicates pulmonary arterial hypertension.

in patients with PAH using univariate Cox proportional hazards analyses (Table S4). Only 6 proteins, namely CRIM1 (also called cysteine-rich motor neuron 1), HGF (hepatocyte growth factor), CLSTN2 (calsyntenin 2), SPON1 (also called F-spondin), PLAUR, and FSTL3 remained independent prognostic factors after adjusting for the 2015 ESC/ERS, the REVEAL 2.0 risk scores, and the refined 4-strata risk assessment (Figure 2 and Table 2). Of interest, CRIM1, HGF, CLSTN2, and FSTL3

were also upregulated in patients with decompensated RV compared with compensated RV and were similarly overexpressed in idiopathic PAH and connective tissue disease-PAH (Figure 2). Moreover, all proteins correlated with mean pulmonary artery pressure, pulmonary vascular resistance, cardiac index, stroke volume, and NT-proBNP at baseline (Figure S1).

Incremental Predicted Performance of Identified Biomarkers and Stratification of Prognosis

In agreement with regression analyses, CRIM1, HGF, SPON1, PLAUR, and FSTL3 significantly discriminated patients with PAH with poorer transplant-free survival, with area under the curve >0.667 (Figure 3). Of note, these proteins had numerically better discriminative properties than NT-proBNP (area under the curve 0.630) (Figure S2). In addition, time-dependent receiver operating characteristics analyses of 1 and 3 years also showed that CRIM1, CLSTN2, FSTL3, SPON1, and PLAUR had significant discriminated capacity for transplant-free survival in patients with PAH (Table S5). We then evaluated the performance of adding these identified biomarkers to the REVEAL 2.0 and the 2015 ESC/ERS risk scores, as well as the refined 4-strata risk assessment for prognosis. Although all predicted transplant-free survival, CRIM1 and SPON1 consistently exhibited significant additional prognostic value on top of all 3 predictive models, as confirmed by improved C-statistics. (Figure 4 and Table S6). FSTL3 and PLAUR also displayed incremental prognostic value on top of the 2015 ESC/ERS and refined 4-strata predictive models (Figure 4 and Table S6).

Expression of SPON1 and FSTL3 in Lung and Right Ventricle From Patients With PAH and Animal Models

Our data showing that PLAUR, FSTL3, SPON1, and CRIM1 improve risk stratification when combined to different models led us to measure their protein expression levels in lungs and RVs from patients with PAH and animal models. Examination of comparative transcriptomic analysis of RVs in human and rat models (PAB and monocrotaline) revealed that PLAUR, FSTL3, and SPON1 but not CRIM1 were increased in decompensated RV.^{15,18} Unfortunately, no suitable anti-CRIM1 antibody was available and the immunoblot for PLAUR yielded inconclusive results due to the presence of multiple bands. Western blot analysis revealed significantly higher levels of SPON1 in the RVs of patients with PAH and in the RVs of monocrotaline- and PAB-challenged rats (Figure 5). No significant difference was observed in dissected PAs from control and patients with PAH (Figure 5). Similarly, protein levels

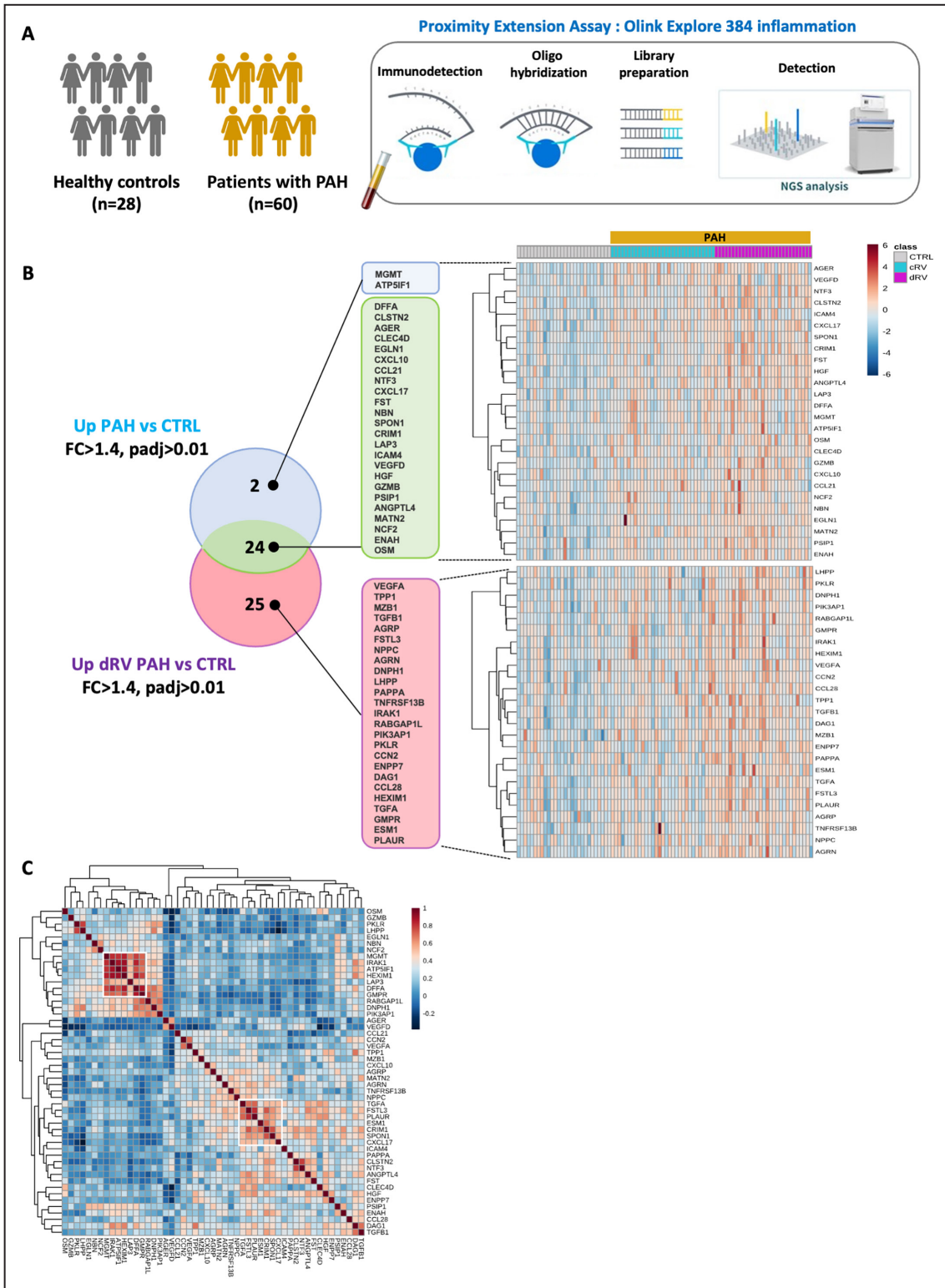


Figure 1. Increased plasma levels of inflammation-related proteins in patients with PAH. **A**, Schematic overview of the study design. **B**, Heatmap of the 51 plasma proteins found differentially expressed (fold change ≥ 1.4 and adjusted P value ≤ 0.01) between patients with PAH and controls. On top of the heatmap are control samples in gray, cRV PAH sample in blue, and patients with dRV PAH in purple. Color key indicates expression value: dark blue: lowest; dark red: highest. **C**, Heatmap of correlation matrix (Pearson) for the 51 circulating proteins significantly increased in patients with PAH (PAH vs CTRL and dRV PAH vs CTRL). Two major clusters of correlating proteins were identified. cRV indicates compensated right ventricle; CTRL, controls; dRV, decompensated right ventricle; FC, fold change; NGS, next-generation sequencing; and PAH, pulmonary arterial hypertension.

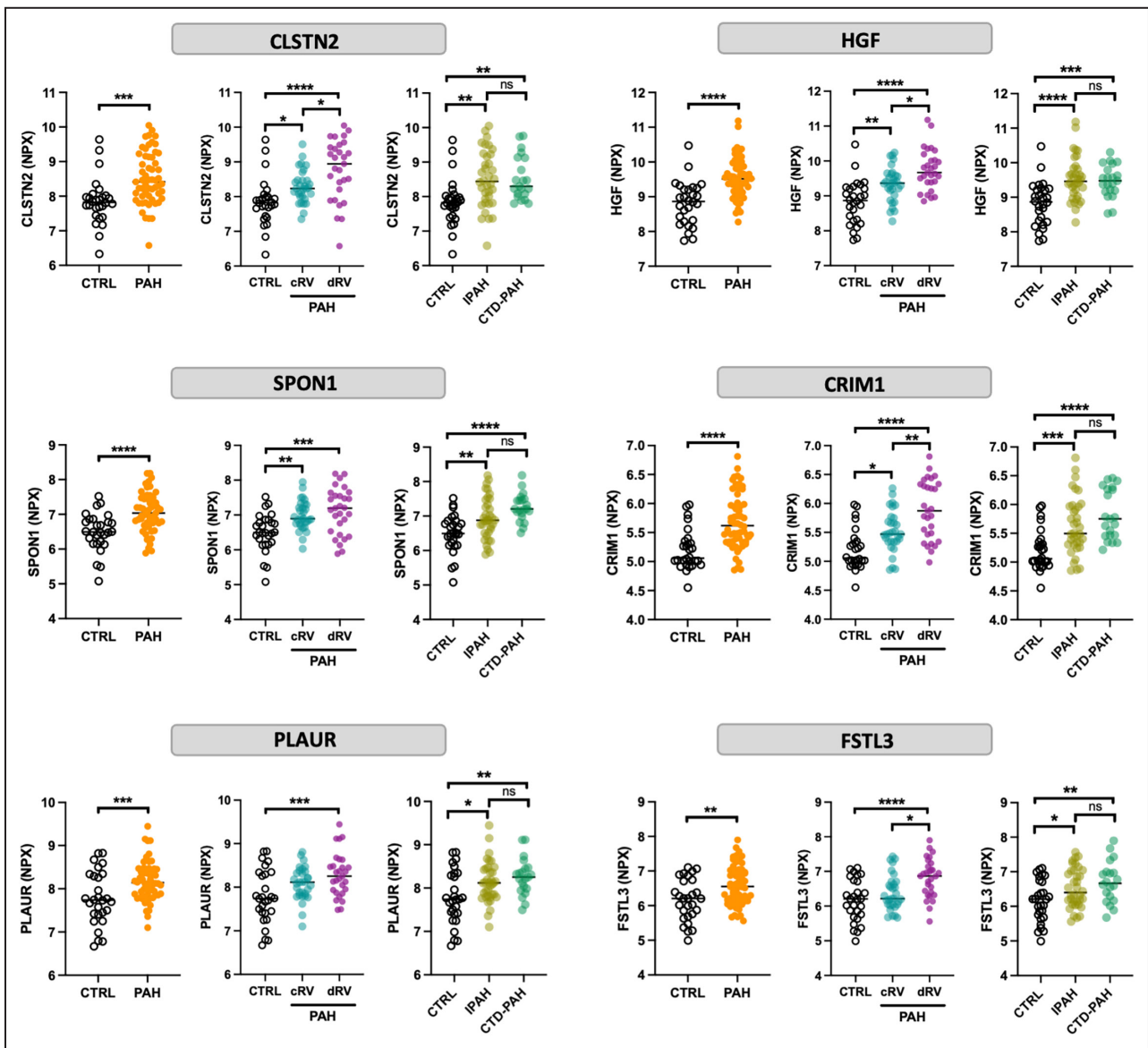


Figure 2. Expression levels (NPX) of CLSTN2, HGF, SPON1, CRIM1, PLAUR, and FSTL3 in plasma from CTRL and patients with PAH.

According to contemporary cardiac index measured by right heart catheterization at the time blood sample was drawn, patients with PAH were categorized as cRV (cardiac index $>2.2\text{L}/[\text{min}\cdot\text{m}^2]$) and dRV (cardiac index $\leq 2.2\text{L}/[\text{min}\cdot\text{m}^2]$). Statistical analyses were performed using the Student's *t* test or 1-way ANOVA followed by Tukey post hoc test. * $P < .05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. CLSTN2 indicates calyntenin 2; cRV, compensated right ventricle; CRIM1, cysteine rich transmembrane bone morphogenetic protein regulator 1; CTD-PAH, connective tissue disease-associated pulmonary arterial hypertension; CTRL, controls; dRV, decompensated right ventricle; FSTL3, follistatin-like 3; HGF, hepatocyte growth factor; IPAH, idiopathic pulmonary hypertension; NPX, normalized protein expression; PAH, pulmonary arterial hypertension; PLAUR, plasminogen activator, urokinase receptor; and SPON1, spondin 1.

of SPON1 within dissected PAs were unchanged between control and monocrotaline rats (Figure 5). For FSTL3, the protein levels were significantly increased in the RV of patients with PAH, whereas no change was observed in the pulmonary vasculature (Figure 6). FSTL3 protein levels were significantly increased in remodeled RVs from monocrotaline and PAB rats (Figure 6). Taken together, these results suggest that the stressed RV is a source of circulating SPON1 and FSTL3 in patients with PAH.

DISCUSSION

Over the past few years, the efforts to identify reliable noninvasive biomarkers to predict outcomes and aid clinical decision-making in PAH were intensified because of the development of high-throughput proteomic technologies.^{15,19,20} The present study is a part of this initiative focusing on inflammation-related circulating factors due to their well-established implication in PAH progression.⁹ Although the nature of the analytes

Table 2. Multivariate Cox Proportional Hazard Analyses for Death or Lung Transplantation in Patients With PAH

Protein	Adjusted for age, sex, and 2015 ESC/ERS risk score			Adjusted for REVEAL 2.0 risk score			Adjusted for age, sex, the 4-strata noninvasive method		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
CRIM1 [†]	3.085	1.020–9.334	0.046	3.494	1.497–8.156	0.004	2.956	1.224–7.139	0.016
HGF [†]	2.368	1.033–5.428	0.042	2.137	1.028–4.444	0.042	2.370	1.092–5.147	0.029
MATN2	1.356	0.424–4.333	0.607	1.558	0.542–4.480	0.411	1.496	0.512–4.371	0.462
EGLN1	1.192	0.849–1.675	0.310	1.037	0.743–1.447	0.830	1.130	0.805–1.587	0.480
ANGPTL4	1.322	0.602–2.902	0.486	1.796	0.884–3.648	0.105	1.501	0.727–3.102	0.272
FST	1.242	0.488–3.160	0.649	1.547	0.662–3.616	0.313	1.211	0.482–3.047	0.684
DNPH1	1.130	0.523–2.444	0.756	0.981	0.488–1.971	0.957	1.021	0.474–2.201	0.958
AGER	0.989	0.583–1.678	0.968	1.382	0.881–2.166	0.159	1.055	0.629–1.769	0.839
DFFA	1.462	0.733–2.765	0.243	1.208	0.650–2.243	0.550	1.410	0.758–2.623	0.278
RABGAP1L	0.786	0.386–1.604	0.509	0.646	0.324–1.288	0.215	0.813	0.396–1.669	0.573
TGF- α	2.201	0.857–5.653	0.101	2.238	0.805–6.226	0.123	1.680	0.610–4.626	0.315
CLEC4D	1.122	0.655–1.922	0.674	1.056	0.628–1.775	0.837	1.150	0.684–1.935	0.598
CLSTN2 [†]	2.056	1.022–4.135	0.043	2.059	1.142–3.711	0.016	2.033	1.126–3.670	0.019
PKLR	0.520	0.233–1.159	0.110	0.380	0.173–0.835	0.016	0.507	0.233–1.102	0.087
NBN	0.949	0.395–2.279	0.907	1.327	0.629–2.799	0.458	0.932	0.386–2.248	0.875
NTF3	1.342	0.865–2.081	0.189	1.220	0.833–1.785	0.307	1.219	0.799–1.858	0.358
ENPP7	1.260	0.914–1.736	0.158	1.228	0.881–1.711	0.225	1.266	0.918–1.746	0.150
DAG1	3.070	0.944–9.984	0.062	2.611	1.036–6.582	0.042	2.957	0.933–9.370	0.065
FSTL3 [†]	2.845	1.352–5.988	0.006	2.918	1.419–6.002	0.004	2.213	1.099–4.458	0.026
TGF- β 1	1.236	0.424–3.601	0.698	1.562	0.587–4.156	0.372	1.500	0.553–4.067	0.425
TPP1	2.373	0.899–6.267	0.081	1.692	0.726–3.944	0.223	2.293	0.884–5.948	0.088
CCL28	1.167	0.543–2.511	0.692	1.215	0.561–2.632	0.622	1.283	0.598–2.752	0.522
OSM	1.089	0.748–1.587	0.656	0.850	0.578–1.250	0.409	0.982	0.648–1.488	0.933
AGRN	0.807	0.399–1.630	0.549	1.296	0.766–2.193	0.334	0.994	0.527–1.876	0.985
PSIP1	1.505	0.738–3.070	0.261	1.396	0.722–2.701	0.322	1.512	0.755–3.027	0.243
TNFRSF13B	1.530	0.809–2.896	0.191	1.729	0.909–3.288	0.095	1.302	0.643–2.635	0.463
PAPPA	1.436	0.695–2.967	0.328	1.532	0.767–3.060	0.227	1.323	0.654–2.680	0.436
CXCL10	0.873	0.554–1.378	0.561	0.976	0.665–1.432	0.901	0.960	0.627–1.471	0.851
SPON1 [†]	3.129	1.268–7.722	0.013	3.675	1.716–7.869	0.001	2.882	1.172–7.086	0.021
VEGFD	1.156	0.595–2.246	0.668	1.750	0.936–3.274	0.080	1.326	0.670–2.627	0.418
CXCL17	1.458	0.637–3.338	0.373	2.041	1.096–3.798	0.024	1.583	0.701–3.577	0.269
NCF2	1.381	0.819–2.328	0.226	1.361	0.833–2.223	0.219	1.362	0.792–2.344	0.264
CCN2	0.512	0.225–1.165	0.110	0.651	0.325–1.304	0.226	0.518	0.235–1.142	0.103
HEXIM1	1.354	0.896–2.044	0.150	1.242	0.822–1.877	0.303	1.370	0.909–2.066	0.133
IRAK1	1.118	0.626–1.998	0.706	1.095	0.649–1.848	0.733	1.113	0.623–1.990	0.718
ESM1	1.617	0.866–3.021	0.132	1.978	1.096–3.572	0.024	1.501	0.800–2.816	0.206
GZMB	0.829	0.496–1.387	0.475	0.782	0.498–1.227	0.284	0.718	0.418–1.234	0.230
CCL21	0.933	0.455–1.912	0.850	1.180	0.695–2.003	0.541	0.997	0.484–2.057	0.995
PIK3AP1	0.994	0.534–1.852	0.986	0.846	0.475–1.506	0.570	0.980	0.541–1.776	0.947
ENAH	1.561	0.662–3.677	0.309	1.667	0.854–3.253	0.134	1.335	0.658–2.712	0.424
LAP3	0.885	0.473–1.656	0.702	0.806	0.436–1.490	0.491	0.883	0.468–1.666	0.701
GMPR	1.325	0.746–2.355	0.337	1.031	0.587–1.810	0.916	1.321	0.747–2.335	0.338
PLAUR [†]	3.780	1.310–10.903	0.014	4.301	1.496–12.362	0.007	2.780	1.022–7.565	0.045
AGRP	1.350	0.716–2.548	0.354	1.300	0.722–2.340	0.382	1.419	0.778–2.589	0.254
NPPC	1.151	0.609–2.177	0.665	1.251	0.712–2.199	0.435	0.988	0.533–1.831	0.970

(Continued)

Table 2. Continued

Protein	Adjusted for age, sex, and 2015 ESC/ERS risk score			Adjusted for REVEAL 2.0 risk score			Adjusted for age, sex, the 4-strata noninvasive method		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
LHPP	0.769	0.402–1.469	0.426	0.531	0.275–1.025	0.059	0.605	0.290–1.261	0.180
ICAM4	1.128	0.688–1.850	0.633	1.148	0.714–1.845	0.569	0.992	0.594–1.657	0.977
VEGFA	0.699	0.360–1.358	0.291	0.684	0.376–1.245	0.214	0.633	0.320–1.249	0.187
MZB1	1.507	0.861–2.638	0.151	1.504	0.904–2.503	0.116	1.452	0.840–2.509	0.181
MGMT	1.100	0.850–1.425	0.469	1.072	0.827–1.390	0.600	1.087	0.837–1.413	0.530
ATP5IF1	1.141	0.831–1.566	0.416	1.073	0.809–1.423	0.623	1.162	0.852–1.586	0.343

AGER indicates advanced glycosylation end-product specific receptor; AGRN, agrin; AGRP, agouti related neuropeptide; ANGPTL4, angiopoietin like 4; ATP5IF1, ATP synthase inhibitory factor subunit 1; CCL21, C-C motif chemokine ligand 21; CCL28, C-C motif chemokine ligand 28; CCN2, cellular communication network factor 2; CLEC4D, C-type lectin domain family 4 member D; CLSTN2, calyntenin 2; CRIM1, cysteine rich transmembrane bone morphogenetic protein regulator 1; CXCL10, C-X-C motif chemokine ligand 10; CXCL17, C-X-C motif chemokine ligand 17; DAG1, dystroglycan 1; DFFA, DNA fragmentation factor subunit alpha; DNP1, 2'-deoxynucleoside 5'-phosphate N-hydrolase 1; EGLN1, Egl-9 family hypoxia inducible factor 1; ENAH, ENAH actin regulator; ENPP7, ectonucleotide pyrophosphatase/phosphodiesterase 7; ERS European Respiratory Society; ESC, European Society of Cardiology; ESM1, endothelial cell specific molecule 1; FST, follistatin; FSTL3, follistatin-like 3; GMPPR, guanosine monophosphate reductase; GZMB, granzyme B; HEXIM1, HEXIM P-TEFb complex subunit 1; HGF, hepatocyte growth factor; HR, hazard ratio; ICAM4, intercellular adhesion molecule 4; IRAK1, interleukin 1 receptor associated kinase 1; LAP3, leucine aminopeptidase 3; LHPP, phospholysine phosphohistidine inorganic pyrophosphate phosphatase; MATN2, matrilin-2; MGMT, O-6-methylguanine-DNA methyltransferase; MZB1, marginal zone B and B1 cell specific protein; NBN, nibrin; NCF2, neutrophil cytosolic factor 2; NPPC, natriuretic peptide C; NTF3, neurotrophin 3; OSM, oncostatin M; PAH, pulmonary arterial hypertension PAPP, pappalysin 1; PIK3AP1, phosphoinositide-3-kinase adaptor protein 1; PKLR, pyruvate kinase L/R; PLAUR, plasminogen activator, urokinase receptor; PSIP1, PC4 and SRSF1 interacting protein 1; RABGAP1L, RAB GTPase activating protein 1 like; SPON1, spondin 1; TGF- α , transforming growth factor alpha; TGF- β 1, transforming growth factor beta 1; TNFRSF13B, tumor necrosis factor receptor superfamily member 13B; TPP1, tripeptidyl peptidase 1; VEGFA, vascular endothelial growth factor A; and VEGFD, vascular endothelial growth factor D.

*Rows identify biomarkers that remain independent prognostic factors after adjusting for the 2015 ESC/ERS, the REVEAL 2.0 risk scores, and the refined 4-strata risk assessment.

tested (ie, inflammation/immune-related factors) makes them unlikely to be specific for PAH, we revealed using proximity extension assay that HGF, SPON1, CRIM1, PLAUR, CLSTN2, and FSTL3 are predictors of death or transplantation. We also demonstrated that SPON1, CRIM1, PLAUR, and FSTL3 add performance to current risk stratification tools.

Among the 384 inflammation-related proteins tested, we identified CRIM1, SPON1, and PLAUR as new plasma proteins closely related to the severity of the disease. CRIM1 is a type I transmembrane glycoprotein bearing an N-terminal IGF1R (insulin-like growth factor-binding protein motif) and 6 cysteine-rich von Willebrand factor C repeats located in the extracellular domain and known to be subjected to a juxtamembrane cleavage that generates a secreted ectodomain.²¹ Although its biological function remains largely unknown, CRIM1 was shown to serve an important role in the development of multiple organs, including the heart, likely via regulating the bioavailability of growth factors and integrin signaling.^{22–24} Interestingly, CRIM1 was documented to be expressed in coronary vascular smooth muscle cells and coronary endothelial cells during cardiac development²⁴ and serum levels of CRIM1 were reported to positively correlate with the severity of left ventricular failure in human.²⁵ SPON1 is a secreted and extracellular matrix-attached protein of the thrombospondin family with obscure functions. Early work on this protein indicated that it stimulates vascular smooth muscle cell proliferation²⁶ and inhibits angiogenesis,²⁷ suggesting that increased

plasma levels in patients with PAH may contribute to RV ischemia. uPAR (urokinase plasminogen activator receptor), encoded by the PLAUR gene, is a component of the plasminogen activation system implicated in a variety of biological processes such as extracellular matrix remodeling, inflammation, and cell behavior.²⁸ Levels of soluble uPAR were repeatedly shown to exhibit predictive and prognostic value in patients with cardiovascular diseases²⁹ and kidney dysfunction.³⁰ Because uPAR regulates many biological processes involved in PAH progression, further studies are required to determine its precise function during maladaptive events and confirm its prognostic significance.

A major challenge to clinical implementation of new biomarkers to complement or improve current approaches for prognosis of PAH is the lack of replicability. In direct connection with this, we validated recent published data showing that HGF and FSTL3 are augmented in blood of patients with PAH and could serve as promising prognostic biomarkers.^{20,31–33} The ability to obtain similar results from different cohorts and using distinctive quantifications of protein concentration is a fundamental step for research credibility. Although FSTL3 was documented to be upregulated in remodeled pulmonary vessels of patients with PAH, we provided additional evidence that FSTL3 protein levels are increased in human PAH RVs. This finding coupled to the fact that FSTL3 is also upregulated in RVs from PAB rats suggests a combined cardiac and pulmonary origin of circulating FSTL3. FSTL3 is known

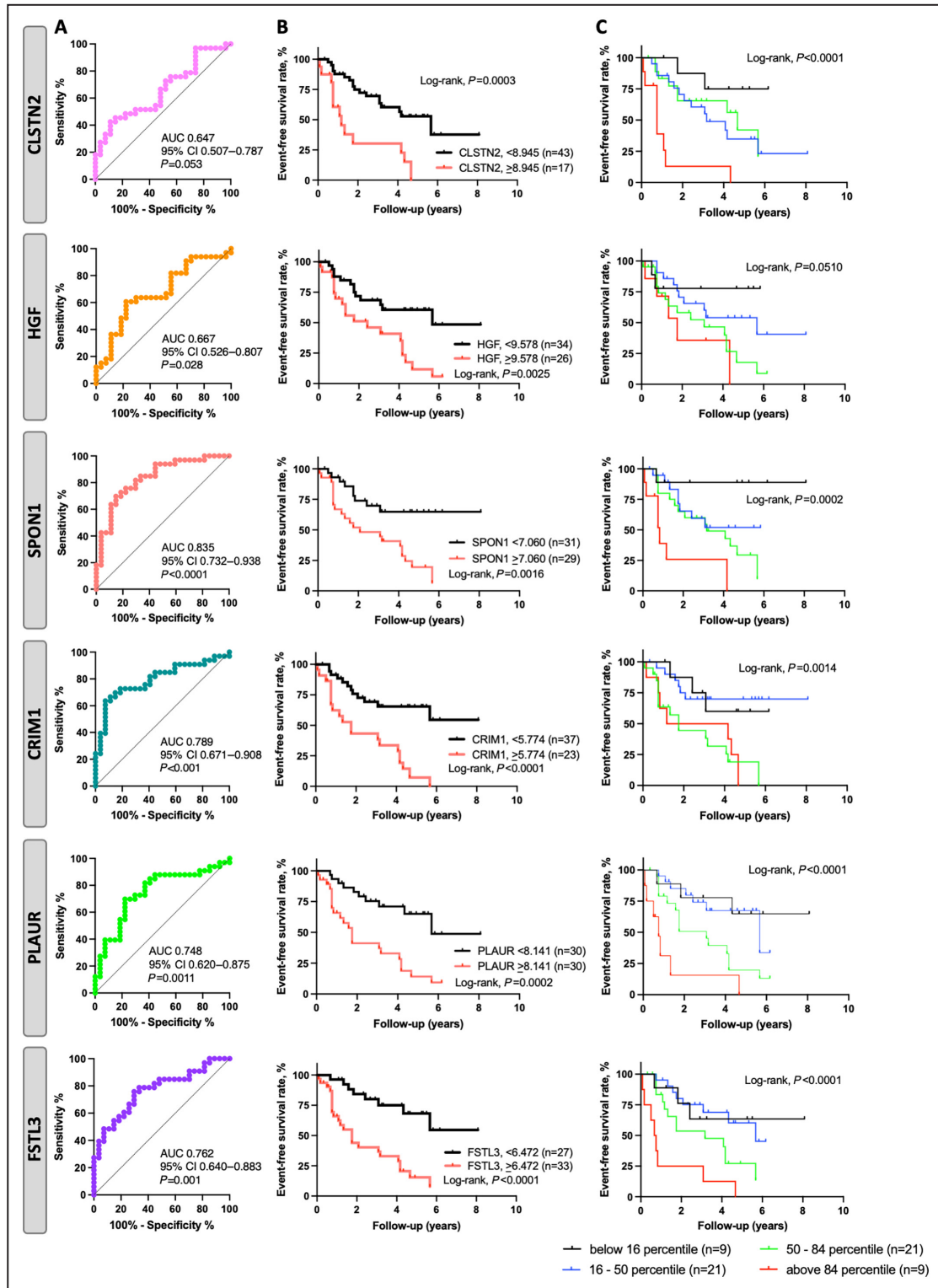


Figure 3. Survival ROC curves and Kaplan–Meier curves showing progression free survival in patients with PAH with baseline biomarker levels above or below the identified threshold.

A. ROC assessing the prognostic accuracy of CLSTN2, HGF, SPON1, CRIM1, PLAUR, and FSTL3. **B.** Kaplan–Meier survival curves according to ROC cutoff values for CLSTN2, HGF, SPON1, CRIM1, PLAUR, and FSTL3. **C.** Higher percentiles of the biomarker levels were also associated with lower event-free survival, suggesting their ability to identify patients at high event risk. AUC indicates area under the curve. CLSTN2, calyntenin 2; CRIM1, cysteine rich transmembrane bone morphogenetic protein regulator 1; FSTL3, follistatin-like 3; HGF, hepatocyte growth factor; PAH, pulmonary arterial hypertension; PLAUR, plasminogen activator, urokinase receptor; ROC, receiver operating characteristics; and SPON1, spondin 1.

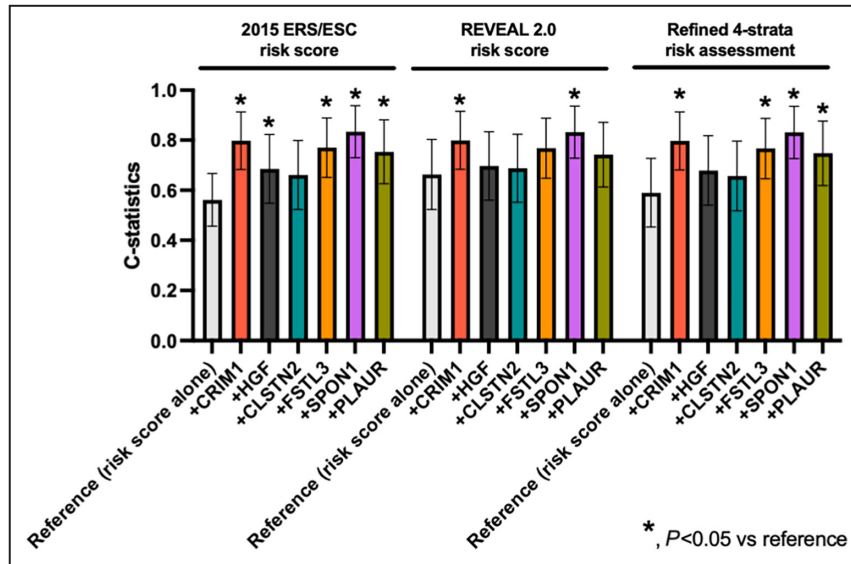


Figure 4. C-statistics of biomarkers with 2015 ERS/ESC risk score, REVEAL 2.0 risk score and the refined four-strata risk assessment method in patients with PAH for death and lung transplantation.

Graph of C-statistic with 95% CI for each prognostic biomarker. C-statistics were compared using the DeLong test with each reference. * $P < 0.05$ vs reference. CLSTN2 indicates calstent 2; CRIM1, cysteine rich transmembrane bone morphogenetic protein regulator 1; ESC/ERS, European Society of Cardiology/European Respiratory Society; FSTL3, follistatin-like 3; HGF, hepatocyte growth factor; PAH, pulmonary arterial hypertension; PLAUR, plasminogen activator, urokinase receptor; REVEAL, Registry to Evaluate Early and Long-Term PAH Disease Management; and SPON1, spondin 1.

to be induced by activin receptor type II activation but also functions as a negative regulator of the pathway activity by antagonizing the action of activin A^{34,35} making it difficult to determine with certainty the impact of its high circulating expression. Although the role of activin A signaling in cardiac remodeling has been the subject of controversy,³⁶ recent findings indicate a detrimental function. Indeed, increasing circulating activin A in mice was shown to induce cardiac activin receptor type II signaling and FSTL3 expression resulting in compromised cardiac function.³⁶ Conversely, genetic or pharmacological inhibition of activin receptor type II signaling preserved cardiac function in several animal models of heart failure.³⁵ In support of this, engagement of activin A signaling was reported to directly impair cardiomyocyte contractile function,³⁷ whereas secretion of FSTL3 by cardiomyocytes promotes proliferation and activation of cardiac fibroblasts.³⁸ Of note, FST (follistatin), an another activin antagonist, was found increased in patients with PAH (Figure 1 and Table S4), stressing the importance of the signaling pathway in PAH pathogenesis. Given that addition of sotatercept, an activin signaling inhibitor, to existing therapy showed promise for the treatment of PAH,³⁹ the clinical significance of circulating FST/FSTL3 in assessing response to therapy will be of great interest.

In addition to validating or identifying promising circulating factors that may serve as prognostic biomarkers in PAH, our work also pinpointed alterations of several plasma proteins that may be involved in PAH pathogenesis. For instance, OSM (oncostatin-M/OSM), a member of the interleukin-6 family cytokines, was found significantly increased in PAH. Although not explored in the setting of PAH, compelling data documenting that OSM stimulates vascular smooth muscle cell proliferation,⁴⁰ induces pulmonary inflammation and fibrosis in mice,⁴¹ and promotes progression of heart failure when continuously activated^{42,43} suggest that increased circulating levels could simultaneously contribute to adverse pulmonary and RV remodeling in PAH. In addition, elevated circulating levels of the serine protease GZMB (granzyme B) were detected in patients with PAH. Because GZMB is essentially stored by immune cells and initiates multiple cell death pathways, it can be envisaged that increased expression of GZMB triggers adverse ventricular remodeling by promoting cardiomyocyte apoptosis and associated interstitial fibrosis.⁴⁴

Several limitations should be acknowledged. First, although our work includes a substantial number of patients, the single-center nature is an inherent limitation of our study. However, the fact that

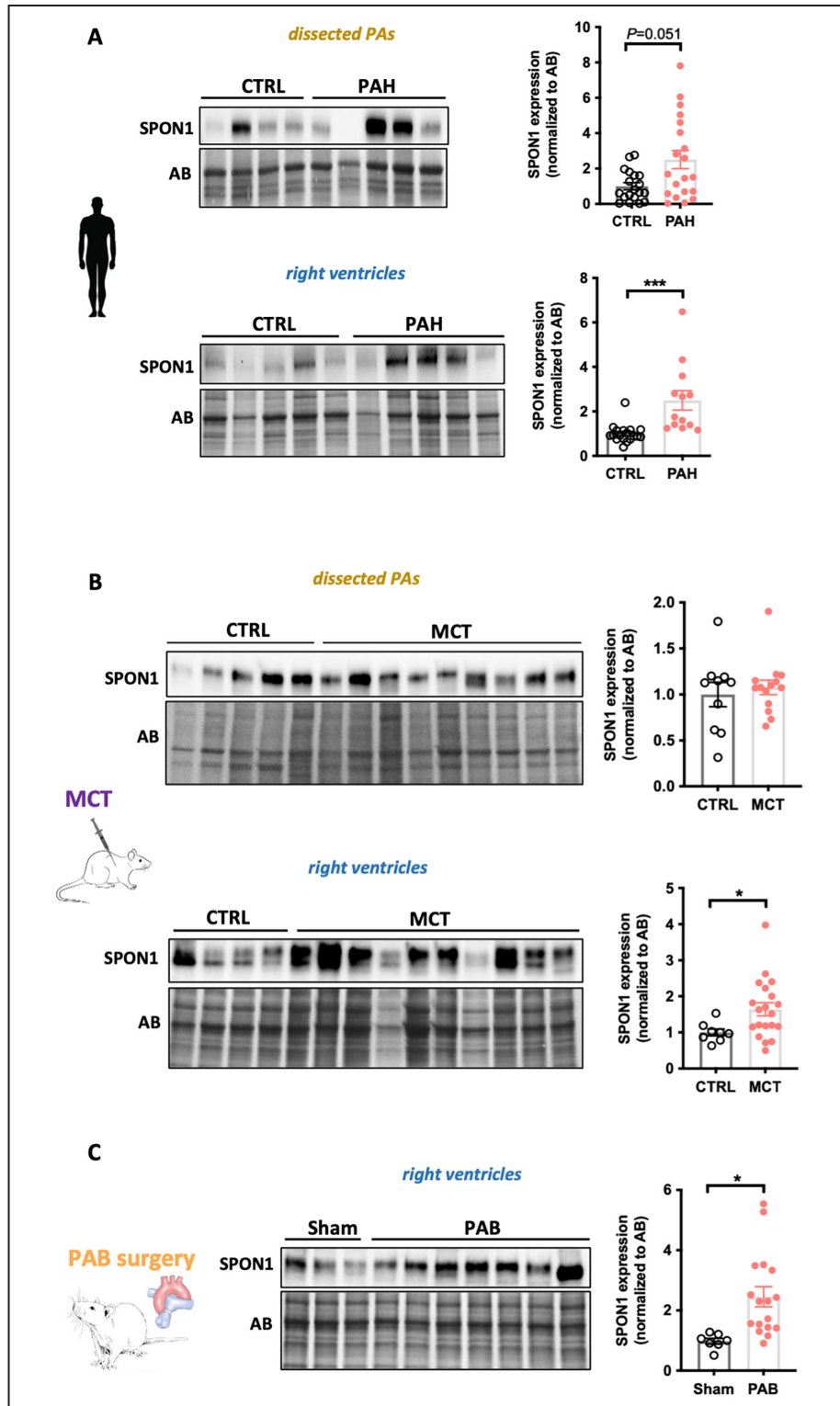


Figure 5. Upregulation of SPON1 in remodeled RVs from patients with PAH and animal models.

A, Representative Western blots and corresponding quantifications of SPON1 in dissected PAs and RVs from CTRL (n=18–19) and patients with PAH (13–20). **B**, Representative Western blots and corresponding quantifications of SPON1 in dissected PAs and RVs from CTRL (n=8–10) and monocrotaline-injected (n=14–20) rats. **C**, Representative Western blot and corresponding quantification of SPON1 in RVs from sham (n=7) and PAB-subjected (n=17) rats. Protein expression was normalized to amido black. Scatter dot plots show individual values and mean±SEM. Statistical analyses were performed using Student’s *t* test. * $P<0.05$ and *** $P<0.001$. AB indicates amido black; CTRL, controls; MCT, monocrotaline; PA, pulmonary arteries; PAB, pulmonary artery banding; PAH, pulmonary arterial hypertension; RV, right ventricle; and SPON1, spondin 1.

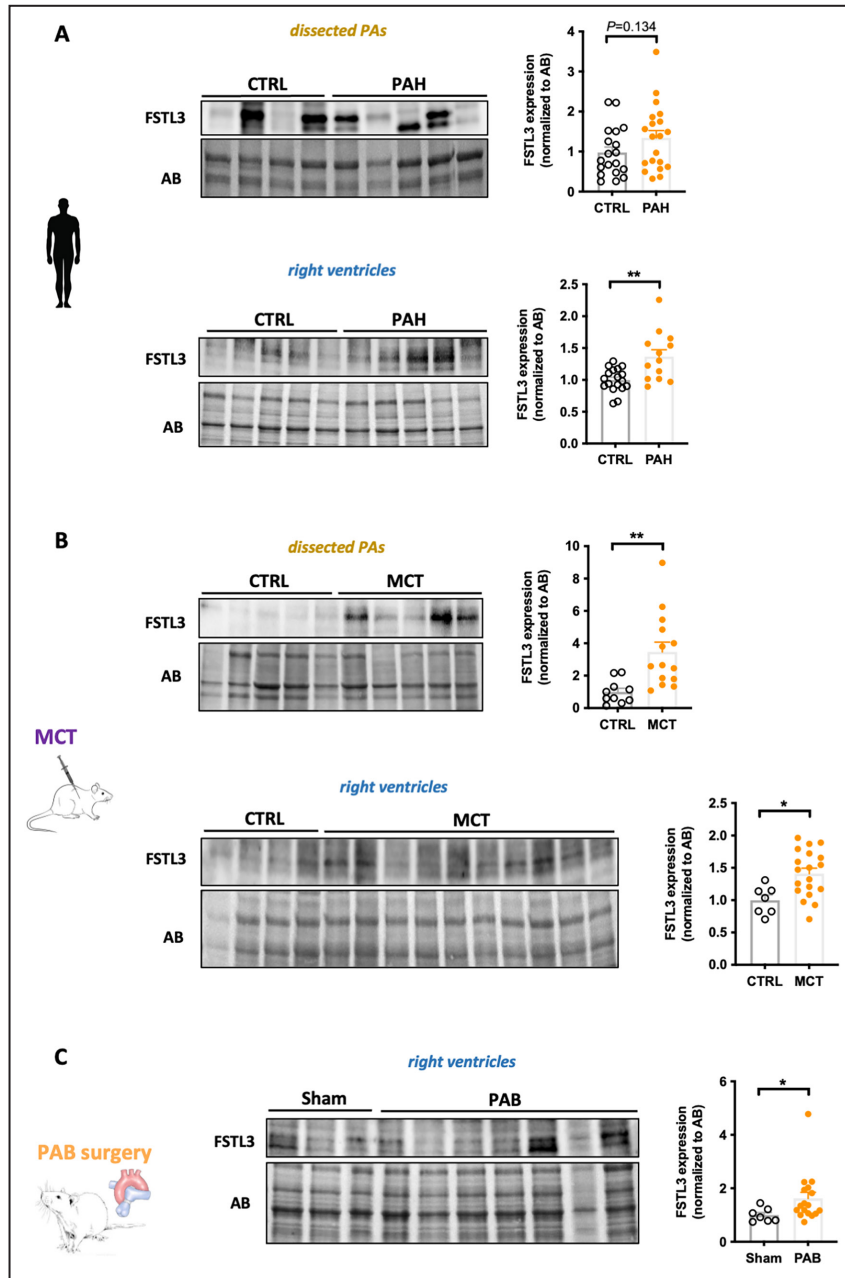


Figure 6. Upregulation of FSTL3 in remodeled RVs from patients with PAH and animal models.

A, Representative Western blots and corresponding quantifications of FSTL3 in dissected pulmonary arteries and RVs from CTRL (n=18–19) and patients with PAH (n=13–20). **B**, Representative Western blots and corresponding quantifications of FSTL3 in dissected PAs and RVs from CTRL (n=7–10) and monocrotaline-injected (n=14–19) rats. **C**, Representative Western blot and corresponding quantification of FSTL3 in RVs from sham (n=7) and PAB-subjected (n=17) rats. Protein expression was normalized to amido black. Scatter dot plots show individual values and mean±SEM. Statistical analyses were performed using Student’s *t* test. **P*<0.05 and ***P*<0.01. AB indicates amido black; CTRL, controls; FSTL3, follistatin-like 3; MCT, monocrotaline; PA, pulmonary arteries; PAB, pulmonary artery banding; PAH, pulmonary arterial hypertension; and RV, right ventricle.

some circulating proteins identified by our study are already documented to provide a prognostic utility in the context of PAH increases the credibility

of our data. Another limitation lies in the fact that uncertainties remain regarding the tissue origin of these circulating biomarkers (pulmonary, cardiac, or

both). In addition, the absence of multiple testing adjustments for Cox proportional hazard analyses may have resulted in type I error. The findings of this study should be interpreted as exploratory. Finally, the evolution of these biomarkers in response to therapies is lacking, a point that needs to be addressed in a future study.

CONCLUSIONS

In conclusion, the present study using high-throughput proteomic technologies enabled us to identify potential novel immune-related plasma biomarkers, which may help improve outcome prediction in PAH if externally confirmed in future studies.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Tables S1–S6
Figures S1–S2

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