

FEATURED PAPERS

Circulating NEDD9 is increased in pulmonary arterial hypertension: A multicenter, retrospective analysis



Andriy O. Samokhin, PhD,^a Steven Hsu, MD,^b Paul B. Yu, MD, PhD,^{a,c}
Aaron B. Waxman, MD, PhD,^{a,d} George A. Alba, MD,^e
Bradley M. Wertheim, MD,^{a,d} C. Danielle Hopkins, BS,^b
Frederick Bowman, BS, ALM,^a Richard N. Channick, MD,^e Ivana Nikolic, MD,^f
Mariana Faria-Urbina, PhD,^{a,d} Paul M. Hassoun, MD,^g Jane A. Leopold, MD,^{a,c}
Ryan J. Tedford, MD,^h Corey E. Ventetuolo, MD, MSc,ⁱ
Peter J. Leary, MD, PhD,^j and Bradley A. Maron, MD^{a,c}

From the ^aDepartment of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts; ^bDivision of Cardiology, Department of Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland; ^cDivision of Cardiovascular Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts; ^dDivision of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts; ^eDivision of Pulmonary and Critical Care Medicine; ^fDivision of Cardiology, Massachusetts General Hospital, Boston, Massachusetts; ^gDivision of Pulmonary and Critical Care Medicine, Department of Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland; ^hDivision of Cardiology, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina; ⁱDivision of Pulmonary, Critical Care & Sleep Medicine, Departments of Medicine and Health Services, Policy and Practice, Brown University, Providence, Rhode Island; and the ^jDivision of Pulmonary, Critical Care & Sleep Medicine, University of Washington, Seattle, Washington.

KEYWORDS:

pulmonary arterial
hypertension;
NEDD9;
biomarker;
hemodynamics;
mortality;
pulmonary vascular
disease

BACKGROUND: Pulmonary arterial hypertension (PAH) is a highly morbid disease characterized by elevated pulmonary vascular resistance (PVR) and pathogenic right ventricular remodeling. Endothelial expression of the prometastatic protein NEDD9 is increased in fibrotic PAH arterioles, and NEDD9 inhibition decreases PVR in experimental PAH. We hypothesized that circulating NEDD9 is increased in PAH and informs the clinical profile of patients.

METHODS: Clinical data and plasma samples were analyzed retrospectively for 242 patients from 5 referral centers (2010–2017): PAH ($n = 139$; female 82%, 58 [48–67] years), non-PAH pulmonary hypertension (PH) ($n = 54$; female 56%, 63.4 $\pm$ 12.2 years), and dyspnea non-PH controls ($n = 36$; female 75%, 54.2 $\pm$ 14.0 years).

RESULTS: Compared with controls, NEDD9 was increased in PAH by 1.82-fold ($p < 0.0001$). Elevated NEDD9 correlated with PVR in idiopathic PAH ($\rho = 0.42$, $p < 0.0001$, $n = 54$), connective tissue disease (CTD)-PAH ($\rho = 0.53$, $p < 0.0001$, $n = 53$), and congenital heart disease-PAH ($\rho = 0.68$, $p < 0.0001$, $n = 10$). In CTD-PAH, NEDD9 correlated with 6-minute walk distance ($\rho = -0.35$, $p = 0.028$, $n = 39$). In contrast to the PAH biomarker N-terminal pro-brain natriuretic peptide ($n = 38$), NEDD9 correlated inversely with exercise pulmonary artery wedge pressure and more strongly with right

ventricular ejection fraction ($\rho = -0.41$, $p = 0.006$, $n = 45$) in a mixed population. The adjusted hazard ratio for lung transplant-free survival was 1.12 (95% confidence interval [CI], 1.02–1.22, $p = 0.01$) and 1.75 (95% CI, 1.12–2.73, $p = 0.01$) per 1 ng/ml and 5 ng/ml increase in plasma NEDD9, respectively, by Cox proportional hazard model.

CONCLUSIONS: In PAH, plasma NEDD9 is increased and associates with key prognostic variables. Prospective studies that include hard end points are warranted to validate NEDD9 as a novel PAH biomarker. *J Heart Lung Transplant* 2020;39:289–299

© Published by Elsevier Inc.

Pulmonary arterial hypertension (PAH) is a severe cardio-pulmonary disease defined, in part, by a fibrotic vasculopathy that increases pulmonary vascular resistance (PVR) and pathogenic remodeling of the right ventricle.¹ Accumulating clinical trial data demonstrate a substantial therapeutic benefit in patients treated using an early-aggressive strategy.² However, dyspnea and other non-specific complaints are often the first presenting symptom in PAH, contributing to delayed or missed diagnosis.³ In addition, longitudinal assessment of PAH progression generally hinges on right heart catheterization, echocardiography, or walk testing, which are invasive or limited by suboptimal precision.^{4–6} For these collective reasons, identifying a PAH-specific biomarker with diagnostic and prognostic relevance has emerged as a major objective in the pulmonary vascular medicine field.⁷

The CAS protein NEDD9 emerged from a recently reported network medicine analysis *in silico* focusing on intermediaries that regulate fibrosis in PAH.⁸ In that study, NEDD9 expression was increased in PAH pulmonary artery endothelial cells *ex vivo* and in the endothelial layer of remodeled arterioles from patients with PAH compared with lung cancer, idiopathic pulmonary fibrosis, and non-diseased controls. Increased NEDD9 activated transcription of *COL3A1*, which is the gene encoding collagen III, to promote fibrotic vascular remodeling. Inhibition of NEDD9 using transgenic animals and small interfering RNA methods prevented the pulmonary arteriopathy of PAH, decreased PVR, and improved right ventricular mass in experimental models *in vivo*.

Importantly, NEDD9 is implicated in the pathogenesis of various solid tumor cancers.⁹ In breast adenocarcinoma, NEDD9 targets transforming growth factor- β ,¹⁰ also implicated in PAH,¹¹ to alter the phenotype of cells and permit blood-borne metastasis.¹² This observation suggests that release of NEDD9 from pulmonary artery endothelial cells into the circulation of patients with PAH is plausible. Taken together, we hypothesized in this study that plasma NEDD9 is increased in PAH and associates with key hemodynamic measurements that are indicative of vascular remodeling extent and prognosis.

Methods

An expanded methods section is provided in the Supplementary Material online at www.jhltonline.org.

Clinical population

This study complies with the Declaration of Helsinki. Plasma samples were recruited based on availability from the following

5 PAH referral centers: Brigham and Women's Hospital (Partners A) (A.B.W. and M.F.U.), Massachusetts General Hospital (Partners B) (P.B.Y.), Rhode Island Hospital/Brown University (C.E.V.), University of Washington (P.L.), and Johns Hopkins University (JHU) (S. H, R.J.T., and P.H.). At each center, consecutive patients were enrolled based on patient consent. This study was approved by the local Institutional Review Board at each participating center. Details on the method for enrolling patients, collecting clinical data, and processing samples are provided in the Supplementary Material online.

Definitions of PAH, other clinical phenotypes, and controls

Standardized clinical definitions were used to classify all patients,¹³ which was informed by each participating center investigator (as above) and reviewed independently by BAM. For PAH, the following hemodynamic criteria were used based on the prevailing definition during the study time period: mean pulmonary artery pressure (mPAP) ≥ 25 mm Hg, PVR ≥ 3.0 Wood units, and pulmonary artery wedge pressure (PAWP) ≤ 15 mm Hg. Patients with World Health Organization (WHO) Group 2 pulmonary hypertension (PH) had mPAP ≥ 25 mm Hg; PAWP > 15 mm Hg; and a diagnosis of heart failure with reduced left ventricular ejection fraction, heart failure with preserved left ventricular ejection fraction, or other primary cardiac pathology.¹ Patients with WHO Group 3 PH had mPAP ≥ 25 mm Hg; PAWP < 15 mm Hg; and a ventilatory defect detected on spirometry, obstructive sleep apnea, or other established risk factor for PH because of hypoxia or sleep disordered breathing. Idiopathic PAH (iPAH), portopulmonary hypertension, human immunodeficiency virus-PAH, pulmonary veno-occlusive disease, and exercise-induced PH were diagnosed by appropriate serological data and/or clinical criteria published previously.^{13,14} Drug- and toxin-induced-PAH was diagnosed mainly in the setting of methamphetamine use according to recently published guidelines.¹⁵ Human immunodeficiency virus-PAH and pulmonary veno-occlusive disease patients ($N = 3$) were considered as a specific subtype of PAH.

NEDD9 measurement assays

Detailed information on liquid chromatography–mass spectrometry (LC-MS) and immunoblot methodologies for detecting NEDD9 is provided in the Supplementary Material online.

Enzyme-linked immunosorbent assay (ELISA) for NEDD9 quantification

Human plasma samples were diluted 1:3 with phosphate buffered saline, and NEDD9 levels were analyzed using a NEDD9 ELISA kit (Aviva Systems Biology, San Diego, CA, #OKEH02459),

according to manufacturer recommendations. All plasma NEDD9 levels were measured and analyzed in the same laboratory by a single investigator (A.O.S.). The manufacturer's reported and the investigator's observed intra-assay coefficient of variance for the ELISA assay used in this study is 4.9% and 8.4%, respectively (Supplementary Table S1 online). The manufacturer's reported interassay coefficient of variance is 8.5%; this metric could not be assessed independently because of insufficient sample quantity.

Statistical methods

All analyses were performed using Origin 9.01 (B.A.M.) or Stata v15.1 (P.J.L.). Data are expressed as mean $\pm$ standard error of the mean or median (interquartile range) for normally and non-normally distributed data, respectively. The unpaired Student *t*-test and one-way analysis of variance (ANOVA) test were used for comparisons between 2 and 2 or more normally distributed groups, respectively. The Mann-Whitney and Kruskal-Wallis non-parametric tests were used to compare 2 or more non-normally distributed groups. The Pearson *r* and Spearman ρ coefficients are reported for linear regression analyses performed using data that was distributed normally and non-normally, respectively. Exploratory analyses of the center-specific relationship between NEDD9 and PVR were performed to evaluate heterogeneity imposed by center in the relationship between NEDD9 and a PAH-specific marker. Linear regression was used to estimate the relationship between NEDD9 and PVR. Unadjusted analyses and models accounting for age or age, sex, and PAH subtype were considered. The area under the curve for the receiver operating characteristic analysis was calculated using methods reported previously.¹⁴ Cox proportional hazards models were used to assess the association between plasma NEDD9 and lung transplant-free survival. Kaplan-Meier event-free curves were plotted for NEDD9 dichotomized using the

median plasma level in patients with PAH using lung transplant or death as the event. Unadjusted group comparisons for time-to-event outcomes were made using the log-rank test.

Results

Verifying plasma NEDD9

The ELISA assay in this study was used previously to quantify NEDD9 in plasma samples from patients with other diseases. However, verification of commercially purchased laboratory reagents is identified increasingly as a critical step toward optimizing scientific rigor,¹⁶ and prior reports used only a single method to analyze plasma NEDD9.¹⁷ In pilot studies, we confirmed detection of NEDD9 in plasma using LC-MS, which is the gold-standard method for proteomic analysis. This approach detected 5 peptides within 3 of the 4 distinct NEDD9 domains ($n \geq 2$ replicates of each peptide per sample; $n = 2$ samples) (Figure 1). This was then confirmed further by anti-NEDD9 immunoblot using an antibody targeting the peptide sequence spanning amino acids 85 to 350 (Supplementary Figure S1 online), which was a region of the protein detected by LC-MS ($n = 3$).

NEDD9 plasma sample throughput

From a total of 269 individual patient plasma samples, 242 (90%) were available for analyzing the clinical relevance of NEDD9 after excluding 1 assay (inclusive only of Partners B samples) that was technically unsound (defined by

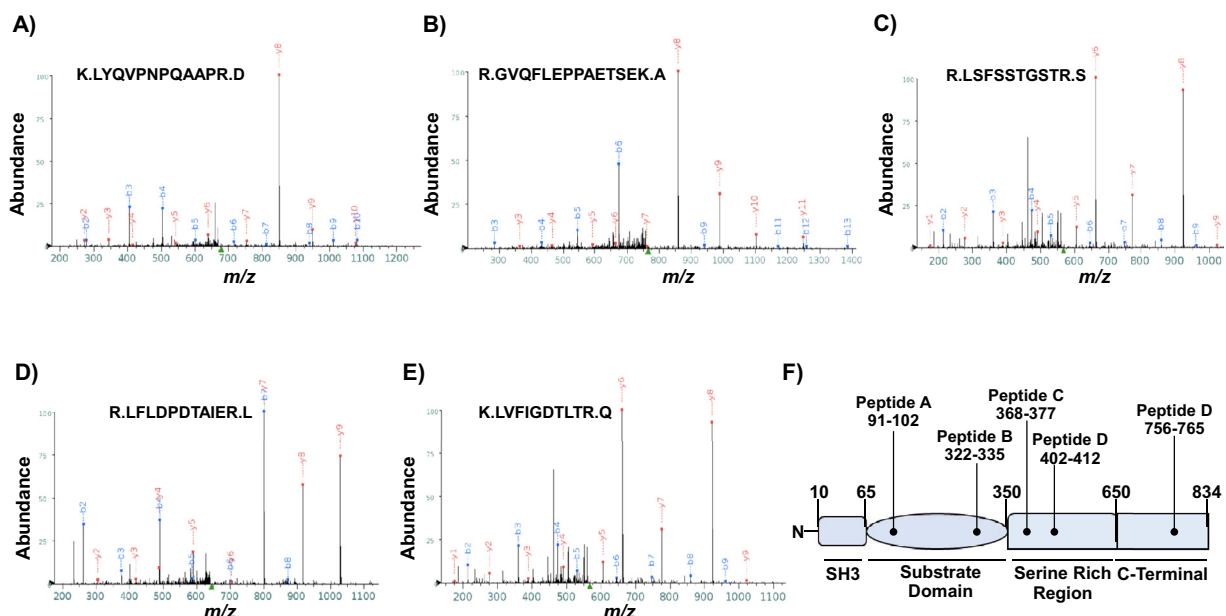


Figure 1 LC-MS confirms detection of NEDD9 in human plasma. Healthy control human plasma was immunoprecipitated with an anti-NEDD9 antibody covering amino acid sequences 82 to 398. In-gel trypsin digestion was performed on samples reduced with dithiothreitol before protein identification by in-tandem LC-MS. The MS1 spectra including the doubly charged y_6 , y_7 , or y_8 ion corresponding to NEDD9 fragments is provided for peptides in the (A, B) protein substrate domain, (C, D) serine rich region, and (E) C-terminal domain, illustrated graphically (F) ($n = 2$ replicates per detected peptide, $n = 2$ samples). Representative spectra are provided. LC-MS, liquid chromatography–mass spectrometry; m/z, mass-to-charge ratio.

Table 1 Summary of Patient Diagnosis for the Study Cohort

Center	Control	PAH	WHO Group 2	WHO Group 3	WHO Group 5/ Mixed phenotype	SSC-no PH	EurI-Physiol	Total
Partners A	9	10	0	0	0	0	0	19
Partners B	24	31	15	12	17	0	3	102
Warren Alpert/ Brown University	0	30	0	0	0	0	0	30
University of Washington	0	43	1	0	1	0	0	45
Johns Hopkins University	3	25	2	3	0	13	0	46
Total	36	139	18	15	18	13	3	242

Abbreviations: EurI-Physiol, exercise-induced pulmonary hypertension; PAH, pulmonary arterial hypertension of any subtype; PH, pulmonary hypertension; SSC, systemic sclerosis; WHO, World Health Organization.

Partners A Is Brigham and Women's Hospital and Partners B Is Massachusetts General Hospital.

biologically implausible values) ($n = 24$) and statistical outliers ($n = 3$) (Table 1 and Supplementary Figure S2 online).

Study population

Compared with controls, patients with PAH (82% female) had higher mPAP (17 [13–19] vs 45 [35–57] mm Hg, $p < 0.0001$), PVR (94.6 [68.0–137] vs 385.8 [229.1–717.3] dyne-s/cm⁵, $p < 0.0001$), and transpulmonary gradient (7 [4–9] vs 35 [24–48] mm Hg, $p < 0.0001$) as expected but were not significantly different in age (52.9 [41.5–66.8] vs 59.0 [46.1–67.3] years, $p = 0.31$) (Table 2 and Supplementary Table S2 online). Clinical comorbidities for each cohort are available in Supplementary Tables S3 to S7 online. The pharmacotherapeutic profile was available for 92.8% of patients with PAH ($n = 129$), in which PAH-specific medication was reported in 42.6% ($n = 55$), indicating a mixture of prevalent and incident patients in the study cohort. Phosphodiesterase type-V inhibitors, endothelin receptor antagonists, and prostacyclin replacement drugs were recorded for 86%, 53%, and 48% of patients with PAH, respectively (Table 3 and Supplementary Tables S8–S12 online).

NEDD9 is increased in PAH and associates with cardiopulmonary hemodynamic severity

Samples were available for control patients from 3 centers, and NEDD9 concentration was significantly different across these cohorts (Supplementary Figure S3A online). Similarly, NEDD9 was different for patients with iPAH across all 5 participating centers (Supplementary Figure S3B online). Despite this variability, however, NEDD9 was increased overall by 1.82-fold in PAH compared with controls ($p < 0.0001$) (Figure 2A). No significant difference for NEDD9 was observed by sex ($p = 0.26$), nor was an association evident between NEDD9 and age ($\rho = 0.09$, $p = 0.20$), creatinine concentration ($\rho = -0.14$, $p = 0.19$), or hemoglobin concentration ($\rho = 0.23$, $p = 0.053$), or when comparing patients with PAH for whom PAH-specific medical therapy was vs was not reported (13.1 ± 4.9 vs 12.8 ± 6.2 ng/ml, $p = 0.75$).

We next evaluated if our finding that NEDD9 is increased in PAH was driven by a particular PAH subtype. Compared with controls, NEDD9 was increased significantly in iPAH ($n = 54$), connective tissue disease (CTD)-PAH ($n = 52$), congenital heart disease-PAH ($n = 11$), portopulmonary

Table 2 Hemodynamic Characteristics of the Initial Control and PAH Cohorts

Variable	Patients (n)			Clinical and hemodynamic variables by group			p -value
	Control	PAH	Non-PAH PH	Control	PAH	Non-PAH PH	
Age, years	37	142	54	52.9 (41.5–66.8)	59.0 (46.1–67.3)	63.0 (54.4–71.1)	0.0027
RAP, mm Hg	33	126	52	3 (2–6)	8 (5–12)	9 (5.5–14)	<0.0001
mPAP, mm Hg	35	134	54	17 (13–19)	45 (35–57)	37.5 (30–46)	<0.0001
TPG, mm Hg	36	134	54	7 (4–9)	35 (24–48)	20 (15–25)	<0.0001
PVR, dyne-s/cm ⁵	35	118	53	94.6 (68.0–137)	624.0 (350.0–808.0)	295.9 (217.0–389.0)	<0.0001
PAWP, mm Hg	28	125	54	10 (6.0–12)	10 (8–13)	18 (11–25)	<0.0001
CO, liter/min	35	114	53	5.3 (4.3–6.7)	4.6 (3.8–5.9)	5.0 (4.0–6.3)	0.1924
CI, liter/min/m ²	32	39	47	2.8 (2.5–3.6)	2.5 (2.1–3.0)	2.7 (2.2–3.2)	0.0511

Abbreviations: ANOVA, analysis of variance; CI, cardiac index; CO, cardiac output; IQR, interquartile range; mPAP, mean pulmonary artery pressure; PAH, pulmonary arterial hypertension; PAWP, pulmonary artery wedge pressure; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; RAP, right atrial pressure; TPG, transpulmonary gradient.

Data are expressed as median (IQR). p -value data are from ANOVA analysis.

Table 3 PAH Medical Regimen for the Overall PAH Cohort

Total PAH cohort (N = 139)	
Medication	n (%)
Calcium-channel antagonist	20 (14)
PDE-Vi	86 (61)
ERA	53 (37)
Prostacyclin therapy	48 (34)
sGC therapy	0 (0)
Supplemental O ₂	16 (11)
ACE-inhibitor/ARB	14 (10)
Anticoagulant	29 (21)
Diuretic	82 (59)
MRA	24 (17)

Abbreviations: ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; ERA, endothelin receptor antagonist; MRA, mineralocorticoid receptor antagonist; PAH, pulmonary arterial hypertension; PDE-Vi, phosphodiesterase-5 inhibitor; sGC, soluble guanylyl cyclase.

hypertension ($n = 12$), and toxin-induced-PAH ($n = 7$) ($6.7 [4.2-9.6]$ vs 12.1 ± 5.6 vs 13.3 ± 5.5 vs 16.4 ± 4.0 vs 9.7 ± 3.0 vs 13.0 ± 3.3 ng/ml, $p < 0.0001$ by ANOVA) (Figure 2B). By contrast, plasma NEDD9 discriminated PAH

from other forms of PH, as no significant difference in levels was observed between controls ($n = 36$) and WHO Group 2 PH ($n = 18$), WHO Group 3 PH ($n = 15$), Group 5/mixed phenotype ($n = 18$), and exercise-induced PH ($n = 3$) (Supplementary Figure S3C online).

NEDD9 and cardiopulmonary hemodynamics

Among controls and patients with PAH, there was a border-line moderate but significant correlation between NEDD9 and mPAP ($\rho = 0.31$, $p < 0.0001$, $n = 166$) and transpulmonary gradient ($\rho = 0.30$, $p < 0.0001$, $n = 166$) (Figure 2C and D). Compared with patients in the lowest PVR quartile, NEDD9 levels were increased in the higher 3 quartiles (8.2 ± 5.6 vs 12.4 ± 5.4 vs 13.1 ± 5.3 vs 12.3 ± 5.5 ng/ml, $p < 0.0001$ by ANOVA) (Figure 2E). However, the strength of the association between NEDD9 and mPAP, transpulmonary gradient, and PVR was increased when considering individual PAH subgroups, particularly among iPAH ($\rho = 0.44$, $p < 0.0001$; $\rho = 0.42$, $p < 0.0001$; and $\rho = 0.44$, $p < 0.0001$, respectively) (Figure 3), CTD-PAH ($\rho = 0.53$, $p < 0.0001$; $\rho = 0.53$, $p < 0.0001$; and $\rho = 0.51$, $p < 0.0001$, respectively) (Figure 4), and congenital heart disease-PAH ($\rho = 0.75$, $p < 0.0001$; $\rho = 0.68$, $p < 0.0001$; and $\rho = 0.43$,

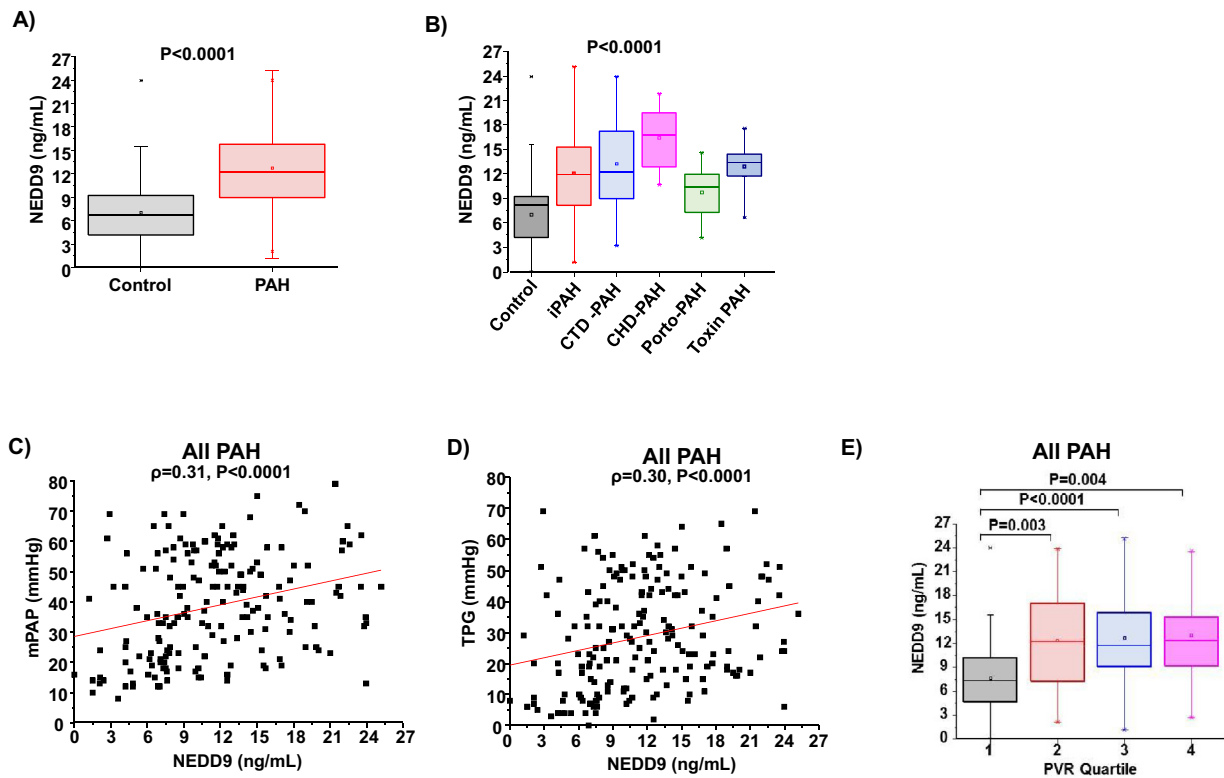


Figure 2 Plasma NEDD9 is increased significantly in PAH and PAH subtypes. (A) Plasma was collected from 5 PAH referral centers, and the NEDD9 concentration for dyspnea non-PH controls ($n = 36$) and patients with PAH ($n = 139$) was quantified by ELISA. (B) Compared with controls, plasma NEDD9 concentration was increased among patients with iPAH ($N = 54$), CTD-PAH ($n = 52$), CHD-PAH ($n = 7$), porto-PAH ($n = 12$), and toxin-induced-PAH ($n = 10$). (C, D) Linear regression analyses correlating NEDD9 with mPAP and TPG. (E) Distribution of NEDD9 concentration by PVR quartile. For box plot analyses: mean, square; median, horizontal line; interquartile range, box distribution; maximum and minimum, y-axis lines. CHD, congenital heart disease; CTD, connective tissue disease; ELISA, enzyme-linked immunosorbent assay; iPAH, idiopathic pulmonary arterial hypertension; mPAP, mean pulmonary artery pressure; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; porto-PAH, portopulmonary hypertension; PVR, pulmonary vascular resistance; TPG, transpulmonary gradient.

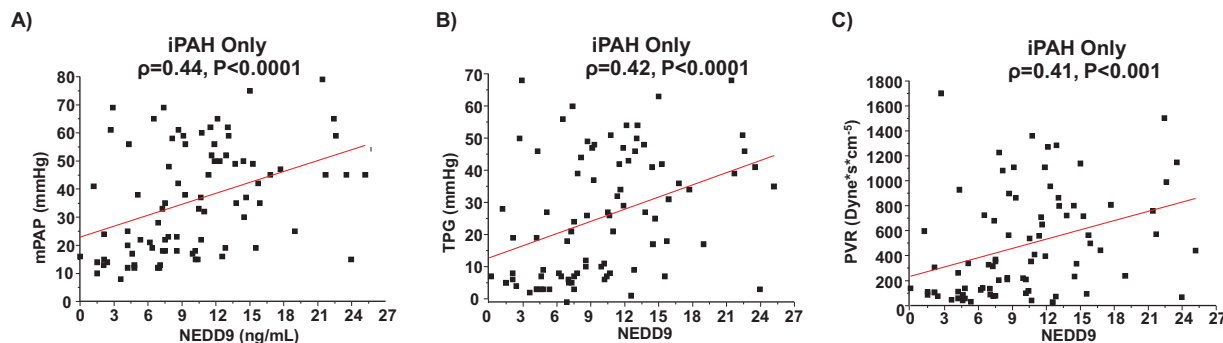


Figure 3 The association between plasma NEDD9 and cardiopulmonary hemodynamic measurements in iPAH. Plasma was collected from 5 PAH referral centers and the NEDD9 concentration for dyspnea non-PH controls ($n = 36$) and patients with iPAH ($n = 54$) was quantified by ELISA. These data were correlated with (A) mPAP, (B) TPG, and (C) PVR. ELISA, enzyme-linked immunosorbent assay; iPAH, idiopathic pulmonary arterial hypertension; mPAP, mean pulmonary artery pressure; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; TPG, transpulmonary gradient.

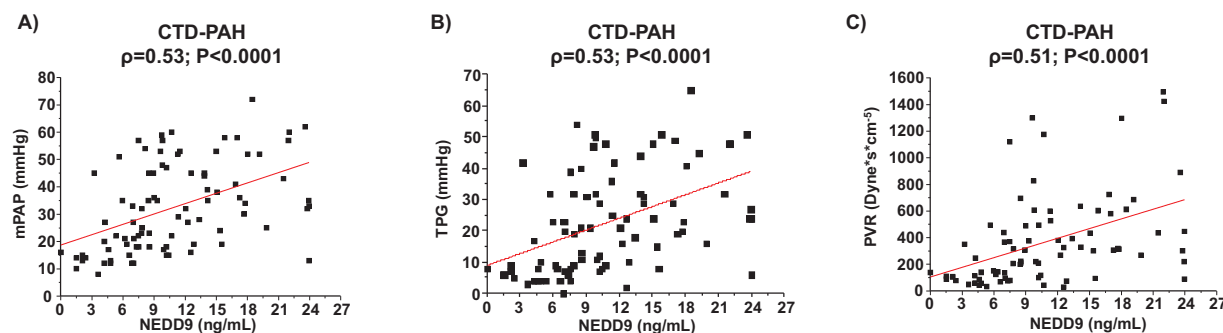


Figure 4 The association between plasma NEDD9 and cardiopulmonary hemodynamic measurements in CTD-PAH. Plasma was collected from 5 PAH referral centers and the NEDD9 concentration for dyspnea non-PH controls ($n = 36$) and patients with CTD-PAH ($n = 53$) was quantified by ELISA. These data were correlated with (A) mPAP, (B) TPG, and (C) PVR. CTD, connective tissue disease; ELISA, enzyme-linked immunosorbent assay; mPAP, mean pulmonary artery pressure; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; TPG, transpulmonary gradient.

$p < 0.0001$, respectively) (Figure 5). Among these PAH subtypes, sufficient data on 6-minute walk distance were available only for CTD-PAH, which correlated modestly with NEDD9 ($\rho = -0.35$, $p = 0.028$, $n = 39$). Akin to the overall PAH cohort, NEDD9 did not associate with cardiac output (CO), cardiac index, or PAWP in the full cohort or in any of these subgroups.

Our findings indicated that NEDD9 levels in patients with iPAH varied by participating center (Supplementary Figure S3C online), and that NEDD9 most strongly associated with PVR among the hemodynamic measurements analyzed in the full cohort. Therefore, we used the association between NEDD9 and PVR to explore center-specific findings and observed a significant increase in PVR per

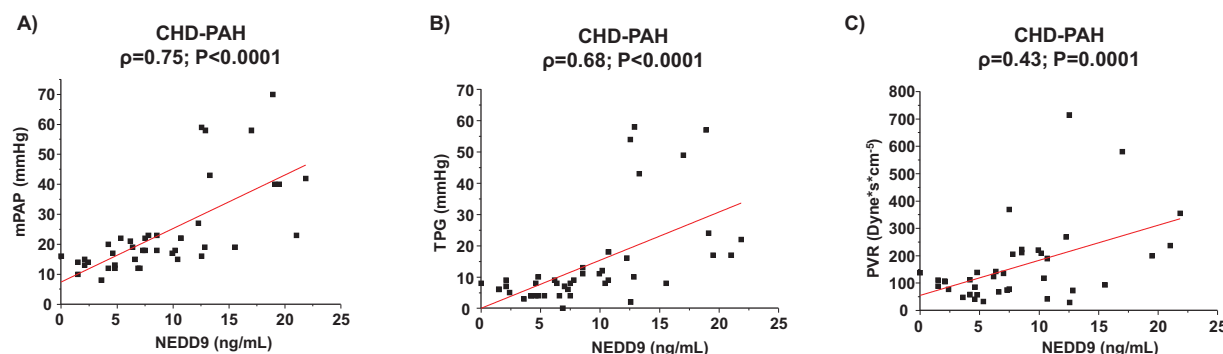


Figure 5 The association between plasma NEDD9 and cardiopulmonary hemodynamic measurements in CHD-PAH. Plasma was collected from 5 PAH referral centers and the NEDD9 concentration for dyspnea non-PH controls ($n = 36$) and patients with CTD-PAH ($n = 10$) was quantified by ELISA. These data were correlated with (A) mPAP, (B) TPG, and (C) PVR. CHD, congenital heart disease; ELISA, enzyme-linked immunosorbent assay; mPAP, mean pulmonary artery pressure; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; TPG, transpulmonary gradient.

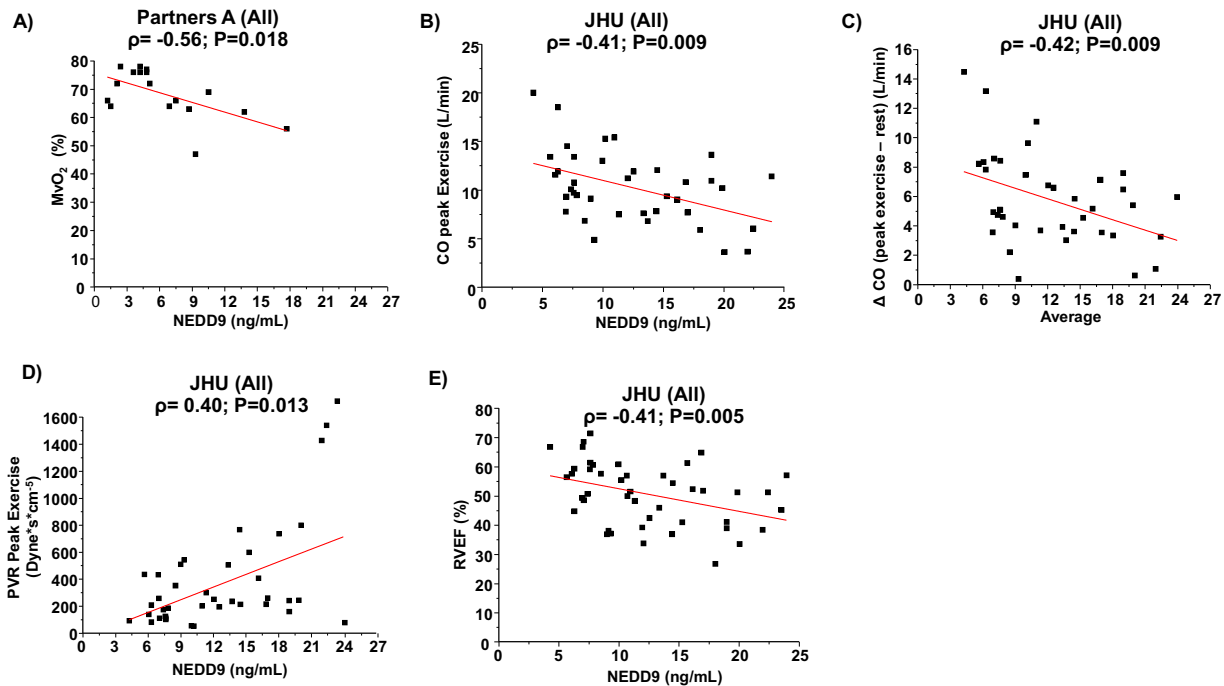


Figure 6 The association between plasma NEDD9 and alternative hemodynamic prognostic measurements at rest and during exercise. From the overall cohort, adjustment for analyses for age, sex, and PAH subtype identified a significant relationship between NEDD9 and PVR among patients in the Partners A and JHU cohorts. In subsequent subgroup analyses focusing on patients from these centers, NEDD9 correlated inversely with (A) resting MvO_2 ($n = 19$), (B) CO at peak exercise, (C) change in CO from rest to peak exercise, (D) PVR at peak exercise on invasive cardiopulmonary exercise testing, and (E) RVEF at rest assessed by cardiac magnetic resonance imaging ($n = 45$ for B–E). CO, cardiac output; JHU, Johns Hopkins University; MvO_2 , mixed venous oxygen saturation level; PAH, pulmonary arterial hypertension; PVR, pulmonary vascular resistance; RVEF, right ventricular ejection fraction.

ng/ml change in plasma NEDD9 concentration for the controls and PAH patients in the Partners A cohort ($n = 19$) of $+28.2$ dyne·s/cm⁵ (95% confidence interval [CI] 3.0–53.4, $p = 0.03$) and JHU cohort ($n = 28$) of $+39.2$ dyne·s/cm⁵ (95% CI 9.6–68.6, $p = 0.01$) (Supplementary Tables S13 and S14 online).

Based on this observation, we focused on the Partners A and JHU cohorts to determine if NEDD9 could inform alternative measurements with prognostic relevance to PAH at these centers specifically. To this end, we identified a modest, inverse correlation between NEDD9 and mixed venous oxyhemoglobin saturation ($\rho = -0.56$, $p = 0.018$, $n = 19$ Partners A), as well as CO at peak exercise ($\rho = -0.41$, $p = 0.009$, $n = 39$ JHU) and change in CO from rest to peak exercise (ΔCO) ($\rho = -0.42$, $p = 0.009$, $n = 39$ JHU) assessed during invasive cardiopulmonary exercise testing. Additionally, NEDD9 correlated moderately with key right ventricular functional and morphological parameters including ejection fraction ($\rho = -0.405$, $p = 0.006$, $n = 45$ JHU) and end-systolic volume ($\rho = 0.313$, $p = 0.036$, $n = 45$ JHU) on cardiac magnetic resonance imaging (Figure 6).

N-terminal pro-brain natriuretic peptide (NT-proBNP), NEDD9, and PAH prognostic measurements

NT-proBNP, released from ventricular cardiomyocytes because of pressure-related stretch, is a bona fide prognostic

biomarker for patients with PAH¹⁸ and was available for all patients in the JHU cohort ($n = 46$). The association between NT-proBNP and New York Heart Association functional class, 6-minute walk distance, and peak volume of oxygen consumption was superior compared with NEDD9. However, similar or stronger associations were observed for NEDD9 and peak exercise PVR, CO, and ΔCO , as well as resting right ventricular ejection fraction and right ventricular end-systolic volume, compared with NT-proBNP. The direction of the association with PAWP at peak exercise diverged for NT-proBNP and NEDD9, in which a positive and inverse correlation of similar magnitude was observed, respectively (Table 4).

Plasma NEDD9 and clinical outcome

The area under the curve for predicting PAH diagnosis from plasma NEDD9 was 0.81 ($p < 0.0001$) (Figure 7A). The event-free curve for the combined end point of lung transplant or all-cause mortality is presented in Figure 7B. The PAH cohort was followed for a median of 874 days. Overall, 21.5% of the cohort had an event, including 28 deaths and 6 lung transplants. The 1-year and 3-year event rates were 8.9% and 15.8%, respectively. The lung transplant-free survival hazard ratio after adjusting for age, sex, referral center, and PAH subtype when modeling plasma NEDD9 as a continuous variable was 1.12 (95% CI 1.02–1.22, $p = 0.01$) and 1.75 (95% CI 1.12–2.73, $p = 0.01$) per 1 ng/ml and 5 ng/ml increase, respectively. Compared with

Table 4 Associations Between NT-proBNP or NEDD9 with Exercise and Cardiac Morphological Parameters				
Variable	NT-proBNP		NEDD9	
	ρ coefficient	<i>p</i> -value	ρ coefficient	<i>p</i> -value
Exercise/functional parameters				
NYHA FC	0.305	0.039	0.185	0.218
6MWD	−0.476	0.001	−0.017	0.909
Peak exercise mPAP	0.317	0.043	0.124	0.440
Peak exercise PAWP	0.391	0.014	−0.335	0.037
Peak exercise CO	−0.542	0.001	−0.413	0.010
Delta CO (exercise-rest)	−0.544	0.001	−0.417	0.009
Peak exercise PVR ^a	0.320 ^a	0.050 ^a	0.399 ^a	0.013 ^a
Peak VO ₂	−0.384	0.023	−0.194	0.265
Ve/VCO ₂	0.446	0.007	−0.026	0.881
Max Watts	−0.518	0.001	−0.007	0.967
Cardiac morphology/function				
RV end-diastolic volume	0.192	0.207	0.146	0.340
RV end-systolic volume ^a	0.156 ^a	0.306 ^a	0.313 ^a	0.036 ^a
RV mass	0.311	0.040	0.140	0.364
RV ejection fraction ^a	−0.147 ^a	0.335 ^a	−0.405 ^a	0.006 ^a

Abbreviations: 6MWD, 6-minute walk distance; CO, cardiac output; mPAP, mean pulmonary artery pressure; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA FC, New York Heart Association Functional Class; PAWP, pulmonary artery wedge pressure; PVR, pulmonary vascular resistance; RV, right ventricular; VO₂, volume of oxygen consumption.

Invasive exercise and cardiac structural and functional data acquired by cardiac magnetic resonance imaging were analyzed for all patients in the Johns Hopkins University cohort (*n* = 46). Values for NT-proBNP and NEDD9 were not normally distributed in this population; therefore, data are presented as Spearman correlation coefficient for each association.

^aStatistically significant associations for NEDD9 that were stronger than NT-proBNP, which is the current pulmonary vascular disease biomarker standard.

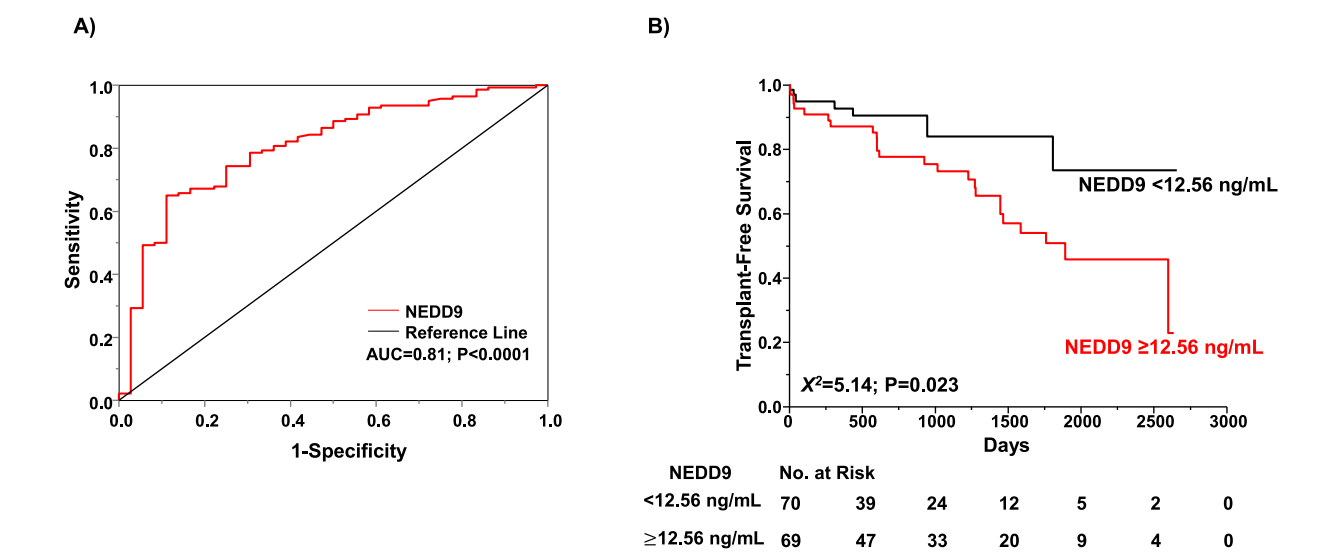


Figure 7 Plasma NEDD9 predicts PAH diagnosis and is associated with adverse clinical events. (A) Receiver operating characteristic curve showing the strength of plasma NEDD9 for predicting PAH diagnosis. (B) Time-to-event plot for unadjusted lung transplantation and mortality-free survival for patients with PAH stratified by the median plasma NEDD9 level from the study cohort (log-rank test, chi-square = 5.14; *p* = 0.023). AUC, area under the curve; PAH, pulmonary arterial hypertension.

patients with a plasma NEDD9 level <12.56 ng/ml, the hazard ratio for event-free survival among patients with NEDD9 ≥ 12.56 ng/ml was 3.28 (95% CI 1.15–9.37, *p* = 0.03).

Discussion

These data show for the first time that circulating levels of the profibrotic protein NEDD9 are increased significantly

in PAH and correlate with key cardiopulmonary hemodynamic and right ventricular parameters that inform prognosis. This effect was maintained despite center-specific differences in control and PAH NEDD9 levels and wide heterogeneity in the clinical profile of the study population for age, sex, and other important clinical covariates. Additionally, NEDD9 associated inversely with left atrial hypertension induced by exercise. This was directionally opposite from NT-proBNP, which is a non-specific

indicator of left- or right-sided myocardial stretch, implying that in this study NEDD9 was not coupled to left atrial hypertension. Further, increased plasma NEDD9 was associated with elevated risk for lung transplantation or mortality. Collectively, these data identify NEDD9 as a potential biomarker that provides important insight on pulmonary vascular—right ventricular pathophysiology and clinical outcome in PAH.

Biomarker discovery has emerged as a major objective in the PAH field owing to delayed and inaccurate diagnosis that is reported commonly by referral centers,¹⁹ and because the optimal strategy for monitoring disease progression requires invasive testing.²⁰ To this end, recent proteomic analyses leveraging multiplex platforms have reported novel independent predictors of adverse outcome, including many proteins unrecognized previously in the pathogenesis of PAH.²¹ Others have reported hypothesis-driven investigations in which the pathophysiological relevance of a putative biomarker was based on data from cardiovascular diseases with overlapping, but not distinct, pathophysiology compared with PAH. These include osteopontin, C-reactive protein, and galectin-3, among others, as well as troponin-T and NT-proBNP that are used commonly in clinical practice today.^{13,22–24} Our work diverges from these prior studies by showing that NEDD9, a protein with specific pathobiological relevance to fibrotic and hypertrophic vascular remodeling in PAH, is also relevant to patients with PAH clinically.⁸

The magnitude of increase in circulating NEDD9 in PAH compared with controls, predictive value of NEDD9 for diagnosing PAH, and the strength of association between NEDD9 and selected hemodynamic predictors of PAH clinical events in this study were equivalent or, in some instances, superior to established biomarkers. For example, although a strong association between NT-proBNP and PVR was reported in one study involving a small cohort of patients with iPAH,¹⁸ subsequent data from cross-sectional or unselected populations suggests that this relationship is comparatively weaker than our findings on NEDD9 and PVR.^{25,26} In addition, the relationship between NEDD9 and mPAP, PVR, and transpulmonary gradient was increased when focusing our analysis on circumspect PAH subtypes, suggesting that pathophysiological or clinical heterogeneity may have contributed to differing results from analyses involving the total PAH population. This observation, in turn, underscores the importance of optimal phenotyping when linking biochemical measurements to patient profile and suggests that additional work on NEDD9 in PAH clinically should consider disease subtypes carefully.

In this study, rising plasma NEDD9 levels were associated with a significant increase in the adjusted risk for mortality or lung transplantation. Taken together with prior work from our group,⁸ we now link NEDD9 to PAH arterial remodeling, abnormal cardiopulmonary hemodynamics, and adverse clinical events. Therefore, a strong framework has been established for NEDD9 as a potentially useful, measurable, and informative indicator of PAH severity relative to pathobiology, pathophysiology, and clinical risk. Our findings support using plasma NEDD9 concentration

as a metric for enrollment in clinical trials studying emergent therapies that aim to inhibit NEDD9 in PAH and other diseases of similar pathobiology²⁷ and suggest that additional studies dedicated to determining the prognostic utility of plasma NEDD9 within the context of patient care are warranted.

A key strength of this work relates to the use of multiple methodologies to verify NEDD9 detection in human plasma, including definitive peptide identification by LC-MS. Results from these experiments imply that full length NEDD9 is present and potentially biologically active in circulation. Thus, although increased NEDD9 is observed from peripheral blood samples in patients with gastric adenocarcinoma¹⁷ and other cancers,²⁸ this work provides additional information that may refine biomarker studies in the future through quantitative proteomic analysis to determine the optimal NEDD9 fragment corresponding to PAH diagnosis, prognosis, and treatment response, for example.

Our prior work demonstrated that pulmonary endothelial NEDD9 is a critical mediator of vascular fibrosis in PAH. It is therefore noteworthy that in CTD-PAH, which is characterized mainly by a fibrotic arteriopathy, NEDD9 was particularly elevated and associated with cardiopulmonary hemodynamics as well as 6-minute walk distance. It is not possible to know from this study if pulmonary endothelial and plasma NEDD9 concentration in any PAH subtype are related quantitatively or mechanistically. However, NEDD9 is reported to translocate to blood in metastatic processes, and transcellular signaling involving pulmonary endothelial NEDD9 has been detailed previously.⁸ Paracrine and endocrine biofunctionality of microRNAs,²⁹ hormones,³⁰ and vasoactive peptides³¹ are recognized increasingly in pulmonary and systemic manifestations of PAH. Findings from this study provide further evidence in support of dedicated studies tracing the afferent and efferent arc of such disease mediators detected systemically.

The mechanisms regulating metabolism of cell-free NEDD9 have not been elucidated. In this study, NEDD9 concentration did not appear to be dependent on renal dysfunction or hemoglobin content, which might have provided insight on its bioelimination. Similarly, an association was not observed between resting CO and NEDD9, implying that regulation of NEDD9 is likely uncoupled from typical neurohumoral signaling pathways in PAH such as the renin-angiotensin axis.¹ By contrast, our findings suggest that NEDD9 regulation may be a pulmonary vascular—right ventricular process, because neither exercise PAWP nor resting PAWP in patients with WHO Group 2 PH (both indicative of elevated left ventricular or left atrial pressure) associated with plasma NEDD9, whereas such an effect was observed between NEDD9 and selected right ventricular—specific measurements. Analyses investigating a potential NEDD9 concentration gradient across differing vascular compartments or across the lung may have provided insight on this issue but were not performed in this study because of the unavailability of samples from appropriate control groups.

This was a retrospective analysis that included a combination of patients with incident and prevalent PAH, is

subject to referral and selection bias, and appears to include batch effects relative to center participation, which, in turn, could have been due to differences in vascular sampling site, changes in protein stability over the study period, or other untested or confounding variables. This was particularly the case for controls, which were not available from all participating centers. Despite improvement in availability of clinical samples through the effort of academic initiatives worldwide, PAH is, nonetheless, a rare disease, making adherence to standardized and tightly controlled collection protocols across institutions challenging.³² This may have affected outcome data in our study, which included patients across a variable and wide follow-up time interval. Similarly, heterogeneity in the diagnostic approach to patients with PAH by center and region may have affected the uniformity of the catheterization (and other clinical) data included in this study. Therefore, additional prospective data are needed to fully characterize the utility of NEDD9 as a bona fide PAH biomarker.

In a multicenter, retrospective, observational study, plasma NEDD9 was increased significantly in PAH. This effect was maintained across 5 PAH subtypes, but elevated NEDD9 was not observed in other forms of PH. Plasma NEDD9 was strongly predictive of PAH diagnosis, correlated with established hemodynamic and right ventricular parameters, and associated with increased hard clinical events. This work, therefore, expands on prior reports implicating NEDD9 in the pathogenesis of PAH and provides important clinical data to suggest NEDD9 may emerge as a useful PAH biomarker with prognostic potential. Future prospective studies utilizing standardized enrollment, NEDD9 measurement, and follow-up protocols are warranted to establish the true clinical utility of NEDD9 in patients with PAH.

Disclosure statement

Dr Maron is a consultant for Actellion. Drs Maron and Alba report co-inventorship on pending patent PCT/US2019/059890. Dr Tedford reports the following conflicts of interest: Merck (hemodynamics core lab), Actellion/J&J (hemodynamics core lab), United Therapeutics (consultant), Arena Pharmaceuticals (consultant), Abiomed (research advisory group), and Abbott (steering committee). Dr Ventetulo reports the following conflicts of interest: Eiger (clinical trial), United Therapeutics (research grant), Acceleron Pharma (Consulting), and CVS Health (spouse is an employee). Dr Leopold is a DSMB committee member for the REHAB-PH study.

The authors thank Ross Tomaino, PhD, at Harvard Medical School for assistance with LC-MS.

Funding to B.A.M.: NIH R56HL131787; 1R01HL139613-01; R21HL145420; National Scleroderma Foundation; and the Cardiovascular Medical Research and Education Foundation (CMREF); B.M.W.: NIH 5T32HL007633-32; J.A.L.: NIH HL125215; American Heart Association 19A1ML34980000; S.H.: NIH K23-HL146889; G.A.A.: Harvard Catalyst 5KL2TR002542-02; P.M.H.: NIH R01 HL114910; and C.E.V.: Institutional Development Award

from the National Institute of General Medical Sciences (P20 GM103652) and NIH R01-HL141268.

Supplementary data

Supplementary data associated with this article can be found in the online version at www.jhltonline.org.

Supplementary materials

Supplementary material associated with this article can be found in the online version at <https://doi.org/10.1016/j.healun.2019.12.002>.

References

1. Maron BA, Galie N. Diagnosis, treatment, and clinical management of pulmonary arterial hypertension in the contemporary era: a review. *JAMA Cardiol* 2016;1:1056-65.
2. Galie N, Barbera JA, Frost AE, et al. Initial use of ambrisentan plus tadalafil in pulmonary arterial hypertension. *N Engl J Med* 2015;373:834-44.
3. McLaughlin VV, Langer A, Tan M, et al. Contemporary trends in the diagnosis and management of pulmonary arterial hypertension: an initiative to close the care gap. *Chest* 2013;143:324-32.
4. Farber HW, Foreman AJ, Miller DP, McGoon MD. REVEAL Registry: correlation of right heart catheterization and echocardiography in patients with pulmonary arterial hypertension. *Congest Heart Fail* 2011;17:56-64.
5. Hemnes AR, Opatowsky AR, Assad TR, et al. Features associated with discordance between pulmonary artery wedge pressure and left ventricular end diastolic pressure in clinical practice: implications for pulmonary hypertension classification. *Chest* 2018;154:1099-107.
6. Weir NA, Brown AW, Shlobin OA, et al. The influence of alternative instruction on 6-m in walk test distance. *Chest* 2013;144:1900-5.
7. Warwick G, Thomas PS, Yates DH. Biomarkers in pulmonary hypertension. *Eur Respir J* 2008;32:503-12.
8. Samokhin AO, Stephens T, Wertheim BM, et al. NEDD9 targets *COL3A1* to promote endothelial fibrosis and pulmonary arterial hypertension. *Sci Transl Med* 2018;10:eaap7294.
9. Zhou RT, He M, Yu Z, et al. Baicalein inhibits pancreatic cancer cell proliferation and invasion via suppression of NEDD9 expression and its downstream Akt and ERK signaling pathways. *Oncotarget* 2017;8:56351-63.
10. Zheng M, McKeown-Longo PJ. Regulation of HGF expression and phosphorylation by TGF- β 1 and cell adhesion. *J Biol Chem* 2002;277:39599-608.
11. Yung LM, Nikolic I, Paskin-Flerlage SD, Pearsall RS, Kumar R, Yu PB. A selective transforming growth factor- β ligand trap attenuates pulmonary hypertension. *Am J Respir Crit Care Med* 2016;194:1140-51.
12. Giampieri S, Manning C, Hooper S, Jones L, Hill CS, Sahai E. Localized and reversible TGF- β signalling switches breast cancer cells from cohesive to single cell motility. *Nat Cell Biol* 2009;11:1287-96.
13. Galie N, Humbert M, Vachiery JL, et al. 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: the Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Heart J* 2016;37:67-119.
14. Oldham WM, Oliveira RKF, Wang RS, et al. Network analysis to risk stratify patients with exercise intolerance. *Circ Res* 2018;122:864-76.
15. Zamanian RT, Hedlin H, Greuenwald P, et al. Features and outcomes of methamphetamine-associated pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2018;197:788-800.
16. Wertheim BM, Lin YD, Zhang YY, et al. Isolating pulmonary microvascular endothelial cells ex vivo: implications for pulmonary arterial

- hypertension, and a caution on the use of commercial biomaterials. *PLOS ONE* 2019;14:e0211909.
17. Karabulut M, Alis H, Afsar CU, et al. Serum neural precursor cell-expressed, developmentally down regulated 9 (NEDD9) level may have a prognostic role in patients with gastric cancer. *Biomed Pharmacother* 2015;73:140-6.
 18. Souza R, Bogossian HB, Humbert M, et al. N-terminal-pro-brain natriuretic peptide as a haemodynamic marker in idiopathic pulmonary arterial hypertension. *Eur Respir J* 2005;25:509-13.
 19. Maron BA, Choudhary G, Khan UA, et al. Clinical profile and underdiagnosis of pulmonary hypertension in US veteran patients. *Circ Heart Fail* 2013;6:906-12.
 20. Maron BA. Hemodynamics should be the primary approach to diagnosing, following, and managing pulmonary arterial hypertension. *Can J Cardiol* 2015;31:515-20.
 21. Rhodes CJ, Wharton J, Ghataorhe P, et al. Plasma proteome analysis in patients with pulmonary arterial hypertension: an observational cohort study. *Lancet Respir Med* 2017;5:717-26.
 22. Lorenzen JM, Nickel N, Krämer R, et al. Osteopontin in patients with idiopathic pulmonary hypertension. *Chest* 2011;139:1010-7.
 23. Geenen LW, Baggen VJM, Koudstaal T, et al. The prognostic value of various biomarkers in adults with pulmonary hypertension; a multi-biomarker approach. *Am Heart J* 2019;208:91-9.
 24. Calvier L, Legchenko E, Grimm L, et al. Galectin-3 and aldosterone as potential tandem biomarkers in pulmonary arterial hypertension. *Heart* 2016;102:390-6.
 25. Allanore Y, Borderie D, Meune C, et al. N-terminal pro-brain natriuretic peptide as a diagnostic marker of early pulmonary artery hypertension in patients with systemic sclerosis and effects of calcium-channel blockers. *Arthritis Rheum* 2003;48:3503-8.
 26. Casserly B, Klinger JR. Brain natriuretic peptide in pulmonary arterial hypertension: biomarker and potential therapeutic agent. *Drug Des Dev Ther* 2009;3:269-87.
 27. Alba GA, Zhang Y-Y, Samokhin AO, et al. NEDD9 is a modifiable target of platelet-pulmonary artery endothelial cell adhesion [abstract]. *Am J Respir Crit Care Med* 2019;199:A4400.
 28. Erturk K, Tas F, Serilmez M, Bilgin E, Duranyildiz D. Significance of serum neural precursor cell-expressed developmentally downregulated protein 9 in melanoma. *Mol Clin Oncol* 2018;8:204-8.
 29. Chen KH, Dasgupta A, Lin J, et al. Epigenetic dysregulation of the dynamin-Related protein 1 binding partners MiD49 and MiD51 increases mitotic mitochondrial fission and promotes pulmonary arterial hypertension: mechanistic and therapeutic implications. *Circulation* 2018;138:287-304.
 30. Maron BA, Opatowsky AR, Landzberg MJ, Loscalzo J, Waxman AB, Leopold JA. Plasma aldosterone levels are elevated in patients with pulmonary arterial hypertension in the absence of left ventricular heart failure: a pilot study. *Eur J Heart Fail* 2013;15:277-83.
 31. Jankowich MD, Wu WC, Choudhary G. Association of elevated plasma endothelin-1 levels with pulmonary hypertension, mortality, and heart failure in African American individuals: the Jackson Heart Study. *JAMA Cardiol* 2016;1:461-9.
 32. Hemnes AR, Beck GJ, Newman JH, et al. PVDOMICS: a multi-center study to improve understanding of pulmonary vascular disease through phenomics. *Circ Res* 2017;121:1136-9.