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Cellular Communication Network Protein 2 in the Right Ventricle of Pulmonary Arterial Hypertension

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Keywords: biomarker | cardiac dysfunction | preclinical model | translational science

ABSTRACT

Cellular communication network 2 (CCN2) is a secreted matricellular protein associated with pulmonary arterial hypertension (PAH) but has not been studied relative to PAH severity, outcomes, or right ventricle (RV) structure and function in a large human cohort and preclinical animal model. This study assessed the associations between CCN2 and PAH severity, survival, hemodynamic measurements, and cardiovascular dysfunction. Serum CCN2 levels were compared in 2548 adults with PAH and 216 controls. CCN2 levels in PAH patients were compared to functional and hemodynamic measurements, and survival outcomes. RV-pulmonary artery coupling and RV morphology were also assessed in a small subset of patients via pressure–volume loops and cardiac magnetic resonance imaging. In a preclinical PAH model, plasma CCN2 levels were compared between ventricles with PAH progression. CCN2 mRNA levels in both ventricles in the preclinical model were measured to compare with morphologic histologic variables. CCN2 serum levels were significantly higher in PAH compared to controls ($p < 0.0001$). Higher CCN2 levels were associated with reduced RV contractility ($p = 0.003$). Higher CCN2 levels were associated with worse 6MWD ($p = 0.035$), and higher risk of mortality or transplant ($p = 0.025$). In the preclinical model, prepulmonary CCN2 plasma levels increased with the progression of disease. CCN2 mRNA levels in the RV were associated with decreased RV

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capillary density ($p = 0.015$) and increased RV fibrosis ($p = 0.045$). Though more investigation is needed, it appears that CCN2 plays a role in the development of PAH and potentially in RV maladaptation in PAH.

Pulmonary arterial hypertension (PAH) is a severe and progressive disease caused by elevations in pulmonary arterial pressures, adverse vascular remodeling, and progressive right ventricle (RV) dysfunction with high morbidity and mortality [1]. Identification of circulating factors associated with disease severity may provide insights into the disease's development and progression [2–6]. Previous studies showed that several insulin-like growth factor binding proteins (IGFBPs) are associated with PAH disease severity including clinical variables, hemodynamics, and mortality [3, 6–10]. Cellular communication network factor 2 (CCN2), also known as IGFBP8 or connective tissue growth factor (CTGF), is a secreted vascular protein with upregulated RV expression in Sugen/hypoxia and monocrotaline rat models of PAH and human PAH RV tissue [11]. CCN2 is important in cell proliferation, differentiation, survival, adhesion, and migration and is found in several tissues, including the heart [7, 12–17]. CCN2 has also been studied previously in a systematic review and found associations with CCN2 levels and PAH [18, 19]. However, both studies were limited to a small subset of PAH (Group 1, congenital heart disease related), and there are few studies of CCN2 in other PAH groups. RV systolic dysfunction complicates PAH but is difficult to measure until late stages of the disease [20–24]. Hence, an early biomarker of early RV changes associated with PAH would guide the approach and therapies in patients with this high-mortality disease.

Right ventricle-pulmonary artery (RV-PA) coupling is an early marker of RV dysfunction which is best measured with pressure–volume (PV) loop analysis [23–25]. PV loops are generated using high-fidelity conductance catheters while altering loading conditions. The slope of the end-systolic PV relation represents the end-systolic elastance (Ees), an indicator of intrinsic contractility that is load independent [23, 24]. Pulmonary effective arterial elastance (Ea) offers a more comprehensive evaluation of RV afterload than pulmonary vascular resistance, as it takes into account both pulsatile and resistive elements [23, 24, 26]. RV-PA coupling is expressed as the ratio of these elastances (Ees/Ea) [23, 24]. Previous studies have revealed that a lower Ees/Ea ratio, or "uncoupling," correlates with worse outcomes in PAH, surpassing traditional RV measurements like RV ejection fraction or the ratio of RV stroke volume to end-systolic volume [23, 24, 26]. However, cardiac MRI and catheterization are not feasible with every visit, which makes a sensitive and specific biomarker that correlates with these findings to be clinically meaningful.

The role of CCN2 at the cellular level is an area of active research. Park et al. looked at the RV transcriptomes in the monocrotaline and Sugen/hypoxia rat and found multiple candidate genes including CCN2 at the basis of pathology [11]. Further, they evaluated the possible mechanism of CCN2 and found evidence of its role in altering cellular metabolism [11]. Tam et al. evaluated CCN2 in a knockout mouse and found reduced fibronectin expression that persisted even with exogenous transforming growth factor ß administration suggesting CCN2 is involved in the fibrotic pathway [27]. Finally, Tejera-Muñoz et al. recently showed CCN2 increased transforming growth factor ß in in vitro smooth muscle cells [15]. These important studies have added to the pathophysiologic understanding of CCN2, but there have been no studies of the role of CCN2 in RV morphologic changes.

In this study, using two independent cohorts of patients with PAH, we determined the relationships between circulating CCN2 and metrics of disease severity, hemodynamics, and measures of intrinsic RV dysfunction. Further, in a preclinical model, we assessed the relationships between CCN2 plasma levels and PAH diagnosis and severity, as well as CCN2 mRNA tissue expression in the RV and measures of pathologic RV remodeling to test the specificity of CCN2 levels to RV dysfunction in PAH development.

1 | Methods

1.1 | Human Study Cohorts

National Biological Sample and Data Repository for Pulmonary Arterial Hypertension (PAH biobank, PAHB) is an NHLBI-funded static repository of adult ($n = 2548$) World Health Organization Group 1 PAH patients. This repository includes biological samples, genetic, and clinical data from 38 centers. Information collected in the biorepository includes hemodynamic data from cardiac catheterization, PAH subtype, clinical severity data including six-minute walk distance (6MWD), New York Heart Association functional class (NYHA FC), and drug therapy. Subjects were included if they were at least 18 years old at the time of diagnosis and the time of enrollment. Most subjects had a prior diagnosis of pulmonary hypertension at the time of enrollment. The median time to enrollment was 3.36 years. The median follow-up time was 3.3 years. PAHB enrollment and informed consent were approved by the University of Cincinnati Institutional Review Board. All participants provided informed consent to participate [8, 10, 28].

Johns Hopkins Pulmonary Hypertension (JHPH) Cohort consists of patients with known or suspected PAH referred to Johns Hopkins University Pulmonary Hypertension Center and who underwent a comprehensive PAH evaluation including same day right heart catheterization, PV loop analyses, cardiac magnetic resonance, and laboratory evaluation with biobanking of samples drawn at the time of the catheterization [23, 24]. Participants were enrolled if they were over 18 years old and provided informed consent. Exclusion criteria included patients found to have pulmonary hypertension due to left heart disease on catheterization, hemodynamically unstable patients (systolic blood pressure < 90 mmHg, or with a vasopressor requirement), patients with RV clot or septal aneurysm, and patients who were pregnant. Any patients meeting criteria for PAH were followed prospectively with serial clinical evaluations [21, 23, 24]. This population of patients were enrolled from 2013 to

2024. Patients in this cohort were followed up at routine clinic appointments with the Johns Hopkins Pulmonary Hypertension Clinic. Transplant-free survival data were obtained by chart review with the censoring date assigned to the patient's most recent clinical contact before July 2024. All participants had follow-up data available to ascertain survival, and the median follow-up time was 8.5 years. This longitudinal database was approved by the Johns Hopkins Institutional Review Board. Thirty-four patients with PAH and PV loop data from this cohort were included for analysis to provide in-depth phenotyping and evaluation of RV-PA coupling that is unavailable in the PAHB cohort.

Control Cohort. In total, 216 samples were obtained from the GeneSTAR study and were used as adult non-PAH controls. The GeneSTAR study enrolled healthy adult siblings, offspring, and spouses of individuals who had early onset (less than 60 years old) myocardial infarction, unstable angina, or coronary artery bypass graft surgery [29, 30].

1.2 | Laboratory Methods

1.2.1 | Enzyme-Linked Immunosorbent Assay

Human CCN2 serum concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) per the manufacturer's protocol in a single batch over a 90-day period. The overall interplate CV% was 2.85% across 45 plates. All assay results were blinded to clinical outcomes and unblinded only for statistical analysis. The following kits were used: ELISA- R-PLEX Human IGFBP-8 Assay, Cat# K151N8R-2, Meso Scale Discovery, Rockville, MD, and Abcam Rat CTGF ELISA Kit Assay, Cat#ab275897, Cambridge, UK.

1.2.2 | Cardiac MR and Right Heart Catheterization in JHPH Cohort

The protocol for obtaining PV loops has been previously described and validated [23–25]. After baseline right heart catheterization, a 4-French balloon wedge catheter and 5-French PV catheter were positioned at the RV apex (SPC-570-2 or RP-CA-41103-PN, Millar, Houston, TX). PV loops were recorded to determine multibeat (MB) Ees, effective Ea, and their ratio (Ees/Ea) as a marker of RV-pulmonary arterial coupling. Signals were analyzed to determine the electrode pairs needed to summate the total volume signal. Steady-state data were acquired during gentle end-expiratory breath hold to generate resting PV loops. Preload was then reduced via Valsalva maneuver and, if needed, manual external inferior vena cava compression, to create a family of PV loops, using a previously validated method. Two experienced investigators reviewed real-time data acquisition to ensure adequate preload reduction such that the end-systolic PV relationship could be determined. Multiple loops from both steady-state and preload measurements were averaged. End-systolic PV points were determined based on the set of loops recorded, and iterative versions of perpendicular regression were used to derive the slope of the PV loops (Ees). Ea was then calculated as a ratio of the end-systolic pressure to stroke volume, and then RV-PA coupling was determined by the ratio of Ees/Ea. This protocol was utilized to determine MB Ees/Ea as a marker of PA-RV coupling [23–25].

1.2.3 | Sugen/Hypoxia PAH Model

Male Wistar rats were used to create a Sugen/hypoxia PAH rat model ($n = 6$) as previously described in detail [31]. All animal experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Animal Care and Use Committee at Johns Hopkins University School of Medicine. CCN2 plasma concentrations were measured by ELISA from pre- and postpulmonary blood samples via RV and left ventricle (LV) puncture at 7, 14, and 21 days. Additionally, tissue samples from the RV (at 14 and 21 days) and LV (at 21 days) were extracted for further analysis, and reverse transcriptase polymerase chain reaction was used to analyze CCN2 mRNA levels from the RV and LV free wall tissue.

1.3 | Statistical Analysis

Baseline characteristics, clinical, and hemodynamic data are presented as number and percentage for categorical variables or median and interquartile range for continuous variables. Continuous and categorical variables were evaluated using Spearman's rank correlation or Wilcoxon rank sum test, respectively. CCN2 serum levels were natural log transformed for the PAHB cohort and log transformed for the JHPH cohort. Linear and logistic regression analyses (adjusted for age and sex) were used for continuous and categorical variables, respectively.

Cox proportional hazard model (adjusted for age and sex) was used to evaluate the risk of composite outcome based on CCN2 concentration. Kaplan–Meier analysis was used with time to a composite outcome (death or transplant). Cohorts were dichotomized at the Youden cutoff from the JHPH cohort. The same Youden cutoff was used for both cohorts for standardization. For all analyses, a p value less than 0.05 was considered statistically significant. Statistical analysis was performed with STATA (Version 15.1; 2018; StataCorp LLC, College Station, TX) and GraphPad Prism (Version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts, USA).

2 | Results

2.1 | Subject Demographics

Demographic characteristics, biometrics, and CCN2 levels at enrollment for each cohort are summarized in Table 1 with median and interquartile ranges or number and percentages as appropriate. The median age was 62 years for the JHPH cohort, 52 years for the PAHB cohort, and 50 years for the GeneStar control group. The PAHB cohort was 79% female, compared to 93% in the JHPH cohort and 64% in the GeneStar cohort. The follow-up time frame for the JHPH cohort was 8.5 years and 3.3 years for the PAHB cohort after censoring for loss to follow-up, lung transplant, or death. All-cause mortality in the JHPH cohort was 38% compared to 16% in the PAHB cohort.

TABLE 1 | Demographics table for PAHB, JHPH, and controls with clinical and hemodynamic characteristics. Numbers are given with medians and interquartile ranges or percentages as denoted in the table.

Variables	PAHB cohort	JHPH	GeneStar control cohort
Demographics			
Subjects, *n*	2548	34	216
Age (years)	52 (39–63)	62 (51–67)	50 (28–74)
Follow-up (years)	3.3 (1.9–4.1)	8.5 (5.9–9.8)	
Sex, *n* female (%)	1996 (79)	27 (93)	139 (64)
Race: EA, AA, other, *n* (%)	2010/323/143 (78/12/10)	29/3/2 (85/9/6)	113/102/0 (52/47/0)
NYHA FC, *n* I/II/III/IV (%III/IV)	115/540/951/149 (43)	0/4/13/17 (50)	
IPAH/CHD-APAH/CTD-APAH/FPAH/ drug induced, *n* (%)	1090/199/763/99/0 (43/ 8/30/4/0)	14/0/19/0/1 (41/0/56/ 0/3)	
6MWD (m)	344 (251–421)	358 (295–453)	
REVEAL 2.0 class (low/intermediate/high)	2471/99/12	19/6/9	
Deaths, *n* (%)	362 (16)	13 (35)	
CCN2 levels (ng/mL)	42.774	42.896	14.719
Hemodynamics			
RAP (mmHg)	8 (5–12)	8 (4–10)	
mPAP (mmHg)	49 (40–58)	39 (29–51)	
mPCWP (mmHg)	10 (7–13)	10 (6–12)	
PVR (Wood units)	9 (6–13)	6 (4–10)	
CO (L/min)	4 (3–5)	4 (3.6–5.1)	
CI (L/min/m^2)	3 (2–3)	3 (1.9–2.8)	
Therapies, n (%)			
No therapy	0 (0)	16 (47)	
PDE5 monotherapy	747 (30)	3 (9)	
PDE5/ERA	950 (37)	15 (44)	
PDE5/ERA/IV/PCA	844 (33)	1 (3)	

Abbreviations: 6MWD, six-minute walk distance; AA, African America; CHD-APAH, congenital heart disease acquired pulmonary arterial hypertension; CI, cardiac index; CO, cardiac output; CTD-PAH, connective tissue disease-acquired pulmonary arterial hypertension; EA, European American (Caucasian); ERA, endothelin receptor antagonist; FPAH, familial pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; mPAP, mean pulmonary arterial pressure; mPCWP, mean pulmonary capillary wedge pressure; NYHA FC, New York Heart Association functional class; PDE5, phosphodiesterase-5; PVR, pulmonary vascular resistance; RAP, right atrial pressure; SC PCA, subcutaneous prostacyclin analog.

2.2 | CCN2 Levels Are Elevated in PAH Diagnosis

Serum CCN2 levels were measured in 2548 adults with PAH from the PAHB and 216 adult controls. CCN2 levels were 2.4 times higher in the PAHB cohort compared to control patients ($p < 0.0001$, Figure 1). CCN2 levels were similar between the PAHB and JHPH cohorts (Table 1). There were no differences in CCN2 levels based on PAH subtype in the PAHB cohort ($p = 0.14$).

2.3 | CCN2 Is Associated With RV Dysfunction

The JHPH cohort underwent PV and hemodynamic analysis. Average baseline hemodynamics for this cohort include a mean right atrial pressure of 8 mmHg, mean PA pressure of 39 mmHg, mean pulmonary capillary wedge pressure of 10 mmHg, pulmonary vascular resistance of 6 Wood units, and cardiac output of 4.4 L/min (Table 1). There was no association between hemodynamics and CCN2 levels (all p values > 0.5). CCN2 levels were associated with lower RV Ees (Spearman rho coefficient –0.46, $p = 0.018$) and impaired RV-pulmonary arterial coupling (Ees/Ea ratio, Spearman rho coefficient –0.45, $p = 0.023$) (Table 2). On linear regression adjusted for age and sex, CCN2 was negatively associated with Ees ($p = 0.003$), but had a nonsignificant association with Ees/Ea ($p = 0.076$) (Table 3). There was no correlation with cardiac magnetic resonance myocardial function variables including RV end diastolic volume, RV stroke volume, RV ejection fraction, LV stroke volume, LV end diastolic volume, or LV ejection fraction (Tables 2 and 3).

2.4 | CCN2 Is Associated With Clinical Variables

In the PAHB cohort, unadjusted analysis demonstrated a significant negative association between CCN2 levels and 6MWD (Spearman rho −0.089, $p = 0.001$), stroke volume (Spearman

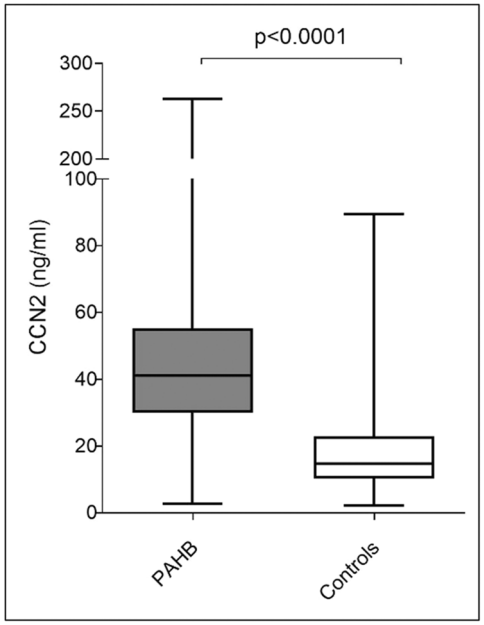

FIGURE 1 | Box and whisker plot of CCN2 concentrations in PAH samples and control samples (ng/mL). PAHB, pulmonary arterial hypertension biobank.

TABLE 2 | Unadjusted associations between CCN2 levels, cardiac magnetic resonance, and pressure–volume loop measurements from the JHPH cohort with spearman rho correlation coefficients presented with *p* values.

Variable	Spearman rho coefficient	*p* value
LV ED vol (mL)	−0.16	0.38
LV ES vol (mL)	−0.092	0.62
LV SV (mL)	−0.17	0.36
LVEF (%)	−0.11	0.57
LV mass (g)	−0.15	0.43
RV ED vol (mL)	−0.21	0.25
RV ES vol (mL)	−0.17	0.37
RV SV (mL)	−0.32	0.083
RV SV/BSA (mL/m^2)	−0.23	0.2
RV mass ED (g)	0.11	0.57
RVEF (%)	0.0024	0.99
Ees (mmHg/mL)	***−0.46***	***0.018***
Ea (mL/mmHg)	0.023	0.91
Ees/Ea	***−0.45***	***0.023***
Beta	0.28	0.19
Eed (mmHg/mL)	0.12	0.57

Significant values are italicized and bolded.
Abbreviations: Ea, arterial elastance; Eed, end diastolic elastance; Ees, end-systolic elastance; LVED vol, left ventricular end diastolic volume; LVEF, left ventricular ejection fraction; LVES vol, left ventricular end-systolic volume; LVSV, left ventricular stroke volume; RVED vol, right ventricular end diastolic volume; RVEF, right ventricular ejection fraction; RVES vol, right ventricular end-systolic volume; RVSV, right ventricular stroke volume; RVSV/BSA, right ventricular stroke volume to body surface area ratio.

TABLE 3 | Adjusted associations between CCN2 levels and cardiac magnetic resonance and pressure–volume loop measurements from the JHPH cohort with linear regression correlation coefficients presented with regression coefficients, confidence intervals (CI), and *p* values.

Variable	Linear regression coefficient (95% CI)	*p* value
LV ED vol (mL)	−8.9 (−40 to 22)	0.55
LV ES vol (mL)	1 (−13 to 15)	0.88
LV SV (mL)	−10 (−31 to 11)	0.33
LVEF (%)	−4.1 (−10.6 to 2.4)	0.21
LV mass (g)	−1.2 (−23 to 21)	0.91
RV ED vol (mL)	5 (−55 to 65)	0.86
RV ES vol (mL)	12 (−39 to 63)	0.63
RV SV (mL)	−7.1 (−28 to 14)	0.49
RV SV/BSA (mL/m^2)	−1.8 (−13 to 9)	0.73
RV mass ED (g)	14 (−0.93 to 28.14)	0.065
RVEF (%)	−4.3 (−16.0 to 7.4)	0.45
Ees (mmHg/mL)	−0.73 (−1.17 to −0.28)	0.003
Ea (mL/mmHg)	−0.15 (−0.88 to 0.57)	0.66
Ees/Ea	−0.63 (−1.32 to 0.07)	0.076
Beta	0.01 (−0.010 to 0.031)	0.3
Eed (mmHg/mL)	0.058 (−0.13 to 0.24)	0.52

Abbreviations: Ea, arterial elastance; Eed, end diastolic elastance; Ees, end-systolic elastance; LVED vol, left ventricular end diastolic volume; LVEF, left ventricular ejection fraction; LVES vol, left ventricular end-systolic volume; LVSV, left ventricular stroke volume; RVED vol, right ventricular end diastolic volume; RVEF, right ventricular ejection fraction; RVES vol, right ventricular end-systolic volume; RVSV, right ventricular stroke volume; RVSV/BSA, right ventricular stroke volume to body surface area ratio.

rho −0.085, $p = 0.001$), and cardiac output (−0.056, $p = 0.006$). Higher CCN2 concentrations in the PAHB cohort were positively correlated with heart rate (Spearman rho 0.061, $p = 0.022$), pulmonary vascular resistance (Spearman rho 0.044, $p = 0.035$), and NYHA FC ($p = 0.008$). REVEAL scores in the PAHB cohort were evaluated as a validated predictive algorithm for 1-year survival in PAH to predict mortality [11, 17]. In the PAHB cohort, higher CCN2 levels were associated with a higher REVEAL score (rank sum $p = 0.009$). There were no statistically significant differences in CCN2 levels based on PAH subtype as shown in Table 4.

On linear regression, when adjusted for age and sex, higher CCN2 levels were associated with decreased 6MWD (β coefficient = −16.67 m, $p = 0.035$), worse NYHA FC (β coefficient = 0.084, $p = 0.026$), stroke volume (β coefficient = −0.005 L, $p = 0.002$), cardiac index (β coefficient = −0.12, $p = 0.014$), and mean right atrial pressure (β coefficient = 0.31 mmHg, $p = 0.012$). CCN2 concentrations were not associated with mean PA pressure, cardiac output, heart rate, or pulmonary vascular resistance after adjusting for age and sex after regression (Table 5).

Using a Cox proportional hazards model (adjusted for age and sex), for every natural log unit higher in CCN2 concentration, there was an increased likelihood of death or transplant with a

TABLE 4 | Median CCN2 levels per subgroup of PAH. There was no difference between the groups with p value = 0.14.

PAH subtype	Median CCN2 level (ng/mL, IQR)
IPAH	42.231 (32.093–56.966)
CHD-APAH	40.324 (27.652–43.942)
CTD-PAH	41.780 (30.889–55.146)
FPAH	42.605 (31.313–56.751)
Kruskal–Wallis p value = 0.14	

Abbreviations: CHD-APAH, congenital heart disease acquired pulmonary arterial hypertension; CTD-PAH, connective tissue disease-acquired pulmonary arterial hypertension; FPAH, familial pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension.

hazard ratio of 1.313 (95% CI 1.034–1.666; $p = 0.025$) in the PAHB cohort. In the JHPH cohort, higher natural log transformed CCN2 levels were associated with lower transplant-free survival with a Cox regression analysis hazard ratio of 11.4 (95% CI 1.04–125; $p = 0.046$).

Kaplan–Meier analysis was completed for visualization of survival outcomes. The JHPH cohort showed a significant difference in composite outcome (death or transplant) when dichotomized at the enrollment CCN2 concentration Youden cutoff (43.039 ng/mL) ($p = 0.036$) (Figure 2B). This is a survival rate at 6 years of 60% for patients above the Youden cutoff and 90% below the Youden cutoff. In the PAHB cohort, there was no difference in composite outcome when dichotomized at the Youden cutoff for the JHPH cohort ($p = 0.50$) (Figure 2A).

TABLE 5 | Adjusted associations between CCN2 and functional variables and hemodynamics in the PAHB cohort. Each regression coefficient represents a separate regression model with adjustment for age and sex.

Linear regression	Coefficient	95% CI	p value
Functional assessments			
6MWD (m)	***−16.673***	***−32.19, −1.16***	***0.035***
Hemodynamics			
Stroke volume (mL)	***−0.005***	***−0.008, −0.002***	***0.002***
mRAP (mmHg)	***0.31***	***0.138, 1.107***	***0.012***
mPAP (mmHg)	0.31	−0.856, 1.476	0.602
mPCWP (mmHg)	−0.053	−0.408, 0.303	0.772
PVR (Wood units)	0.409	−0.099, 0.918	0.114
CO (L/min)	−0.095	−0.274, 0.085	0.302
CI (L/min/m^2)	***−0.124***	***−0.223, −0.025***	***0.014***
Logistic regression	**Coefficient**	**95% CI**	***p* value**
NYHA -FC	***0.084***	***0.01, 0.158***	***0.026***

Significant variables are bolded and italicized.
Abbreviations: CI, cardiac index; CO, cardiac output; 6MWD, 6 minute walk distance; mRAP, right atrial pressure; mPAP, mean pulmonary arterial pressure; mPCWP, mean pulmonary capillary wedge pressure; NYHA-FC, New York Heart Association functional class; PVR, pulmonary vascular resistance. Other abbreviations as noted in Table 2 legend.

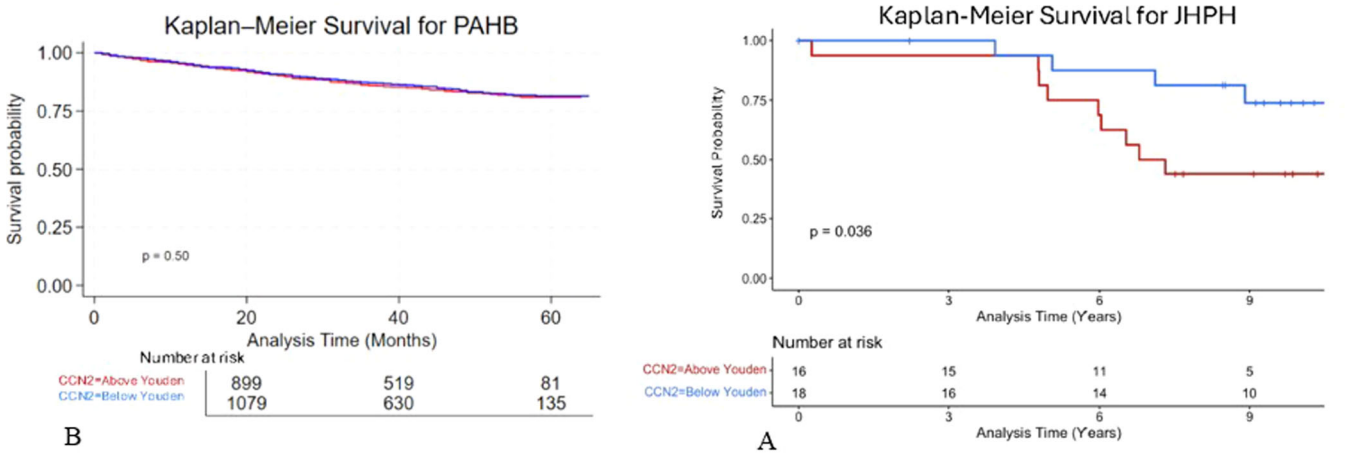

FIGURE 2 | Kaplan–Meier analysis of survival of JHPH cohort (A) and PAHB cohort (B). Cohorts are dichotomized at the Youden cutoff from the JHPH cohort.

TABLE 6 | Association of CCN2 mRNA in the RV and LVs of Sugen–hypoxia animals versus Sham. There were no statistically significant differences between RV and LV mRNA levels within the same animal, but there were significant differences between models for the RV only.

Animal	Mean RV CCN2 mRNA levels ($2^{-(\Delta\Delta Ct)}$)	Mean LV CCN2 mRNA levels ($2^{-(\Delta\Delta Ct)}$)	*p* value comparing RV to LV
Sugen–hypoxia	6.61 (± 1.77)	12.3 (± 9.45)	0.28
Sham	1.04 (± 0.30)	1.16 (± 0.52)	0.75
p value comparing models by RV or LV	0.0008	0.0571	

2.5 | CCN2 Levels Associated With RV Morphologic Changes in a Preclinical Model

Sugen/hypoxia animals were compared to Sham animals and had increased right heart pressures and increased Fulton index when assessed at 21 days. Pre- and postpulmonary plasma samples were collected in both PAH animals and Sham animals, and CCN2 levels were measured by ELISA. There were statistically significant differences between the two models RV CCN2 mRNA with mean mRNA level of 6.61 in the Sugen–hypoxia animal versus 1.04 in the Sham animal ($p = 0.008$). There were no statistically significant differences between CCN2 levels in the RV and LV within the same animal (Table 6).

CCN2 mRNA tissue expression was then measured in the RV and LV of the Sugen/hypoxia PAH model by quantitative PCR. RV CCN2 mRNA expression was inversely correlated with RV capillary density (Spearman rho –0.81, $p = 0.015$) and positively correlated with RV fibrosis (percent area) (Spearman rho 0.59, $p = 0.045$), RV systolic pressure (Spearman rho 0.721, $p = 0.002$), Fulton's index (Spearman rho 0.735, $p = 0.001$), and RV weight indexed to body weight (Spearman rho 0.703, $p = 0.002$) (Table 7). There were no significant correlations between CCN2 mRNA expression and LV tissue variables.

TABLE 7 | Associations of RV CCN2 mRNA levels in the Sugen/hypoxia PAH rat model and RV tissue variables at 14 and 21 days and LV tissue variables at 21 days. Spearman rho correlation coefficients presented with *p* values.

RV variables	Spearman rho	*p* value
Fulton index	***0.735***	***0.001***
RV/body weight ratio (mg/g)	***0.703***	***0.002***
RV fibrosis (% area)	***0.587***	***0.045***
RVSP (%)	***0.721***	***0.002***
RV weight (g)	***0.685***	***0.003***
RV capillary density (capillaries/mm^2)	***−0.810***	***0.015***
LV/body weight ratio (mg/g)	0.6429	0.0962
LV fibrosis (% area)	0.4286	0.2992
LV weight (g)	0.2857	0.5008
LV capillary density (capillaries/mm^2)	−0.4524	0.2675

Significant variables are bolded and italicized.
Abbreviations: Fulton index = RV/(LV + S), RVSP = right ventricular systolic pressure.

3 | Discussion

Our study used multiple patient cohorts to investigate the role of CCN2 in PAH. Our study demonstrates that CCN2 correlates with PAH diagnosis and prognosis with specificity for the RV. This RV specificity was demonstrated in both human cardiac catheterization data and a preclinical model of disease. We found that CCN2 concentrations were markedly higher in PAH patients compared to controls and that CCN2 levels in adult PAH patients correlate with hemodynamics and markers of severity including NYHA FC and 6MWD.

RV-PA coupling is an invasive measure of RV function that is deranged even in subjects with preserved RV ejection fraction suggesting that RV-pulmonary arterial uncoupling can be an early signal of impending RV dysfunction in patients with PAH [23, 24]. In a small cohort of patients from Johns Hopkins Hospital with available PV loop assessments, we found that CCN2 levels correlated with impaired load-independent RV contractility (Ees), which remained significant on regression analysis. RV-PA coupling (Ees/Ea) was significant on initial analysis, but lost significance with regression although trended towards it. This analysis is limited by a small but deeply phenotyped cohort. The small sample size could limit the ability to detect a statistically significant difference in RV-PA coupling. These results suggest that CCN2 could be a noninvasive biomarker for early RV systolic dysfunction in patients with PAH and could be a potential therapeutic target for PAH related RV dysfunction.

The relationship of CCN2 in the PAHB and JHPH cohorts and invasive hemodynamic variables was less robust with low correlation coefficients, but the result remains significant, nonetheless. Stroke volume, right atrial pressure, and cardiac index worsened with increasing CCN2 levels. The clinical utility of these hemodynamic associations remains unknown with the small correlation coefficients seen in this study. However, the role of CCN2 in PAH may be more related to early RV dysfunction and therefore may not have strong associations with other hemodynamic variables.

Regarding prognostication, this study showed that the CCN2 levels correlate with a composite outcome of death or transplant on Cox proportional hazards models for both the JHPH and PAHB cohort. On Kaplan–Meier analysis, in the JHPH cohort, there was a statistically significant difference in outcomes.

However, there was no difference in outcomes in the PAHB cohort when CCN2 was dichotomized at the JHPH Youden cutoff. The same cutoff point was used between groups to standardize the survival analyses as best as possible. We suspect several reasons underpinning this difference in Kaplan–Meier analysis. First, the PAHB cohort was followed for a shorter period compared to the JHPH cohort (3.3 years vs. 8.5 years). The lack of extended follow-up time for the PAHB cohort limits the ability to interpret longer term outcome analyses, and this difference in follow-up period could account for the differences in outcome seen across the two groups. It also limits our ability to understand CCN2 trends with time, RV recovery, and therapy initiation. Second, the JHPH cohort had higher REVEAL 2.0 scores compared to the PAHB and likely sicker patients (Table 1). Third, the JHPH cohort is enriched with patients who have connective tissue diseases and more specifically scleroderma which confers a higher mortality in PAH [32]. Finally, the PAHB cohort is younger than JHPH (52 years vs. 62 years, respectively). These are potential reasons that likely lead to the differences in the Kaplan–Meier analysis.

CCN2 has been shown to increase transforming growth factor-β receptor expression in vascular smooth muscle cells in vitro [12]. In the Sugen/hypoxia rat model of pulmonary hypertension, one study found that CCN2 knockout animal resulted in a significant decrease in pulmonary vasculature remodeling as well as improvement in PA and right ventricular hemodynamics [27]. That study also evaluated the role of CCN2 in pulmonary fibrosis and found that gene deletion significantly reduced pulmonary interstitial scarring and fibrosis via modulation of transforming growth factor-β signaling [27].

In our study of Sugen/hypoxia rats, we found a direct association between CCN2 tissue mRNA, RV systolic pressure, and RV fibrosis with an inverse relationship with RV capillary density. Our data demonstrated that CCN2 in the RV is expressed more highly in the PAH model than the Sham model. This finding was not seen in the LV, and there was no difference in CCN2 levels between the RV and the LV within each model suggesting that the origin of CCN2 is the RV, and differential RV expression of CCN2 exists between Sham and PAH models. These associations were not seen in the LV which suggests a larger role of CCN2 in RV maladaptation in PAH progression. Previous murine data with a preclinical animal model support the role of CCN2 in the development of lung fibrosis [27], pulmonary vascular remodeling, and right ventricular specificity [11, 15]. As CCN2 is a modulator of the transforming growth factor-β pathway, further studies should focus on the cellular mechanisms of action of CCN2 and its full effects on the transforming growth factor-β signaling pathway as a potential therapeutic target for PAH.

Collectively, these results suggest that CCN2 could be a non-invasive biomarker for early RV systolic dysfunction and developing RV fibrosis in patients with PAH and could be a target for potential future treatments in RV dysfunction. Given that RV dysfunction is a poor prognostic indicator in PAH, more studies should be done to further elucidate the biological pathways between CCN2 and impaired RV-PA coupling and RV systolic dysfunction. In addition, future studies should evaluate genotype associations of CCN2 levels and PAH outcomes by investigating single-nucleotide polymorphisms in a PAH biorepository with access to genetic data. Many studies are ongoing to find biomarkers of PAH and CCN2 could be a candidate for inclusion in panels in the future pending further research.

Author Contributions

Carly E. Byrd: data analysis, interpretation, manuscript writing. **Jennifer E. Schramm:** data interpretation, manuscript preparation and editing. **Jun Yang:** data analysis, interpretation, manuscript editing. **Allan E. Barnes:** data acquisition, laboratory assays. **Megan Griffiths:** data interpretation, data analysis, manuscript editing. **Anjira S. Ambade:** data interpretation, data analysis. **Darin T. Rosen:** data analysis and interpretation, manuscript writing. **Ilton M. Cubero Salazar:** data interpretation, data analysis. **Catherine E. Simpson:** data acquisition, data interpretation. **Ryan J. Tedford:** data interpretation, data analysis. **Steven Hsu:** data interpretation, data analysis. **Dhananjay Vaidya:** data analysis and interpretation. **Todd M. Kolb:** data acquisition, data interpretation. **Michael W. Pauciulo:** data acquisition. **William C. Nichols:** data acquisition, data interpretation. **David D. Ivy:** data acquisition, data interpretation. **Eric D. Austin:** data acquisition, data interpretation. **Paul M. Hassoun:** data interpretation, data analysis. **Rachel L. Damico:** data acquisition, data interpretation, data analysis, manuscript editing. **Allen D. Everett:** data acquisition, data interpretation, data analysis, manuscript editing.

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Disclosure

Allen D. Everett takes responsibility for the content of the manuscript including the data and analysis.

Ethics Statement

Approval for this study was given by the Johns Hopkins School of Medicine IRB (IRB00185087). Approval for the PAHB cohort registry was given by the University of Cincinnati IRB. Approval for the JHPH cohort registry was given by the Johns Hopkins School of Medicine IRB.

Conflicts of Interest

Dr. Byrd, Dr. Schramm, Dr Yang, Allan Barnes, Dr. Griffiths, Dr. Ambade, Dr. Rosen, Dr. Cubero Salazar, Dr. Simpson, Dr. Hsu, Dr. Vaidya, Michael W. Pauciulo, Dr. Nichols, and Dr. Damico have no conflicts to disclose. Dr. Tedford reports no disclosures relative to this manuscript. Dr. Tedford is Deputy Editor for the *Journal of Heart and Lung Transplantation*. He reports general disclosures to include consulting relationships with and receiving honorarium from Abbott, Acorai, Adona, Aria CV Inc., Acceleron/Merck, Alleviant, Boston Scientific, Cytokinetics, Edwards LifeSciences, Gradient, Medtronic, Morphic Therapeutics, Restore Medical, and United Therapeutics. Dr. Tedford serves on steering committee for Abbott, Edwards, Endotronix, and Merck as well as a research advisory board for Abiomed. He also does hemodynamic core lab work for Merck. Dr. Kolb reports no disclosures relative to this manuscript. Dr. Kolb reports general disclosures to include a leadership role on the medical advisory committee of Team Phenomenal Hope and stock options in Oxiwear Inc. Dr. Ivy reports no

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