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Cellular sources of IL-6 and associations with clinical phenotypes and outcomes in PAH

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Summary:
Circulating interleukin-6, a pro-inflammatory cytokine produced by pulmonary arterial smooth muscle cells, is significantly associated with clinical phenotypes and survival in pulmonary arterial hypertension, which may guide individualized disease management.

Abstract

The pro-inflammatory cytokine interleukin-6 (IL-6) has been associated with outcomes in small pulmonary arterial hypertension (PAH) cohorts composed largely of patients with severe idiopathic PAH (IPAH). It is unclear whether IL-6 is a marker of critical illness or a mechanistic biomarker of pulmonary vascular remodeling. We hypothesized that IL-6 is produced by pulmonary vascular cells and sought to explore IL-6 associations with phenotypes and outcomes across diverse subtypes in a large PAH cohort.

IL-6 protein and gene expression levels were measured in cultured pulmonary artery smooth muscle cells (PASMCs) and endothelial cells (PAECs) from PAH patients and healthy controls. Serum IL-6 was measured in 2017 well-characterized PAH subjects representing each PAH subgroup. Relationships between IL-6 levels, clinical variables, and mortality were analyzed with regression models.

Significantly higher IL-6 protein and gene expression levels were produced by PASMCs than by PAECs in PAH ($p<0.001$), while there was no difference in IL-6 between cell types in controls. Serum IL-6 was highest in PAH related to portal hypertension and connective tissue diseases (CTD-PAH). In multivariable modeling, serum IL-6 was associated with survival in the overall cohort (HR 1.22, 95% CI 1.08-1.38, $p<0.01$) and in IPAH, though not in CTD-PAH. IL-6 remained associated with survival in low-risk subgroups of subjects with mild disease.

IL-6 is released from PASMCs, and circulating IL-6 is associated with specific clinical phenotypes and outcomes in various PAH subgroups, including subjects with less severe disease. IL-6 is a mechanistic biomarker, and thus a potential therapeutic target, in certain PAH subgroups.

Abstract Word Count: 250

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by abnormal cellular proliferation, pulmonary vascular remodeling, and increased pulmonary vascular resistance [1,2]. Perivascular inflammation with lymphocyte and macrophage infiltration has been observed across PAH subtypes [3], and preclinical studies support direct involvement of inflammatory mechanisms in PAH pathobiology [4–7]. Cytokines known to drive abnormal proliferation of pulmonary vascular cells are easily measurable in human serum. Thus, pro-inflammatory cytokines may serve as mechanistic biomarkers that provide insights into phenotypic differences in PAH pathobiology, disease severity, and survival across different disease subtypes [8,9].

Interleukin-6 (IL-6) is a circulating pro-inflammatory cytokine [10]. Among tissue sources, the lung has the second highest expression of IL-6 at the RNA level [11]. The predominant cellular source of IL-6 in the pulmonary vasculature is unclear, though the membrane-bound IL-6 receptor is upregulated in pulmonary artery smooth muscle cells (PASMCs) in patients with idiopathic PAH (IPAH) [12]. Previous studies have shown that IL-6 is elevated in PAH and independently associated with indices of right ventricular function [5,13–15]. However, prior investigations of IL-6 as a prognostic biomarker have found inconsistent associations with mortality [13,15].

Most prior studies of IL-6 in PAH have been undertaken in small, single-center cohorts composed primarily of IPAH patients with severe disease, calling into question whether IL-6 is reflective of PAH or critical illness, and whether associations with clinical outcomes are generalizable to other PAH subtypes. In one small PAH cohort, IL-6 was an independent predictor of mortality in a subgroup of patients with normal brain natriuretic peptide (BNP) levels (<180 pg/mL) [16], suggesting prognostic value in mild disease.

To date, there has been no large-scale, multicenter study of associations between IL-6 levels and patient phenotypes and outcomes across diverse PAH clinical subgroups, including patients with mild

PAH. Whether IL-6 reflects cellular processes occurring in the pulmonary vasculature or merely reflects severe disease remains uncertain. Given the diverse array of mechanisms known to contribute to PAH pathogenesis [2], markers identifying specific pathobiology in particular subgroups of patients may inform clinical phenotyping and could link phenotypes to tailored PAH-specific therapies. This study addresses several knowledge gaps by investigating the cellular sources of IL-6 in the pulmonary vasculature at both the protein and RNA level, and by examining relationships between IL-6 levels, detailed clinical metrics, and outcomes in a multi-center, deeply phenotyped, heterogeneous PAH cohort.

METHODS

Cohort Data Collection

This study was conducted in accordance with the Declaration of Helsinki and approved by the Johns Hopkins University Institutional Review Board (IRB) (NA_00069663, Baltimore, MD). Samples and clinical data were obtained from the National Institutes of Health and National Heart, Lung, and Blood Institute PAH Biobank (www.pahbiobank.org), which includes data aggregated from 34 enrollment centers across North America. Specimen collection was approved by the IRBs at each center, and informed consent was obtained for all subjects prior to their enrollment. Eligible enrollees are patients with World Health Organization (WHO) Group 1 PAH. Clinical data is extracted from the electronic medical records of each patient, and de-identified data is managed by the PAH Biobank. Patients provide whole blood specimens via venipuncture at enrollment, which are stored as serum in secure freezers at Cincinnati Children's Hospital Medical Center. PAH Biobank specimens and data from enrollees 21 years of age or older (N=2017) and serum samples from adult controls without PAH from Vanderbilt University (N=60) were studied. Serum IL-6 levels were measured by a commercial

electrochemiluminescence immunoassay (ELISA) in a 96-well plate based format (Meso Scale Discovery [MSD], Gaithersburg, MD). The average lower limit of detection was 0.152 pg/mL.

Cell Line Data Collection

Cell lines were obtained from the Cardiovascular Medical Research and Education Fund Pulmonary Hypertension Breakthrough Initiative (PHBI), which included PASMCs and pulmonary artery endothelial cells (PAECs) from transplanted patients with severe PAH (N=22) or from non-transplanted donors (N=11) [11,17,18]. Cells were maintained in normal culture conditions, and IL-6 levels from the conditioned media for each cell type were measured by ELISA. Cells were subjected to RNA extraction when they reached 80-90% confluence in order to perform RNA sequencing (RNAseq). IL-6 gene expression levels were measured in fragments per kilobase of exon model per million reads mapped (FPKM) and compared across cell types. Full methodology for performance of cell culture, RNAseq, and ELISA is available in the Supplement.

Statistical Analysis

IL-6 comparisons were made using t-tests, Wilcoxon rank-sum tests, or Kruskal-Wallis tests, as appropriate. Relationships between IL-6 levels and clinical variables were analyzed using linear and logistic regression models adjusted for age and sex. IL-6 levels were right-skewed and log-transformed for analyses. Associations between IL-6 levels and survival were studied in the overall cohort and in pre-specified disease subtypes using Kaplan-Meier analysis, in which subjects were dichotomized based on the median IL-6 level, and using Cox proportional hazard models adjusted for potential confounders of the relationship between IL-6 and survival. The proportional hazards assumption was examined for all covariates on the basis of Schoenfeld residuals. Thirty-three subjects for whom survival data were not available were not included in time-to-event analyses. A *p* value of less than 0.05 was considered

statistically significant. Bonferroni correction for multiple testing was performed for variable associations with IL-6 in Table 2 (n = 12), yielding a threshold for significance of 0.0042. All analyses were performed with Stata (Version 15.1, StataCorp, College Station, TX).

RESULTS

Patient Demographics

A total of 2017 subjects were included in the PAH cohort. The cohort was 80% female and 82% white with a mean age at enrollment of 55 and median six-minute walk distance (6MWD) of 348 meters (Table 1). Subjects had moderate to severe PAH, with average mean pulmonary arterial pressure (mPAP) of 50 mmHg, pulmonary vascular resistance (PVR) of 10 Wood units, and cardiac output of 4.7 L/min. The median time from right heart catheterization (RHC) to enrollment was 48 months (IQR 14-92 months). Most subjects received treatment with phosphodiesterase-5 (PDE5) inhibitors and endothelin receptor antagonists (ERAs). The majority of subjects had IPAH (43%) or connective tissue disease-associated PAH (CTD-PAH) (31%). Other subtypes included portal hypertension-associated PAH (5%), familial PAH (4%), and congenital heart disease associated PAH (2%), among others. Overall, 324 of 1,984 subjects with survival data died (16.3% mortality). Subjects were followed for a median of 41 months (IQR 28-55 months) from the time of enrollment to the time of death or censor. Demographic data for the control cohort, PHBI cell line donors, and the 33 subjects for whom survival data were not available are provided in the Supplement, Tables S1-S3.

IL-6 in PAH Subtypes and Associations with Clinical Phenotypes

The median IL-6 level in the overall PAH cohort was 1.82 pg/mL (IQR 0.86-3.34 pg/mL) compared with 0 pg/mL (IQR 0-2.72 pg/mL) in controls ($p<0.001$) (Supplemental Table S1). Subjects with portal

hypertension-associated PAH (3.02, IQR 1.68-5.78 pg/mL) and CTD-PAH (2.25, IQR 1.09-4.33 pg/mL) had significantly higher serum IL-6 levels than those with IPAH (1.62, IQR 0.72-2.94 pg/mL) ($p<0.001$) (Figure 1). Among subjects with CTD-PAH, subjects with rheumatoid arthritis (3.38, IQR 1.19-9.96 pg/mL) had higher IL-6 levels than subjects with systemic sclerosis (2.34, IQR 1.19-4.19 pg/mL) or systemic lupus erythematosus (1.53, IQR 0.77-4.01 pg/mL). Each log-unit higher IL-6 concentration was associated with 22% greater odds of having PAH associated with connective tissue disease (OR 1.22, 95% CI 1.13-1.31, $p<0.001$) and 37% greater odds of having PAH associated with portopulmonary hypertension (OR 1.37, 95% CI 1.18-1.59, $p<0.001$) (Table 2).

As shown in Table 2, each log-unit higher IL-6 was associated with higher right atrial pressure (RAP), pulmonary artery wedge pressure (PAWP), and cardiac output and with lower PVR. Each log-unit higher IL-6 was also associated with lower RV stroke work index and higher RV power output. Functionally, each log-unit higher IL-6 was associated with 17% greater odds of having dyspnea at rest (OR 1.17, 95% CI 1.07-1.28, $p=0.001$), 8% greater odds of requiring treatment with prostacyclin analogs (OR 1.08, 95% CI 1.01-1.15, $p=0.019$), and a 16.0 meter shorter 6MWD (95% CI 10.2-21.7, $p<0.001$). Overall, higher IL-6 was associated with a more severe New York Heart Association Functional Class (NYHA FC) ($p<0.001$) (Supplemental Figure S1a) and a higher REVEAL risk score ($p<0.001$) (Supplemental Figure S1b), a multivariable score that predicts 1-year survival based on a combination of patient demographics, etiologic factors, and physical exam and laboratory results [19–21].

IL-6 Associations with Survival in the Overall Cohort and in PAH Subgroups

As shown in the Kaplan-Meier plot in Figure 2, 5-year survival was shorter among subjects with IL-6 levels above the cohort median, 1.82 pg/mL (log-rank $p<0.0001$). In Cox proportional hazard modeling, each log-unit higher IL-6 was associated with a 35% greater risk of death, with an unadjusted hazard ratio (HR) of 1.35 (95% CI 1.25-1.46, $p<0.01$). This relationship remained significant when

adjusted for age, sex, PAH subtype, PAH-specific therapy drug class, NYHA FC, 6MWD, body mass index (BMI), and hemodynamic variables (RAP, mPAP, PVR, CI) (HR 1.22, 95% CI 1.08-1.38, p=0.002).

Kaplan-Meier analysis conducted within the two largest disease subtypes demonstrated shorter survival in both IPAH and CTD-PAH among subjects with IL-6 levels above the median (each log-rank p<0.001, Figure 3a-b). Cox multivariable analysis of the IPAH subgroup demonstrated that each log-unit higher IL-6 was associated with a 31% greater risk of death (HR 1.31, 95% CI 1.01-1.71, p=0.039). However, the significance of the relationship between IL-6 and survival was attenuated in multivariable analysis of the CTD-PAH subgroup (HR 1.18, 95% CI 0.98-1.42, p=0.074).

In Kaplan-Meier analysis, IL-6 above the median was associated with worse survival in subjects in REVEAL risk categories 1 (N=123, log-rank p<0.01), 2 (N=73, log-rank p<0.001), and 3 (N=62, log-rank p<0.01). IL-6 was not significantly associated with survival in REVEAL risk categories 4 (N=57, log-rank p=0.06) or 5 (N=9, log-rank p=0.31), though sample sizes were smaller in higher risk categories. Univariable associations between log-transformed IL-6 levels and survival for each REVEAL risk category (Table 3) align with the results of Kaplan-Meier analysis, with significant relationships demonstrated in lower risk categories and significance of associations lost in higher risk categories.

As shown in Table 4, higher IL-6 was associated with worse survival in subgroups of subjects with low-risk clinical features as defined by European Society of Cardiology and European Respiratory Society (ESC/ERS) guidelines [22], including NT-proBNP <300 pg/mL (N=623, HR 1.41, 95% CI 1.08-1.83, p=0.011), 6MWD >440 m (N=1192, HR 1.43, 95% CI 1.29-1.58, p<0.001), RAP <8 mmHg (N=867, 1.43, 95% CI 1.26-1.62, p<0.001), and cardiac index >2.5 L/min/m^2 (N=1053, HR 1.36, 95% CI 1.23-1.52, p<0.001).

IL-6 in Pulmonary Artery Cell Lines

Median IL-6 concentrations in conditioned media were significantly higher in PASMCs (12,301, IQR 1694-21,822 pg/mL) than in PAECs (398, IQR 298-525 pg/mL) ($p<0.0001$) in PAH cell lines (Figure 4a). No significant difference in IL-6 concentrations existed between PASMCs and PAECs in controls (Figure 4a) or between PAH subtypes among either PASMCs (Figure 4b) or PAECs (Figure 4c). IL-6 concentrations were higher in PASMCs from PAH patients (12,301, IQR 1694-21,822 pg/mL) than in PASMCs from controls (2,445, IQR 2253-14,799 pg/mL), though this difference did not reach statistical significance (Figure 4a). RNASeq results aligned with these findings, with significantly higher IL-6 gene expression in PASMCs compared to PAECs in PAH, and a trend toward higher IL-6 gene expression in PASMCs in PAH patients compared to controls (Figure 4d). Further, cellular IL-6 gene expression levels by PAH subtype closely mirrored IL-6 protein concentrations in conditioned media (Figures 4e-f).

DISCUSSION

IL-6 is a pro-inflammatory cytokine shown in animal models to mediate the pulmonary vascular remodeling and progressive occlusion of the pulmonary vessels that characterizes PAH in humans [1,6–8]. Our study is the largest to date to investigate clinical associations with circulating IL-6 levels across diverse PAH subtypes. Our results confirm that IL-6 is higher in PAH compared to controls and demonstrate that IL-6 levels are highest in PAH associated with portal hypertension and connective tissue diseases.

Importantly, we demonstrate significant associations between IL-6 and mortality in multivariable models in both the overall PAH cohort and in the IPAH subgroup. This is in contrast to previous studies that have demonstrated inconsistent associations with mortality. Soon et al. previously demonstrated unadjusted IL-6 associations with mortality in 57 subjects with severe disease; however,

the significance of the relationship was lost with adjustment for important covariates [13]. Cracowski et al. examined a panel of pro-inflammatory cytokines, including IL-6, in 74 PAH patients, though the significance of the association between IL-6 and mortality was borderline (p=0.06) [15].

We found significant relationships between serum IL-6 levels and phenotypic variables across disease subtypes, including higher NYHA FC, shorter 6MWD, and the presence of dyspnea at rest. In alignment with previous studies [13,14], we found null or unexpected associations between serum IL-6 levels and hemodynamic variables indicative of PAH, such as mPAP and PVR. We did find associations between IL-6 and decreased RV stroke work index and increased RV power, two metrics of RV function [24–27]. One potential explanation for this is that IL-6 may be a poor marker of hemodynamic impairment and instead a better marker of RV dysfunction. Prins et al. also found no difference in hemodynamics in PAH patients with high versus low IL-6, but did find significant associations between IL-6 and measures of RV dysfunction and impaired RV-pulmonary arterial coupling [14]. RV dysfunction is the major determinant of mortality in PAH, thus these associations align with the strong relationships between IL-6 levels and mortality observed in our cohort. Unfortunately, we do not have echocardiographic or imaging data available for our cohort to recapitulate Prins' specific findings.

Our study re-demonstrates the prognostic utility of IL-6 among PAH patients with mild disease, including among patients with low-risk features designated by current ESC/ERS guidelines, such as lower RAP, longer 6MWD, and low/normal NT-proBNP levels [22], and among patients in low REVEAL risk categories. These clinical results, together with prior animal studies, suggest IL-6 is a mechanistic marker of pulmonary vascular disease, rather than a nonspecific marker of critical illness in severe PAH. Perivascular inflammation precedes pulmonary vascular remodeling in experimental models of pulmonary hypertension [23], and it is provocative to speculate that IL-6 may be a biomarker of upstream pathobiologic events in PAH, in contrast to NT-proBNP, which reflects cardiomyocyte stretch that occurs once pathologic pulmonary vascular remodeling has evolved significantly [28–30]. NT-

proBNP levels were only weakly correlated with IL-6 levels in our cohort (Spearman correlation coefficient 0.25, $p<0.01$), implying that IL-6 reflects different pathobiologic mechanisms than NT-proBNP, and therefore may provide additional, multi-dimensional prognostic information. Establishing markers of mild disease is particularly relevant in light of the recent re-definition of PAH at the 6th World Symposium on Pulmonary Hypertension (with revision of the mPAP threshold from 25 mmHg to 20mmHg) [31].

Our study shows that, in addition to known production of IL-6 by pulmonary macrophages and other inflammatory cells of the lung, PAMSCs release µg/mL quantities of IL-6 in PAH, which may contribute to circulating IL-6 levels measured in the serum (typically measured in pg/mL) or have local effects. Preclinical studies have demonstrated ectopic upregulation of the IL-6 receptor in PASMCs in experimental PH. Moreover, deletion of the IL-6 receptor in the smooth muscle layer of animal PASMCs prevents development of hypoxia-induced PH [12].

Collectively, our results corroborate an important role for IL-6 in PAH pathobiology. These findings support the dual potential of IL-6 as a biomarker of a dysfunctional pulmonary circulation and as a possible therapeutic target. Importantly, a pharmacologic IL-6 inhibitor, tocilizumab, is currently under investigation for efficacy in PAH (NCT02676947). Notably, the designated co-primary endpoints for this phase 2 trial are: 1) change in PVR and 2) incidence of adverse events [32]. In light of the clinical associations demonstrated in our study, special attention should be paid to the trial's secondary outcome measures, especially changes in 6MWD, functional class, and quality of life, when interpreting the results. Future studies of anti-IL-6 therapies should consider incorporation of endpoints such as changes in RV function, time to clinical worsening, or changes in IL-6 levels with therapy, and should be powered to analyze results within distinct pre-specified disease subtypes. Selectively enriching study populations by preferentially enrolling subjects with high IL-6 pre-intervention could also be considered in designing future efficacy trials.

A major strength of our study is the large overall sample size of subjects with detailed hemodynamic, functional, and phenotypic data, enabling a thorough analysis of clinical variables in relation to IL-6 levels. Moreover, this study pairs a large-scale epidemiologic investigation of serum IL-6 levels in PAH with cell culture and RNAseq experiments to examine IL-6 production by cells of the pulmonary vasculature. The study was somewhat limited by the composition of the cohort. Some subgroups of interest (for example, high-risk REVEAL categories) had relatively small sample sizes. Further, the majority of subjects were prevalent patients on PAH-specific therapy at the time of enrollment. Therapies may have affected IL-6 measurements in serum, as treatment with ERAs has been shown to reduce circulating IL-6 levels [33]. However, our large overall sample size allowed for adjustment of multiple covariates in Cox proportional hazard models, including adjustment for PAH-specific therapies.

In conclusion, IL-6 is produced by pulmonary vascular cells, is variably upregulated across diverse PAH subtypes, and is strongly associated with clinical features of disease, including specific phenotypes and survival times. IL-6 may be a more upstream, mechanistic biomarker of disease development than other biomarkers currently in clinical use, and therefore may aid in efforts toward diagnosis and phenotyping of mild PAH. Serum IL-6 measurements offer insights into disease pathobiology and prognosis. In the future, measurements of mechanistic biomarkers like IL-6 may aid in accurately phenotyping patients, selecting patients most likely to benefit from novel therapies, and monitoring therapeutic effects of tailored therapies.

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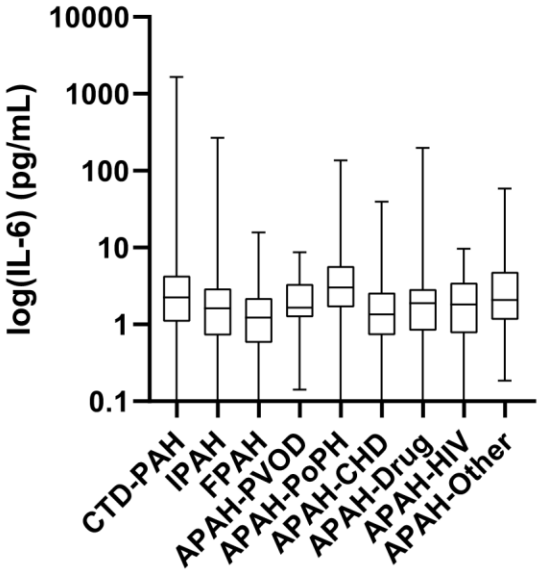
Figure Legends

Figure 1. Comparison of interleukin 6 (IL-6) levels by pulmonary arterial hypertension (PAH) subtype: connective tissue disease-associated PAH (CTD-PAH), idiopathic PAH (IPAH), familial PAH (FPAH), pulmonary veno-occlusive disease-associated PAH (APAH-PVOD), portopulmonary hypertension-associated PAH (APAH-PoPH), congenital heart disease-associated PAH (APAH-CHD), drug-associated PAH (APAH-Drug), HIV-associated PAH (APAH-HIV), and other non-specified disease-associated forms of PAH (APAH-Other).

Figure 2. Kaplan-Meier survival analysis among pulmonary arterial hypertension (PAH) subjects with available survival data with interleukin 6 (IL-6) levels above vs. below the median (log rank $p<0.0001$).

Figure 3. Kaplan-Meier survival analyses among subjects with a) idiopathic pulmonary arterial hypertension (IPAH) and b) connective tissue disease-associated pulmonary arterial hypertension (CTD-PAH) with interleukin 6 (IL-6) levels above vs. below the median of the respective subgroup (log rank $p<0.0001$ for each).

Figure 4. Comparison of interleukin 6 (IL-6) levels in conditioned media from a) smooth muscle cells (SMC) and endothelial cells (EC) from transplanted pulmonary arterial hypertension (PAH) subjects and non-transplanted donor controls ($p<0.0001$ for SMC-PAH vs. EC-PAH), b) SMCs in controls and PAH subtypes, and c) ECs in controls and PAH subtypes. Concentrations for IL-6 in the conditioned media are reported in pg/mL. Comparison of IL-6 gene expression levels by fragments per kilobase of exon model per million reads mapped (FPKM) between d) SMCs and ECs from PAH subjects and controls ($p<0.0001$ for SMC-PAH vs. EC-PAH), e) SMCs in controls and PAH subtypes, and f) ECs in controls and PAH subtypes.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log(IL-6) (pg/mL)
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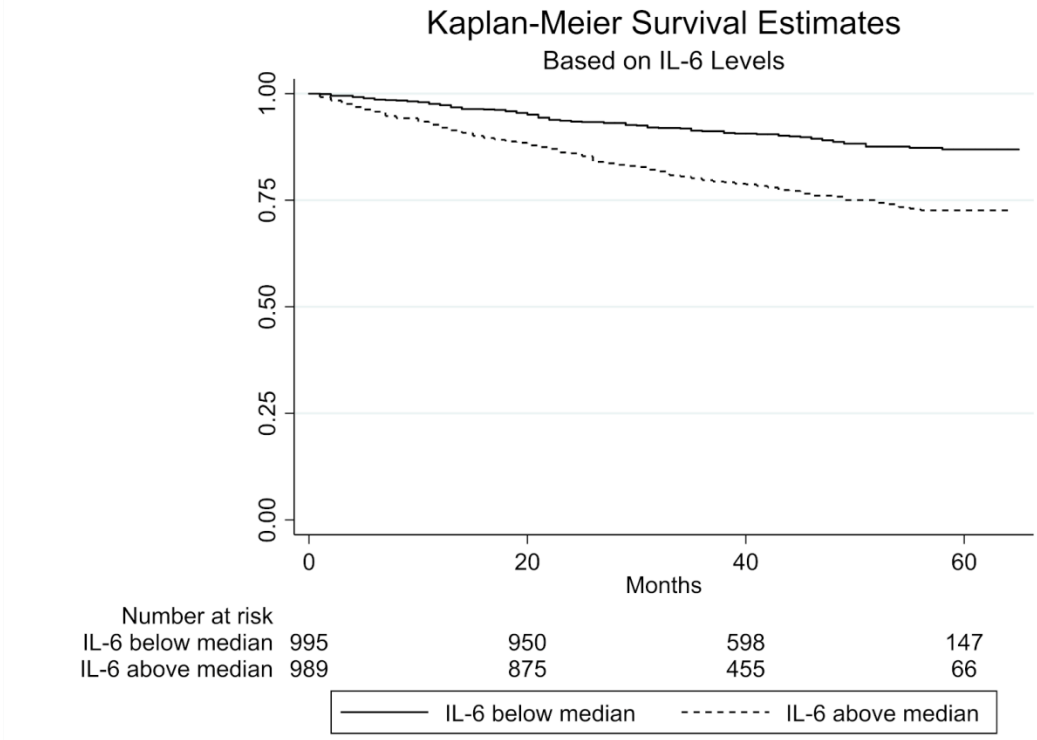
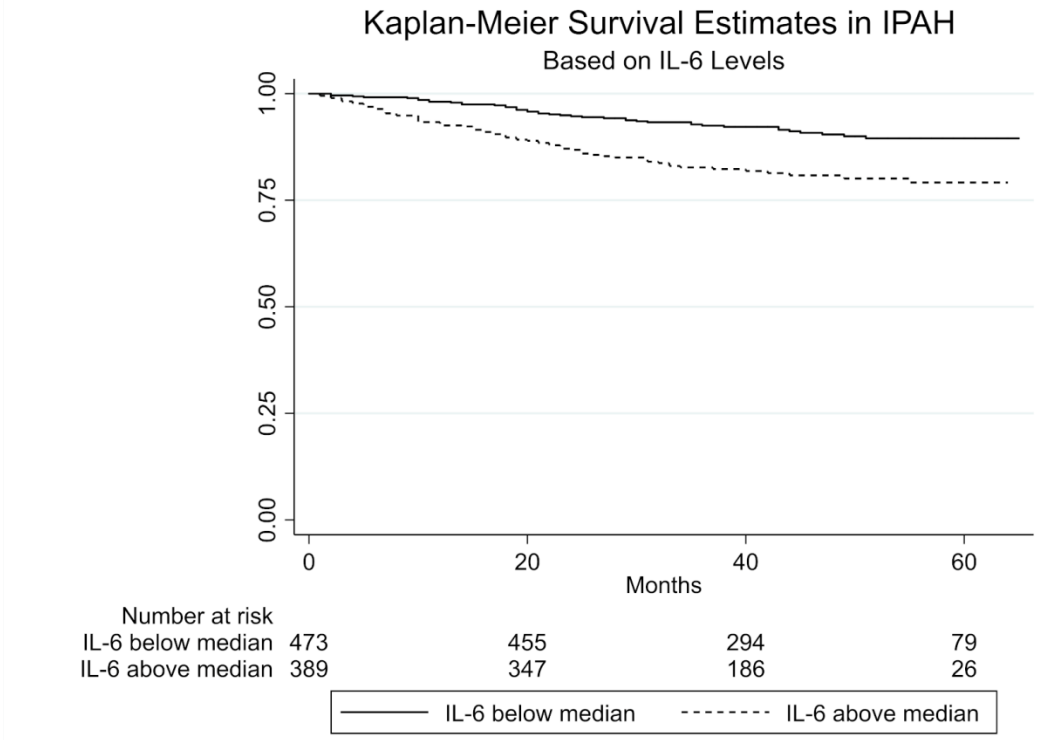
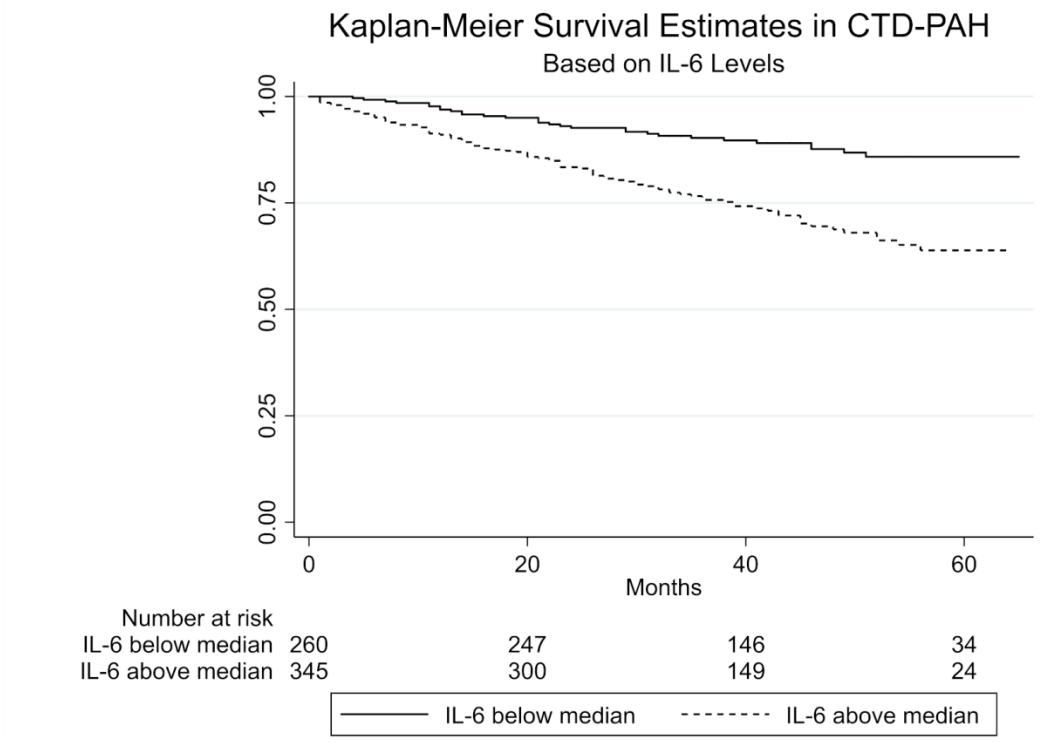
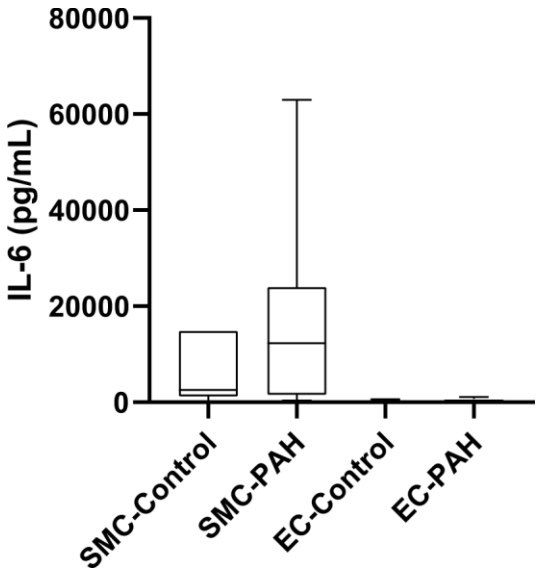
IPAH
FPAH
APAH-PVOD
APAH-PoPH
APAH-CHD
APAH-Drug
APAH-HIV
APAH-Other


Kaplan-Meier Survival Estimates
Based on IL-6 Levels
1.00
0.75
0.50
0.25
0.00
0
20
40
60
Months
Number at risk
IL-6 below median 995 950 598 147
IL-6 above median 989 875 455 66
IL-6 below median
IL-6 above median


Kaplan-Meier Survival Estimates in IPAH
Based on IL-6 Levels
1.00
0.75
0.50
0.25
0.00
0
20
40
60
Months
Number at risk
IL-6 below median 473 455 294 79
IL-6 above median 389 347 186 26
IL-6 below median
IL-6 above median


Kaplan-Meier Survival Estimates in CTD-PAH
Based on IL-6 Levels
1.00
0.75
0.50
0.25
0.00
0
20
40
60
Months
Number at risk
IL-6 below median 260 247 146 34
IL-6 above median 345 300 149 24
IL-6 below median
IL-6 above median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60000
40000
20000
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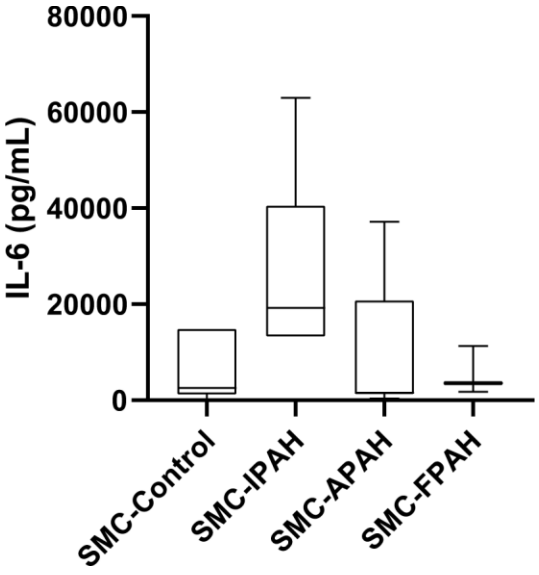
IL-6 (pg/mL)
SMC-Control
SMC-PAH
EC-Control
EC-PAH


80000
60000
40000
20000
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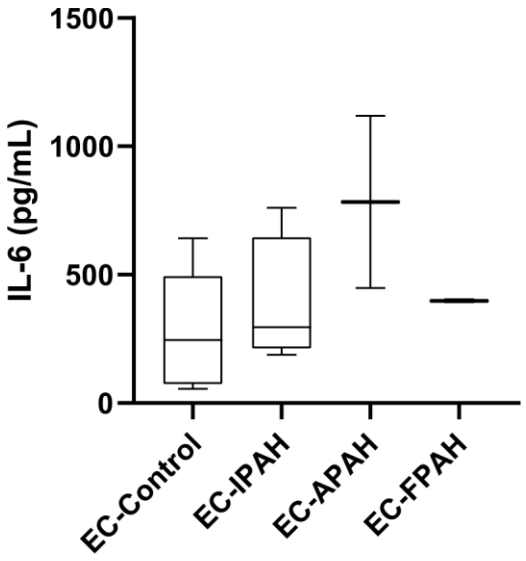
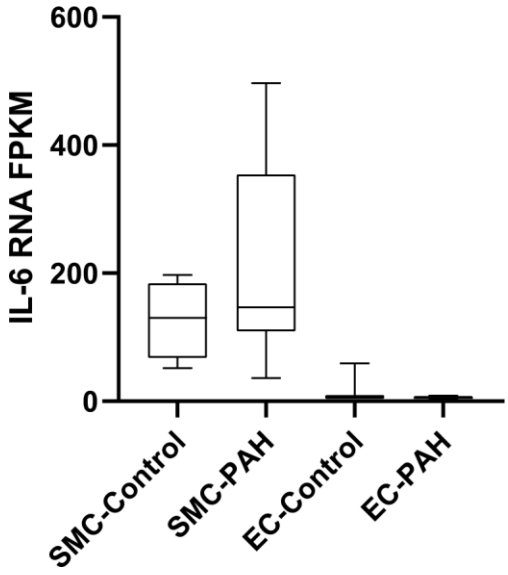
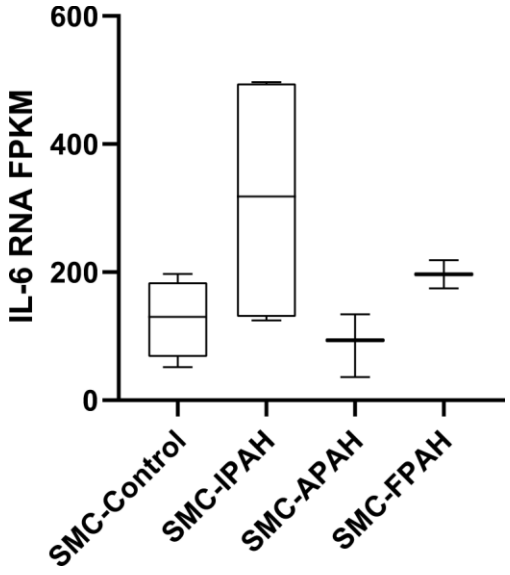
IL-6 (pg/mL)
SMC-Control
SMC-IPAH
SMC-APAH
SMC-FPAH


1500
1000
500
0
IL-6 (pg/mL)
EC-Control
EC-IPAH
EC-APAH
EC-FPAH


600
400
200
0
IL-6 RNA FPKM
SMC-Control
SMC-PAH
EC-Control
EC-PAH


600
400
200
0
IL-6 RNA FPKM
SMC-Control
SMC-IPAH
SMC-APAH
SMC-FPAH

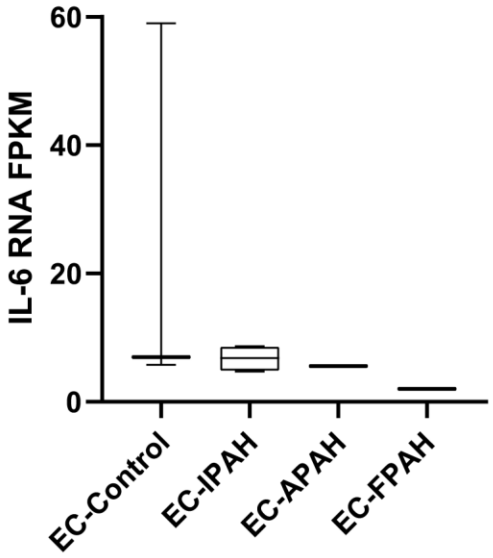

60
40
20
0
IL-6 RNA FPKM
EC-Control
EC-IPAH
EC-APAH
EC-FPAH

Table 1. Demographics and Clinical Characteristics of the Overall Cohort and CTD-PAH and IPAH Subgroups

	Overall	CTD-PAH	IPAH	p value
Demographics				
Subjects, n	2017	623	870	
Age, years	55 (15)	59 (14)	55 (15)	<0.01
Sex, n female (%)	1611 (80)	565 (91)	698 (80)	<0.01
Race, n white (%)	1662 (82)	564 (91)	780 (90)	NS
NYHA FC, n I/II/III/IV (%III/IV)	90/451/789/118 (45)	24/140/266/34 (48)	38/188/340/56 (45)	NS
6MWD, m	347 (141)	327 (160)	351 (136)	<0.01
BMI	30 (10)	29 (12)	31 (9)	<0.01
Deaths, n (%)	338 (17)	138 (22)	110 (13)	<0.01
Etiology, n CTD-PAH/IPAH/FPAH/PVOD/ PortoPulm/Cong/Drug/HIV/Other	623/870/81/8/ 111/171/93/42/18	-	-	
IL-6 Levels				
IL-6, pg/mL (median, IQR)	1.82 (0.86-3.34)	2.24 (1.09-4.33)	1.62 (0.72-2.94)	<0.01
Hemodynamics				
RAP, mmHg	9 (5)	9 (5)	9 (6)	<0.01
mPAP, mmHg	50 (15)	44 (11)	51 (14)	<0.01
PAWP, mmHg	10 (4)	10 (4)	10 (4)	NS
PVR, Wood units	10 (6)	8 (5)	10 (6)	<0.01
Cardiac output, L/min	4.7 (1.7)	4.7 (1.6)	4.6 (1.6)	0.01
Cardiac index, L/min/m^2	2.7 (1.2)	2.8 (0.9)	2.6 (1.1)	<0.01
Therapies, n (%)				
PDE5 inhibitor	1546 (77)	470 (75)	641 (74)	NS
ERA	1205 (60)	370 (59)	515 (59)	NS
IV/SC prostacyclin	699 (35)	161 (26)	355 (41)	<0.01
CCB	199 (10)	51 (8)	99 (11)	0.05

All data presented as mean (SD) unless otherwise specified. p values reflect comparisons between CTD-PAH and IPAH.
Definition of abbreviations: CTD-PAH: connective tissue disease-associated pulmonary arterial hypertension; IPAH: idiopathic PAH; NYHA FC: New York Heart Association Functional Class; 6MWD: six-minute walk distance; BMI: body mass index; FPAH: familial PAH; PVOD: pulmonary veno-occlusive disease; PortoPulm: portopulmonary hypertension; Cong: congenital; IL-6: interleukin-6; RAP: right atrial pressure; mPAP: mean pulmonary arterial pressure; PAWP: pulmonary artery wedge pressure; PVR: pulmonary vascular resistance; PDE5: phosphodiesterase-5; ERA: endothelin receptor antagonist; IV: intravenous; SC: subcutaneous; CCB: calcium channel blocker.

Table 2. IL-6 Associations with Clinical Variables

	Regression Coefficient (95% CI, p value)
RAP, mmHg	0.50 (0.33 – 0.67, <0.001)
mPAP, mmHg	-0.04 (-0.46 – 0.37, NS)
PAWP, mmHg	0.17 (0.05 – 0.30, 0.007)
PVR, Wood units	-0.24 (-0.42 – -0.06, 0.010)
Cardiac output, L/min	0.08 (0.03 – 0.13, 0.004)
Cardiac index, L/min/m^2	0.01 (-0.03 – 0.05, NS)
Stroke volume, mL	0.00 (0.00 – 0.00, NS)
PA compliance, mL/mm Hg	0.00 (-0.04 – 0.03, NS)
Heart rate, beats/min	1.17 (0.59 – 1.75, <0.001)
6MWD, m	-15.99 (-21.74 – -10.25, <0.001)
RV stroke work index	-0.41 (-0.81 – -0.01, 0.042)
RV power	4.84 (1.28 – 8.40, 0.008)
	Odds Ratio (95% CI, p value)
CTD-PAH	1.22 (1.13-1.31, <0.001)
PortoPulm PAH	1.37 (1.18-1.59, p<0.001)
Dyspnea at rest	1.17 (1.07-1.28, 0.001)
IV/SC prostacyclin	1.08 (1.01-1.15, 0.019)

Definition of abbreviations: PA: pulmonary arterial; RV: right ventricle. See Table 1 for all other abbreviations.
All regression coefficients and odds ratios were adjusted for age and sex.
Associations with p less than 0.0042 are significant after Bonferroni correction for multiple testing.

Table 3. IL-6 Associations with Survival in REVEAL Risk Categories

REVEAL Risk Category	Univariable Hazard Ratio (95% CI, p value)
Category 1	1.29 (1.13-1.48, <0.001)
Category 2	1.31 (1.10-1.57, 0.003)
Category 3	1.21 (1.02-1.44, 0.02)
Category 4	1.12 (0.93-1.35, 0.22)
Category 5	1.12 (0.36-3.46, 0.85)

Table 4. IL-6 Associations with Survival in Low-Risk PAH Clinical Features

Clinical Feature	Univariable Hazard Ratio (95% CI, p value)
NT-proBNP <300 ng/mL	1.41 (1.08-1.83, 0.011)
6MWD >440 m	1.43 (1.29-1.58, <0.001)
RAP <8 mmHg	1.43 (1.26-1.62, <0.001)
Cardiac index >2.5 L/min/m^2	1.36 (1.23-1.52, <0.001)
Definition of abbreviations: NT-proBNP: N-terminal pro b-type natriuretic peptide. See Table 1 for all other abbreviations.	

Supplement

Cellular sources of IL-6 and associations with clinical phenotypes and outcomes in PAH

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[5]Johns Hopkins University Department of Medicine, Division of General Internal Medicine
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[7]Children's Hospital Colorado, Department of Pediatric Cardiology
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Baltimore, MD 21287
USA
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Supplemental Methods

Cell Lines and Cell Culture:

Primary pulmonary artery smooth muscle (PASMC) and endothelial cells (PAEC) were obtained from the Pulmonary Hypertension Breakthrough Initiative (PHBI) cell core facility (University of Pennsylvania) funded by the Cardiovascular Medical Research and Education Fund (CMREF). The cells were isolated from small pulmonary arteries of transplanted patients with severe PAH (N=22) or non-transplanted donor lungs (N=11). All cells were maintained at low passage (passage 3-8) under normal culture conditions in a humidified 5% CO_2-supplemented incubator at 37°C (Napco 8000 DH, ThermoFisher Scientific). Each cell type was cultured in a specific medium: PASMC in VascuLife SMC Medium (Cat# LL-0014, Lifeline Cell Technology, Frederick, MD) and PAEC in VascuLife VEGF-Mv Endothelial Medium (Cat# LL-0005, Lifeline Cell Technology, Frederick, MD). The cell conditioned media were harvested and centrifuged at 3000 rpm for 5 minutes at 4°C to remove cell debris then aliquoted and stored at -80°C.

RNA Extraction and RNAseq Analysis:

PASMCs and PAECs in normal culture conditions were subjected to RNA extraction after reaching 80-90% confluence. TRIzol reagent (Cat# 15596026, ThermoFisher Scientific) was used for total RNA extraction then the manufacturer's instructions (ArrayStar 6G RNAseq service, Rockville, MD) were followed to perform the RNAseq experiments. After RNAseq library preparation, libraries were sequenced for 150 cycles for both ends on an Illumina NovaSeq 6000 instrument. Image analysis and base calling were performed using the Solexa pipeline v1.8 (Off-Line Base Caller software, v1.8). Sequence quality was examined using the FastQC software [1]. The trimmed reads [2] were aligned to

reference genomes using Hisat2 software [3]. The transcript abundances for each sample were estimated with StringTie [4], and the fragments per kilobase of exon model per million reads mapped (FPKM) values for gene and transcript levels were calculated with R package Ballgown [5–7]. IL-6 gene expression levels (FPKM values) were extracted from the data analysis results. RNAseq raw data are currently being uploaded to Gene Expression Omnibus (GEO).

ELISA for IL-6:

Electrochemiluminescent sandwich immunosorbent assays for measuring IL-6 were performed using robotically spotted capture antibodies (Cat# D21AK-3, Meso Scale Discovery [MSD], Gaithersburg, MD) on an MSD 96-well plate. Capture antibody-spotted plates were blocked with 5% BSA-PBS complemented with .05% TWEEN (PBS-T) and incubated at room temperature on an orbital shaker (500 rpm) for 60 minutes. Calibrator for IL-6 (Cat# C0049-2, MSD) was used at a concentration range of 0.024-100 pg/mL via a 1:4 series dilution with diluent II (Cat# R51BB-3, MSD). Samples were diluted 15x in the same diluent II (Cat# R51BB-3, MSD) before being added onto the ELISA plate. The plates containing samples/calibrators were incubated at room temperature with shaking for 2 hours then washed with PBS-T 3 times. The IL-6 sulfo-tagged detection antibody cocktail (Cat# D21AK, 50X, MSD) was diluted in diluent III (Cat# R51BA-5, MSD) to 1X and added onto the plates. Finally, after one hour of incubation followed by PBS-T washing, 150 µl of 1X read buffer (Cat# R92TC-1, MSD) was added into each well, and the plate was promptly read in an MSD Sector Imager 2400. Inter-assay reliability as measured by percent coefficient of variation was 6.5 $\pm$ 3.2% (mean $\pm$ SD). All assays were performed in a single laboratory and by the same laboratory specialist.

Supplemental References

1. Andrews S. FastQC: A quality control tool for high throughput sequence data. Babraham Bioinformatics. 2010.
2. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011;17(1).
3. Kim D, Langmead B, Salzberg SL. HISAT: A fast spliced aligner with low memory requirements. Nat Methods. 2015;12(4):357–60.
4. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol. 2015;33(3):290–5.
5. Fu J, Frazee AC, Collado-Torres L, Jaffe, Andrew E. Leek JT. ballgown: Flexible, isoform-level differential expression analysis. 2016.
6. Frazee AC, Pertea G, Jaffe AE, Langmead B, Salzberg SL, Leek JT. Ballgown bridges the gap between transcriptome assembly and expression analysis. Nat Biotechnol. 2015;33(3):243–6.
7. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc. 2016;11(9):1650–67.

Supplemental Tables and Figures

Table S1. Demographics and IL-6 Levels of Subjects with PAH versus Healthy Controls

	PAH	Controls	p value
Demographics			
Subjects, n	2017	60	
Age, years	55 (15)	35 (9)	<0.01
Sex, n female (%)	1611 (79.9)	47 (78.3)	0.45
Race, n Caucasian (%)	1662 (82.4)	49 (81.7)	0.91
IL-6 Levels			
IL-6, pg/mL (median, IQR)	1.82 (0.86-3.34)	0 (0-2.72)	<0.01

All data presented as mean (SD) unless otherwise specified. p values reflect comparisons between PAH cases and controls.
See Table 1 for abbreviations.

Table S2. Demographics and IL-6 Levels of PAH Patients and Non-Transplanted Donors of PHBI Cell Lines

	SMC-PAH*	EC-PAH*	SMC-Control	EC-Control
Demographics				
Subjects, n	16	8	5	6
Age, years (median)	36	37	52	43
Sex, n female (%)	10 (62.5)	3 (37.5)	2 (40.0)	4 (66.7)
Race, n Caucasian (%)	12 (75.0)	7 (87.5)	5 (100.0)	6 (100.0)
PAH subtypes, n IPAH/APAH/FPAH	6/7/3	4/2/2		
IL-6 Levels				
IL-6, pg/mL (median, IQR)	12,301 (1694-21,822)	398 (298-525)	2554 (2253-14,799)	245 (116-400)

Definition of abbreviations: SMC: smooth muscle cell; EC: endothelial cell; APAH: disease-associated PAH. See Table 1 for abbreviations.
** Demographic information was missing for one PAH patient who provided both SMC-PAH and EC-PAH samples*

Table S3. PAH Subtypes for Subjects without Available Survival Data

Diagnosis	Number of Patients (overall n=33)
Disease Subtype	
CTD-PAH	18
IPAH	8
FPAH	2
Congenital heart disease	3
Portopulmonary hypertension	1
APAH, other	1

Definition of abbreviations: APAH: disease-associated PAH. See Table 1 for abbreviations.

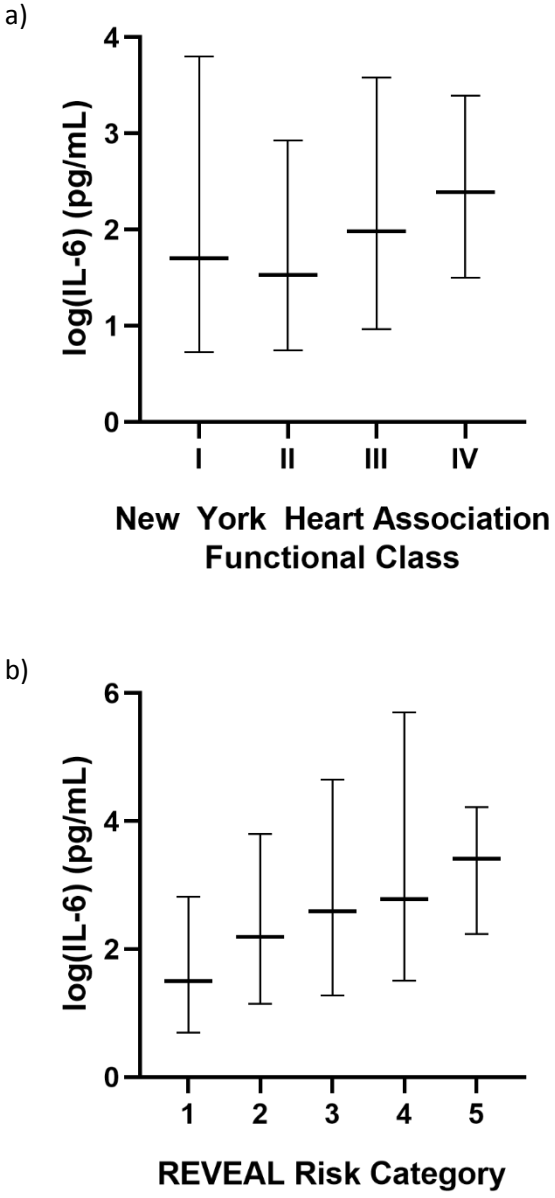

Figure S1. Comparison of interleukin 6 (IL-6) levels by: a) New York Heart Association Functional Class (NYHA FC), and b) REVEAL risk category ($p<0.001$). 25^{th} percentile, median, and 75^{th} percentile values are presented for each functional class and risk category.