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# Transcriptomic profiles in pulmonary arterial hypertension associate with disease severity and identify novel candidate genes

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## Abstract

Using RNAseq, we identified a 61 gene-based circulating transcriptomic profile most correlated with four indices of pulmonary arterial hypertension severity. In an independent dataset, 13/61 (21%) genes were differentially expressed in lung tissues of pulmonary arterial hypertension cases versus controls, highlighting potentially novel candidate genes involved in pulmonary arterial hypertension development.

## Keywords

pulmonary arterial hypertension, transcriptome profiling, PBMC, RNAseq

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## Introduction

Group 1 pulmonary arterial hypertension (PAH) is rare and often fatal disease characterized by excessive vasoconstriction and obliterative vascular remodeling contributing to elevated pulmonary vascular resistance (PVR), right ventricular failure, and death.<sup>1,2</sup> While several studies reported individual genes/pathways associated with severity or disease risk,<sup>3–5</sup> mechanisms remain unclear. Previously, expression profiling of peripheral blood mononuclear cells (PBMC) isolated from patients with PAH have searched for biomarkers of disease risk, PH Group classification, and have led to insights of disease mechanisms.<sup>6,7</sup> However, most of these profiles have not been replicated.

Explanations for this shortcoming is likely due, in part, to the dynamic changes in expression profiles over time, profiling in different tissues (e.g., circulating tissue versus target vascular tissue), as well as the quality and heterogeneity of clinical phenotypes. Given the ease of access and the role of inflammation/immunity in PAH, studying PBMC

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expression profiles remains an appealing method for developing biomarkers for disease progression and prioritizing candidate genes in PAH. In the current study, we developed a single multi-gene classifier based on RNA-seq-based expression profiles from PBMCs derived from patients that associate with four disease severity markers. To gain insight into disease pathology, we next evaluated differential regulation of the gene classifier in target lung tissues available from both PAH patients and non-PAH controls. Taken together, these data represent both possible biomarkers for disease severity as well as robust candidate genes potentially involved with PAH development.

## Materials and methods

### University of Arizona (UA) cohort

PAH patients receiving care at our University Pulmonary Hypertension clinic between 2012 and 2015 were prospectively recruited in accordance with institutional guidelines and provided informed consent. The cohort was composed of 84 subjects with Group 1 PAH (43 associated, 32 idiopathic, 4 anorexigen/drug-associated, 5 congenital-associated or HIV-associated PAH). For each subject, demographics and multiple measures of clinical severity were collected from four separate clinical tests including right heart catheterization (RHC), transthoracic echocardiogram (TTE), six-minute walk distance (6MWD, m), and brain natriuretic peptide levels (BNP, ng/L), all acquired during their first visit (or as part of their initial collections). Clinical severity was represented by four quantitative traits representing PVR (WU), tricuspid annular plane systolic excursion (TAPSE, cm), BNP, and 6MWD.

### RNA processing in UA cohort

PBMCs were stored in RNAItr as described.<sup>8</sup> In total, approximately 3600 million clusters with paired-end 75 bp reads (~35M cluster per sample) were generated from PBMC-derived RNA. Spearman correlations were performed and adjusted for age, sex, ethnicity, and treatment status (on PAH-specific medication). Only transcripts with  $r > |0.2|$  and  $p$ -value  $< 0.05$  thresholds with any one of the four clinical variables (TAPSE, BNP, PVR, and 6MWD) were selected for annotation using Ensembl (GRCh38). Transcripts that met this threshold across at least three of four clinical severity traits were further evaluated in the PHBI cohort.

### Pulmonary hypertension breakthrough initiative (PHBI) cohort

The PHBI cohort was comprised of 83 lung samples (58 PAH patients/25 control lung failed donors (FD)). Details of these patients have been recently reported<sup>9</sup> and deposited at NCBI/GEO as accession GSE117261. Patient profiles

including their clinical data are all previously described.<sup>10</sup> The PHBI cohort was analyzed by Affymetrix GeneChip Human Gene 1.0 ST microarray.<sup>9</sup> Using .cel files, an ANOVA model was used to identify differentially regulated genes between the PAH and FD lung transcriptomes after correcting for sex imbalance in PAH disease population and batch effects (false discovery rate (FDR)  $q$ -value  $< 0.001$ , yielding 1140 transcripts).

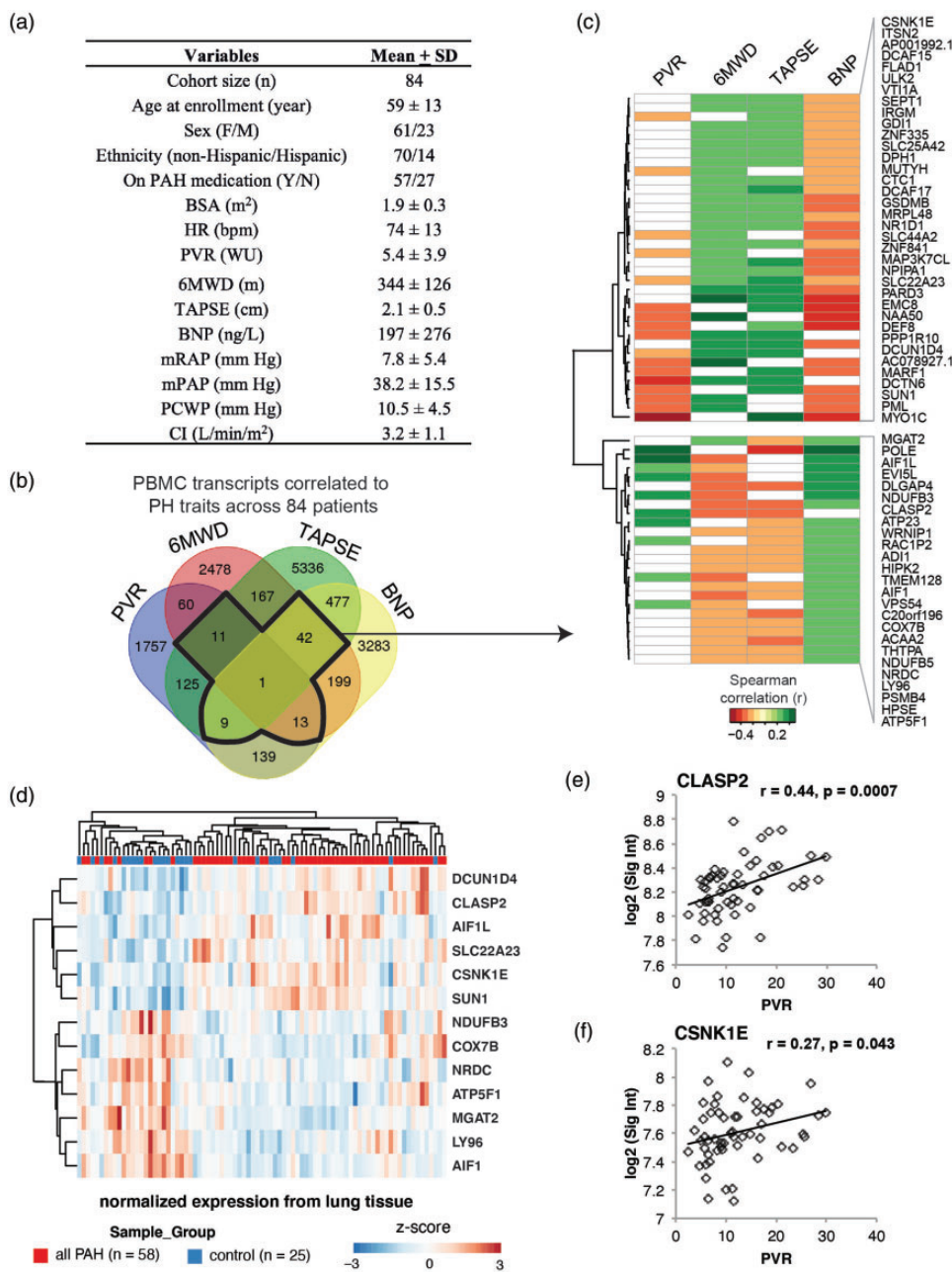
## Results

A total of 84 Group 1 PAH patients were included in the UA cohort (Fig. 1a). The mean age was 59 years and the cohort was composed of 75% women, consistent with the sexual dimorphism of PAH. All four independent clinical measures (PVR, BNP, TAPSE, and 6MWD) were characterized in the cohort to provide multiple quantitative diagnostic measures that together bolster stratifications of PAH severity.

To identify genes whose expression is indicative of PAH severity, we first correlated the normalized expression values per individual with individual PAH traits. Using a Spearman correlation coefficient threshold of  $> 0.2$ , 61 transcripts demonstrated correlation with at least three of the four PAH severity measures (Fig. 1b and c). Zinc finger protein 841 (*ZNF841*) RNA levels were uniquely correlated with all four PAH measured traits ( $p < 0.05$ ). Lower expression levels of *ZNF841* from PBMCs correlated with higher PVR and BNP levels as well as lower TAPSE and 6MWD results. We then confirmed that *ZNF841* was down-regulated in human pulmonary artery endothelial cells (HPAECs, data not shown) isolated from patients with PAH versus those from controls. In combination with PBMC data, these data suggest *ZNF841* deficiency may have a causal role in PAH risk and development.

In an independent cohort, we tested whether any of the 61 genes identified were differentially expressed in whole lung tissues between those collected from patients with PAH and those from non-PAH controls. Beyond discovery of putative genomic biomarkers of PAH severity, the PHBI cohort prioritized gene sets that may be involved in the development or in advanced disease. Based on available microarray data, we identified that 13 of the 61 gene set were differentially expressed (Fig. 1d; FDR  $< 10\%$ ).

We also evaluated association of gene expression levels of individual transcripts of the top 13 genes filtered from the Replication cohort against two available measures of severity—PVR and a quantitative measure of percent lung inflammation. Specifically, *CLASP2* (cytoplasmic linker associated protein 2) and *CSNK1E* (casein kinase 1 epsilon) displayed significant correlation and consistent directionality as the Discovery cohort with PVR (Fig. 1e and f,  $r > 0.2$ ,  $p < 0.05$ ). Given their reproducibility across two cohorts from two tissue sources, these data suggest that *CLASP2* and *CSNK1E* may represent novel candidate genes that modify PAH severity and possibly involved in inflammatory signaling in PAH lungs.



**Figure 1.** 61-gene signature associated with PAH severity and risk. (a) Table depicts demographic and clinical characteristics of the UA cohort. (b) Venn diagram for the numbers of transcripts shared among four correlation analyses with correlation coefficient  $r \geq 0.2$  ( $p < 0.05$ ). A single transcript (*ZNF841*) shows significant correlation with all four clinical parameters. (c) The heat map depicts correlation coefficients for the 61 genes correlated with PVR, BNP, TAPSE, or 6MWD ( $r > 0.2$ ,  $p < 0.05$ ). (d) The heatmap depicts 13 of the 61 genes from the original classifier, which is significantly differentially regulated between lungs of patients with PAH ( $n = 58$ ) versus non-PAH controls ( $n = 25$ ). (e and f) Two of the 13 filtered genes in lungs were also significantly correlated with PVR in the Replication cohort (*CLASP2*, *CSNK1E*). BSA: body surface area; HR: heart rate; PVR: pulmonary vascular resistance; WU: wood units; 6MWD: six-minute walk distance; TAPSE: tricuspid annular plane systolic excursion; BNP: brain natriuretic peptide; mRAP: mean right atrial pressure; mPAP: mean pulmonary artery pressure; PCWP: pulmonary capillary wedge pressure; CI: cardiac index; PA saturation: pulmonary artery saturation. Descriptive statistics are presented as mean  $\pm$  SD.

## Discussion

We report novel RNAseq profiles from PBMCs that associate with PAH disease severity. For the first time, we integrate expression profiles associated with multi-modality

assessment of severity to generate a single multi-gene classifier. In an independent testing cohort, a subset (~21%) of these genes consistently mirrored expression profiles in target lung tissues from patients with and without PAH

with moderate correlation to disease severity. This latter observation likely reflects the heterogeneity of whole lung tissue samples and provides initial perspectives into the ability of the circulating PBMC transcriptome to provide insights into the expression of disease-causing candidate genes in target tissues. Replication in larger cohorts will validate the robustness of potentially causal biomarkers, highlighting promising targets for novel therapies.

Our analysis also highlights *ZNF841*, *CLASP2*, and *CSNK1E* as some of the top genes associated with selected markers of disease severity. These genes were not observed to be differentially regulated in a recent meta-analysis<sup>11</sup> of circulating PBMCs from patients and non-PAH controls. Part of this likely stems from differences in the utility of microarray (used in the meta-analysis) versus RNAseq (current work) technologies as well as the focus of their analyses on disease risk versus disease severity in the current work. Furthermore, while the function *ZNF841* is unknown, other zinc fingers are reported to play a role in lung diseases such as asthma.<sup>12</sup> Zinc fingers are structurally diverse and are present among proteins that perform a broad range of functions in various cellular processes, such as replication and repair, metabolism, cell proliferation, and apoptosis.<sup>13</sup> *CLASP2* belongs to a family of microtubule plus-end tracking proteins that localizes to the distal ends of microtubules and regulates microtubule dynamics.<sup>14</sup> *CLASP2* functions in various microtubule-dependent processes, including cell division, cytoskeletal remodeling for cell migration, podosome regulation, and stabilization of adherens junctions.<sup>15,16</sup> *CLASP2* is also involved in epithelial-mesenchymal transition and progression in cancer.<sup>17</sup> And finally, *CSNK1* is a group of ubiquitous serine/threonine kinases that are involved in normal cellular functions and several pathological conditions, such as DNA repair, cell cycle progression, and apoptosis.<sup>18</sup> Recent studies indicate that *CSNK1E* expression has a role in human cancers.<sup>19,20</sup> Based on these reports, all three genes are involved in hyperproliferative cellular processes, which is highly relevant to vascular remodeling observed in PAH, and may represent novel biomarkers of PAH severity.

Limitations of this study include a small sample size reflecting low severity of disease and heterogeneity in PAH subtypes. We do not have the power to evaluate differences in patients with advanced PAH or in homogenous cases such as idiopathic PAH alone due to incrementally smaller sample sizes during subgroup analysis. We also present a cross-sectional view of gene expression patterns and not serial patterns over time, which may better reflect progression of disease. While there are many phenotypes in PAH that provide a measure of severity, we chose one representative and well-studied variable from each diagnostic test (i.e., PVR from RHC) for association analyses, limiting interpretation to other markers of severity.

In summary, the current study demonstrates the utility of PBMC transcriptomes to identify molecular classifiers of

disease risk and severity in PAH. They further highlight how PBMCs may shed insights into both target tissue expression profiles as well as possible roles of inflammation in the molecular pathogenesis of PAH.

## Scientific Knowledge on the Subject

Pulmonary arterial hypertension is a complex disease with multiple molecular mediators that contribute to disease predilection and severity. While a number of studies have reported individual genes/pathways associated with severity or disease risk, mechanisms remain unclear.

## What This Study Adds to the Field

This study has identified novel transcripts that are potential valuable biomarkers of both pulmonary arterial hypertension severity assessed via multi-modality testing. Importantly, a subset of these gene expression profiles may play a pathological role in disease development.

## Author Contributions

CER: contributed to the concept of this study, data collection, drafting of the manuscript, and data analysis; XQ: contributed to the concept of this study, data collection, drafting of the manuscript, and data analysis; SS: contributed to the data collection and drafting of the manuscript; RRV: contributed to the data collection; RSS: contributed to the data collection and analysis and drafting of the manuscript; AC: contributed to the data analysis; MGG: contributed to the data analysis; FR: contributed to the data collection; RJA: contributed to the data collection; JW: contributed to the drafting of the manuscript; TS: contributed to the drafting of the manuscript; AB: contributed to the data collection and analysis; YS: contributed to the data collection; HT: contributed to the data collection and analysis and drafting of the manuscript; AM: contributed to the data collection and drafting of the manuscript; YK: contributed to the drafting of the manuscript; MWG: contributed to the data collection and drafting of the manuscript; JGG: contributed to the data collection and drafting of the manuscript; JXJ: contributed to the design of this study, data collection, drafting of the manuscript, and data analysis; AAD: contributed to the concept and design of this study, data collection, drafting of the manuscript, and data analysis.

## Conflict of interest

The author(s) declare that there is no conflict of interest.

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