Transcriptome-wide analysis associates \textit{ID2} expression with combined pre- and post-capillary pulmonary hypertension

Meghan J. Arwood\textsuperscript{1,4}, Nasim Vahabi\textsuperscript{1,4}, Christelle Lteif\textsuperscript{2}, Ravindra K. Sharma\textsuperscript{2}, Roberto F. Machado\textsuperscript{3} & Julio D. Duarte\textsuperscript{1*}

Heart failure with preserved ejection fraction (HFpEF) patients who develop pulmonary hypertension (PH) have an increased risk of death, with combined pre- and post-capillary PH (CpcPH) having the highest risk. However, the mechanism behind PH development in HFpEF is poorly understood. We aimed to identify transcriptomic associations with PH development in HFpEF. Blood was collected from 30 HFpEF patients: 10 without PH, 10 with isolated post-capillary PH, and 10 with CpcPH. Gene expression measurements were completed using transcriptome-wide RNA sequencing. Gene expression differences were compared using a quasi-likelihood method adjusting for age, sex, race, and smoking-status. Biological pathways were compared using global gene expression differences. A replication in 34 additional heart failure patients and a validation in lung tissue from a representative mouse model were completed using quantitative PCR. Six differentially expressed genes were identified when comparing transcriptomics between subjects with CpcPH and those without PH. When tested in additional subjects, only the association with \textit{ID2} replicated. Consistent with clinical findings, \textit{Id2} expression was also upregulated in mice with HFpEF and PH. Pathway analysis identified proliferative and mitochondrial pathways associated with CpcPH. Thus, these patients may possess systemic pathophysiological differences similar to those observed in pulmonary arterial hypertension patients.

Pulmonary hypertension (PH) is a complex condition, with the persistence of elevated mean pulmonary arterial pressure (MPAP) leading to right-sided heart failure and ultimately death. PH due to left heart disease (also termed Group 2 PH by the World Health Organization) is one of the most common types of PH, stemming from left ventricular dysfunction and/or valvular heart disease. In Group 2 PH (historically defined as a MPAP $\geq 25$ mmHg and pulmonary arterial wedge pressure (PAWP) $>15$ mmHg), elevated left-sided filling pressures lead to passive post-capillary pulmonary venous congestion and may lead to additional remodeling effects. PH is common in left heart failure (HF), with researchers estimating that 55 to 80% of HF patients develop PH. Importantly, both the presence and severity of PH confer additional morbidity and mortality risk in HF patients. In general, the five-year mortality rate for HF-PH patients is about 50%.

HF-PH has, in recent years, been categorized into two main hemodynamic phenotypes based on calculations of diastolic pulmonary gradient (DPG) and/or pulmonary vascular resistance (PVR): isolated post-capillary PH (IpcPH; DPG $<7$ mmHg and/or PVR $\leq 3$ Wood Units) and combined pre- and post-capillary PH (CpcPH; DPG $\geq 7$ mmHg and/or PVR $>3$ Wood Units). While most HF-PH patients present with IpcPH, CpcPH is observed in up to 20% of HF-PH patients. CpcPH patients likely have intrinsic pulmonary vascular disease and MPAP that is disproportionate to the initial increase of left-sided filling pressures. Right ventricular to pulmonary vascular coupling is poor in these patients, which is thought to be one reason for the more than two-fold increased mortality risk compared to IpcPH patients.
Since a high percentage of HF patients develop PH and the mortality rate is alarming, effective treatment strategies for this disease are needed, yet no established treatment guidelines currently exist\(^1\). The current standard of care is to treat comorbid disorders and concentrate on improving volume status and left ventricular relaxation properties\(^2\). This is particularly problematic for patients with heart failure with preserved ejection fraction, who have few treatment options available to treat their underlying disease. Thus, it is vital to understand the mechanisms underlying PH development in HFpEF patients in order to personalize their treatment regimens and uncover novel treatment strategies. One way to better understand these mechanisms is to identify biological pathways that are associated with HFpEF-PH development. Although recent studies have identified limited candidate genes for HF-PH pathogenesis\(^3-15\), there is a paucity of research in this area and a lack of robust gene expression analyses. Therefore, the aim of this study was to conduct a transcriptome-wide association study to identify gene expression signatures associated with IpcPH and CpcPH development in HFpEF patients.

**Methods**

**Study population.** The discovery and replication cohorts used in this study consisted of HF patients with and without PH recruited from the University of Illinois Hospital and Health Science System (UI Health) heart failure and pulmonary hypertension clinics. All patients provided informed, written consent before participation, and protocols for recruitment were approved by the University of Illinois at Chicago Institutional Review Board in accordance with federal and local regulations. Data available for abstraction included patient demographics, past medical history, medication regimen, as well as echocardiogram and right heart catheterization measurements. Additionally, blood samples were collected at the time of enrollment, and RNA was isolated from peripheral blood mononuclear cells (PBMCs) in a subset of subjects.

HFpEF patients were diagnosed with heart failure, had an ejection fraction >45%, and a history of diastolic dysfunction on echocardiogram. In the discovery cohort, PBMCs were available from 30 HFpEF patients: 10 with HFpEF without PH (MPAP ≤ 20 mmHg or tricuspid regurgitation velocity ≤ 3 m/s measured on echocardiogram), 10 with IpcPH, and 10 with CpcPH.

**Gene expression with RNA-seq.** RNA was isolated from PBMCs that were extracted from peripherally drawn blood. After bead-based rRNA depletion was performed, cDNA was synthesized from RNA, and then amplified to create a sequencing library. Sequencing was performed on the Illumina NextSeq 500 platform (Illumina, San Diego, CA, USA), and paired reads of approximately 75 bases were generated. Sequencing was completed in four separate pools, with alignments to each pool being randomized to minimize potential biases from sequencing pool or RNA extraction batch. FASTQ files from sequencing were aligned to the reference genome (hg38) with STAR\(^24\). STAR was also used to derive the number of reads mapped to each gene/transcript. Genes with low expression were filtered out and data were normalized using the trimmed mean of M-values (TMM) method\(^25\).

Gene expression levels were compared in pairwise analyses among the three HFpEF groups: IpcPH vs. no PH, CpcPH vs. no PH, and CpcPH vs. IpcPH. These analyses were performed using a negative binomial generalized linear model with a quasi-likelihood method in edgeR\(^26\), adjusting for age, sex, race, and smoking status. Genes were considered differentially expressed if the false discovery rate (FDR) adjusted q-value ≤ 0.05. A network analysis was completed to determine co-expressed genes using GeneMANIA\(^27\). A pathway analysis using gene set enrichment was also performed in GAGE\(^28\), using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways\(^29\). Additional information on RNA-seq procedures can be found in the Online Data Supplement.

**Replication analysis.** In an attempt to replicate the differentially expressed genes identified using RNA-seq, PBMC gene expression was compared in subjects that were not included in the RNA-seq discovery cohort but were from the same research protocols used to recruit patients at UI-Health. Thus, similar clinical data as described in the discovery cohort were also collected in the replication cohort. These subjects were categorized into groups of no PH, IpcPH and CpcPH using the criteria described above. However, unlike the discovery cohort above, this replication cohort contained both HFpEF and heart failure with reduced ejection fraction (HFrEF) subjects.

Expression was measured in each of the differentially expressed genes identified in RNA-seq analysis using quantitative real-time PCR (qRT-PCR). RNA was isolated and reverse transcribed to complementary DNA. Gene expression was then measured using TaqMan\(^9\) gene expression assays (Thermo Fisher Scientific, Waltham, MA, USA). The comparative 2\(^{-ΔΔCt}\) method was used for calculating relative quantitation of gene expression\(^30\), normalized to the expression of the housekeeping gene RPLP0. Pairwise analyses of the no PH, IpcPH and CpcPH groups were completed using a similar regression model as described above, adjusting for identical covaraites (age, sex, race, and smoking status). Because our aim was to confirm findings in the discovery cohort, associations with a similar direction of effect and a one-sided P-value ≤ 0.05 were considered significant.

**Tissue-specific validation.** Male AKR/J mice (The Jackson Laboratory, Bar Harbor, ME) between 3–6 weeks old were randomized (N = 4–5 per group) to receive a high-fat diet (HFD; 60% lipid/kcal; Research Diets, New Brunswick, NJ) or normal chow (control mice) for 19 weeks. This mouse model has been previously shown to consistently develop HFpEF-PH\(^11\). After 19 weeks, mice were weighed, a blood sample was taken, and hemodynamics were measured via left and right heart catheterization, as previously described\(^32\). Mice were then euthanized, and a lung tissue sample was immediately taken and flash-frozen. After tissue samples were homogenized, RNA was isolated and gene expression was measured using the qRT-PCR methods described above, except normalizing expression to the housekeeping gene Actb. Gene expression was compared between HFD and control mice via a two-sample t-test, and due to the small number of mice within each group, P-values were calculated...
Overall, clinical characteristics were similar between the discovery cohort used for the RNA-seq analysis and the replication of gene expression associations.

Pathway and network analyses. In an attempt to identify additional genes that might be involved in CpcPH pathway known to interact with genes differentially expressed CpcPH, we conducted a network analysis. A single cluster was created with 5 of the 6 genes (Fig. 2), as NCBP2-AS2 did not cluster with any other genes. Within the network of co-expressed genes, a total of 19 additional genes were identified as part of the network and also detected in our RNA-seq analysis. Of these, over half (11 genes) were also differentially expressed in CpcPH compared to no PH with marginal associations \( (P < 0.05) \), and three of the 11 differentially expressed genes \( (DUSP2, MAP3K8, IER5) \) had associations with FDR < 0.1.

Based on enrichment analysis of all 21,680 genes detected, the “cell cycle” and “oxidative phosphorylation” KEGG pathways were significantly upregulated in CpcPH compared to subjects without PH, with nine other pathways marginally upregulated (Table 3). Similar to individual gene results, no significant pathway associations were observed when comparing IpcPH to CpcPH or IpcPH to CpcPH.

Replication of gene expression associations. We attempted to replicate associations identified by RNA-seq within the ID1, ID2, RYR1, ZNF772, and ZNF132 genes in 34 additional subjects with HF (Table 4). Overall, clinical characteristics were similar between the discovery cohort used for the RNA-seq analysis and the replication of gene expression associations.

Table 1. Baseline characteristics of the discovery cohort. DPG- diastolic pulmonary gradient; LVEF- left ventricular ejection fraction; mPAP- mean pulmonary artery pressure; OSA- obstructive sleep apnea; PCWP- pulmonary capillary wedge pressure; PVR- pulmonary vascular resistance; SD- standard deviation; TPG- transpulmonary gradient. *Includes asthma, chronic obstructive pulmonary disease, restrictive lung disease, latent tuberculosis, parenchymal lung disease, history of pulmonary embolism.

| Baseline Characteristics | HFpEF without PH (n = 10) | IpcPH (n = 8) | CpcPH (n = 10) | P-value |
|--------------------------|---------------------------|--------------|---------------|---------|
| Age (years), mean ± SD   | 58.0 ± 6.0                | 63.1 ± 12.4  | 55.0 ± 8.8    | 0.16    |
| Female sex, n (%)        | 8 (80)                    | 5 (62.5)     | 5 (50)        | 0.38    |
| Race/ethnicity, n (%)    |                           |              |               |         |
| African American         | 7 (70)                    | 3 (37.5)     | 7 (70)        | 0.31    |
| Latino American          | 0 (0)                     | 3 (37.5)     | 1 (10)        |         |
| European American        | 2 (20)                    | 2 (25)       | 2 (20)        |         |
| Unknown                  | 1 (10)                    | 0 (0)        | 0 (0)         |         |
| mPAP (mmHg), mean ± SD   | N/A                       | 33.6 ± 6.4   | 49.7 ± 7.5    | 0.001   |
| PCWP (mmHg), mean ± SD   | 25.0 ± 8.5                | 22.5 ± 3.3   | 0.89          |
| PVR (Wood units), mean ± SD | 1.2 ± 0.7            | 4.1 ± 1.4    | 0.002         |
| TPG (mmHg), mean ± SD    | 8.8 ± 4.1                 | 27.2 ± 5.7   | 0.0004        |
| DPG (mmHg), mean ± SD    | 0.5 ± 1.4                 | 11.5 ± 3.3   | 0.0003        |
| LVEF (%), mean ± SD      | 58.5 ± 5.7                | 65.0 ± 4.8   | 0.07          |
| Systemic hypertension, n (%) | 9 (90)                 | 8 (100)      | 10 (100)      | 1.00    |
| Obstructive Sleep Apnea, n (%) | 4 (40)                | 3 (37.5)     | 5 (50)        | 0.90    |
| History of other lung disease*, n (%) | 6 (60)            | 1 (12.5)     | 6 (60)        | 0.08    |
| Hemodialysis, n (%)      | 1 (10)                    | 1 (12.5)     | 2 (20)        | 1.00    |
| Smoker                   |                           |              |               |         |
| Never                    | 5 (50)                    | 3 (37.5)     | 1 (10)        | 0.01    |
| Previous                 | 2 (20)                    | 5 (62.5)     | 9 (90)        |         |
| Current                  | 3 (30)                    | 0 (0)        | 0 (0)         |         |

In the adjusted analysis, four genes were significantly upregulated (ID1, ID2, RYR1, NCBP2-AS2), and two were significantly downregulated (ZNF772, ZNF132), in CpcPH patients compared to HFpEF patients without PH (FDR ≤ 0.05; Fig. 1 and Table 2). No differentially expressed genes meeting the pre-defined significance threshold were observed when comparing the no PH to IpcPH or CpcPH to IpcPH groups.

Results
In total, 28 subjects were included in the RNA-seq analysis, as two IpcPH samples failed quality control and were excluded from all analyses. Demographics were similar among the three groups, as well as prevalence of hypertension, obstructive sleep apnea, lung disease, and hemodialysis use. As anticipated based on study selection criteria and diagnostic differences between PH groups, mean PVR, MPAP, TPG, and DPG were significantly different between patient groups (Table 1). Smoking status also significantly differed among all groups, since the majority of PH patients were previous smokers and roughly a third of patients without PH were current smokers.

Differential gene expression analysis. An average of 49.6–68.0 million paired-end reads per sample were mapped to the human reference genome (hg38), of which approximately 85% were uniquely mapped. Additional information on alignment statistics and normalization are found in the Online Data Supplement. In the adjusted analysis, four genes were significantly upregulated (ID1, ID2, RYR1, NCBP2-AS2), and two were significantly downregulated (ZNF772, ZNF132), in CpcPH patients compared to HFpEF patients without PH (FDR ≤ 0.05; Fig. 1 and Table 2). No differentially expressed genes meeting the pre-defined significance threshold were observed when comparing the no PH to IpcPH or CpcPH to IpcPH groups.
replication cohort, with the exception of a lower LVEF in the replication cohort (likely due to the inclusion of HFrEF patients). Out of the five genes tested for replication, only ID2 expression was significantly upregulated in CpcPH patients compared to HF patients without PH (Table 5). Similar to the original transcriptomic analysis, none of the genes tested were associated with IpcPH (when compared to no PH or CpcPH).

**Tissue-Specific validation of gene expression associations.** To confirm that gene expression signatures discovered in PBMCs were consistent in lung tissue, expression was measured in the lungs using a mouse-model of HFpEF-PH. Compared to control mice, HFpEF-PH mice had numerically higher left ventricular end diastolic pressure (LVEDP) and significantly higher right ventricular systolic pressure (RVSP), body weight, and hemoglobin A1c (Fig. S3). Because murine homologs of NCBP2-AS2, ZNF772, and ZNF132 could not be identified, only Id1, Id2, and Ryr1 were tested. Out of the three genes tested, only Id2 was significantly upregulated in HFpEF-PH mice versus control mice (Fig. 3).

**Discussion**

We conducted a transcriptome-wide analysis followed by replication and validation studies to identify genes and biological pathways that may be involved in the development of PH in HFpEF patients. To our knowledge, this is the first such study to investigate transcriptomic differences in HF-PH. Using total RNA extracted from PBMCs, we used RNA-seq to compare pairwise gene expression values among three groups: CpcPH vs. HFpEF without PH, CpcPH vs. IpcPH, and IpcPH vs. HFpEF without PH. Of these, differentially expressed genes were only discovered between CpcPH vs. HFpEF subjects without PH. Six genes were identified, including two from the “inhibitor of DNA binding” (ID) family (ID1 and ID2). From a network analysis of previously established co-expressed genes, we found that 11 of 19 genes also had at least marginal associations with CpcPH, suggesting that our transcriptomic associations are not likely due to chance. In the gene set enrichment analysis, pathways involved in “cell cycle” and “oxidative phosphorylation” were significantly upregulated in CpcPH
compared to subjects without PH. Following the transcriptome-wide analysis, we replicated the observed association between increased \( \text{ID2} \) expression and CpcPH in a separate, similar-sized cohort of both HFpEF and HFrEF patients. Finally, we completed a tissue-specific validation showing \( \text{Id2} \) is also upregulated in the lungs of HFpEF-PH mice.

Our results suggest that ID signaling may be involved in the development of CpcPH. The ID family consists of four members (ID1 – ID4) that are part of the helix-loop-helix (HLH) group of transcription factors. ID proteins bind with other HLH transcription factors to form heterodimers, preventing the complex binding to their specific binding motif. Previous evidence shows that ID1 – ID3 are important in the cardiovascular system, have functions closely linked to vascular endothelial growth factor (VEGF), and are associated with muscle cell differentiation as well as endothelial cell activation, differentiation, and proliferation\(^{33}\). ID2 in particular has been associated with promotion of cell death, prevention of differentiation, regulating myogenesis, vascular smooth muscle cell phenotype, and vessel plasticity\(^{34-37}\). Moreover, ID1 - ID3 are all expressed in endothelial and vascular smooth

---

**Figure 2.** Network analysis completed for genes with significant differential expression identified in RNA-seq analysis (shaded with gray diagonal lines).

| Pathway (Homo sapiens)                  | \( P \)-value |
|----------------------------------------|---------------|
| Cell cycle (hsa04110)                  | 0.0001*       |
| Oxidative phosphorylation (hsa00190)   | 0.0003*       |
| Phagosome (hsa04145)                   | 0.0036        |
| Oocyte meiosis (hsa04114)              | 0.0044        |
| Gap junction (hsa04540)                | 0.0069        |
| p53 signaling pathway (hsa04115)      | 0.0071        |
| Proteasome (hsa03050)                  | 0.0100        |
| Protein processing in endoplasmic reticulum (hsa04141) | 0.0171 |
| Ribosome (hsa03010)                    | 0.0180        |
| Regulation of actin cytoskeleton (hsa04810) | 0.0323 |
| Progesterone-mediated oocyte maturation (hsa04914) | 0.0387 |

**Table 3.** KEGG pathways upregulated in CpcPH compared to HFpEF subjects without PH. *FDR \( \leq \) 0.05.
muscle cells derived from pulmonary arteries and microvasculature. ID expression is regulated by transforming growth factor beta (TGFβ)-related growth factors, such as the bone morphogenetic proteins (BMPs). BMP signaling is fairly well-described in the pathogenesis of pulmonary arterial hypertension (PAH), primarily through clinical data associating BMP receptor type II (BMPR2) gene mutations with familial PAH.

### Table 4. Demographic and clinical characteristics of the replication cohort.

| Baseline Characteristics | HF without PH (n = 14) | IpCPH (n = 11) | CpcPH (n = 9) | P-value |
|--------------------------|-----------------------|---------------|--------------|---------|
| Age (years), mean ± SD   | 55 ± 11               | 55 ± 13       | 56 ± 9       | 1.0     |
| Female sex, n (%)        | 10 (71.4)             | 7 (63.6)      | 6 (66.7)     | 1.0     |
| Race/ethnicity, n (%)    |                       |               |              |         |
| African American         | 12 (85.7)             | 10 (90.9)     | 8 (88.9)     |         |
| Latino                   | 2 (14.3)              | 1 (9.1)       | 1 (11.1)     |         |
| European American        | 0 (0)                 | 0 (0)         | 0 (0)        |         |
| mPAP (mmHg), mean ± SD   | 32.2 ± 5.2            | 37.8 ± 8.5    | 0.13         |
| PCWP (mmHg), mean ± SD   | 27.5 ± 8.5            | 20.1 ± 9.0    | 0.08         |
| PVR (Wood units), mean ± SD | 1.7 ± 0.7          | 5.2 ± 2.0     | 0.0008       |
| TPG (mmHg), mean ± SD    | 5.1 ± 3.0             | 17.7 ± 3.3    | 0.0002       |
| DPG (mmHg), mean ± SD    | 0.1 ± 0.3             | 8.6 ± 4.3     | <0.0001      |
| LVEF (%), mean ± SD      | 40.4 ± 12.7           | 38.9 ± 9.2    | 0.94         |
| Systemic hypertension, n (%) | 13 (92.9)          | 9 (81.8)      | 9 (100)      | 0.46    |
| Obstructive Sleep Apnea, n (%) | 1 (7.1)            | 5 (45.5)      | 4 (44.4)     | 0.05    |
| History of other lung disease*, n (%) | 6 (42.9)         | 3 (27.3)      | 3 (33.3)     | 0.90    |
| Hemodialysis, n (%)      | 0 (0)                 | 0 (0)         | 0 (0)        | N/A     |
| Smoker, n (%)            |                       |               |              |         |
| Never                    | 5 (35.7)              | 4 (36.4)      | 4 (44.4)     | 0.91    |
| Previous                 | 5 (35.7)              | 4 (36.4)      | 4 (44.4)     | 0.91    |

### Table 5. Replication of six differentially expressed genes comparing HF subjects with CpcPH to those without PH.

| Gene     | Fold change (CpcPH vs. no PH) | P-value (one-sided) |
|----------|-------------------------------|---------------------|
| ZNF132   | 0.89                          | 0.410               |
| ID1      | 0.81                          | 0.546               |
| ZNF772   | 0.75                          | 0.156               |
| RYR1     | 1.29                          | 0.329               |
| NCRP2-AS2| 0.94                          | 0.619               |
| ID2      | 1.24                          | 0.047               |

### Figure 3. Comparison of (A) Id1, (B) Ryr1, and (C) Id2, pulmonary gene expression in HFpEF-PH mice vs. control mice. N = 3–5 in each group. *P ≤ 0.05 compared to control mice.
PAH subjects with left HF, since there is currently no practical way to clinically differentiate between these associations. Additional mechanistic studies are needed to better parse out the cell-specific mechanism of ID pulmonary vascular tissue which may have also decreased sensitivity for validating additional gene expression similarly upregulated in the lungs. However, total lung tissue was used in our validation rather than isolated ID2 is involved in PH development. In order to consider ID2 expression levels in PBMCs to be a reliable predictor of CpcPH, perhaps in a similar fashion as in PAH. This theory is supported by the work of Assad et al., who have demonstrated that CpcPH patients have a pulmonary vascular physiology that more closely resembles that of PAH patients than that of IpcPH patients. However, additional research is needed to better outline the mechanisms underlying ID function in CpcPH.

Evidence suggests that PH is a multifaceted pan-vasculopathy that mirrors characteristics observed in cancer, including resistance to apoptosis, inflammation, increased proliferation, and fibrosis caused by remodeling of the extracellular matrix. Dysregulation in the ability of pulmonary vascular cells to progress through the cell cycle stages could result in apoptosis resistance and cell proliferation. Thus, it is not surprising that our pathway analysis identified significant upregulation of the “cell cycle” pathway in CpcPH patients. In fact, ID proteins have been associated with promotion of proliferation and migration of endothelial progenitor cells, and markers of smooth muscle cell proliferation.

A popular theory contends that early in PH development, initial endothelial cell apoptosis causes a separate population of pathogenic and hyperproliferative endothelial cells that drive later PH pathogenesis. In a phenomenon discovered in cancer called the Warburg effect, cells rely upon glycolysis and shift away from oxidative phosphorylation for production of cellular energy. However, aerobic glycolysis is not limited to malignancy, as the Warburg effect has also been described to play a role in PAH pathogenesis. Interestingly, evidence shows that ID2 regulates mitochondrial function and contributes to maintenance of mitochondrial membrane potential, oxidative respiration, and mitochondrial electron transport chain functions. Specifically, ID2 has shown the ability to suppress mitochondrial oxidative respiration and ATP production. However, our pathway analysis showed that the “oxidative phosphorylation” pathway was significantly upregulated in CpcPH patients compared to those without PH. Thus, our findings seem to contradict this theory and contrast previous evidence showing a repression of endothelial oxidative phosphorylation in PH development. This apparent discrepancy could simply reflect differences in gene expression patterns between the pulmonary vasculature and circulating immune cells. Further research is needed to clarify these differences.

Another gene identified in the RNA-seq analysis, RYR1 (which encodes the rynodyne receptor 1), was consistently upregulated in each analysis. RYR1 is primarily expressed in skeletal muscle, but is also expressed to a lesser extent in vascular smooth muscle. Its role in calcium-dependent muscle contraction makes it a potential candidate gene for PH development. However, neither the replication nor validation analysis showed significant associations and network analysis did not identify any additional genes with even nominal associations between CpcPH and subjects without PH. Thus, we did not find sufficient evidence associating RYR1 with CpcPH pathogenesis.

While we identified multiple differentially expressed genes in our transcriptome-wide analysis, we found no associations with any comparisons including IpcPH subjects. This lack of significant associations could suggest that those analyses were underpowered. This could be due to either the loss of two IpcPH samples that were excluded because of a failure to meet quality control standards. Or, as discussed above, perhaps a much larger sample size is needed to detect differentially expressed genes when comparing IpcPH to subjects without PH because those phenotypes appear more related to each other than to CpcPH.

This study has several strengths and limitations. First, we identified through transcriptome-wide analysis that ID2 expression is associated with CpcPH development. While the exact mechanism is unclear, the association of ID proteins with BMP downstream signaling and PAH development is reasonably well-established. Thus, our finding of differential ID2 expression in CpcPH patients suggest that alterations in the BMP signaling pathway (via at least ID2) could play a role in development of CpcPH, perhaps in a similar fashion as in PAH. This theory is supported by the work of Assad et al., who have demonstrated that CpcPH patients have a pulmonary vascular physiology that more closely resembles that of PAH patients than that of IpcPH patients. However, additional research is needed to better outline the mechanisms underlying ID function in CpcPH.

Our relatively small sample size in each PH group could also be a limitation, potentially underpowering us to detect additional gene expression associations. Because of the difficulty obtaining lung biopsies from PH patients, we opted to complete our transcriptome-wide analysis in PBMCs, which is not likely the primary tissue involved in PH development. In order to consider ID2 expression levels in PBMCs to be a reliable predictor of CpcPH development, additional studies would be required showing that changes in ID2 expression are associated with corresponding alterations in PH phenotype. Our data from a murine model of HfPef-PH indicate that ID2 is similarly upregulated in the lungs. However, total lung tissue was used in our validation rather than isolated pulmonary vascular tissue which may have also decreased sensitivity for validating additional gene expression associations. Additional mechanistic studies are needed to better parse out the cell-specific mechanism of ID signaling in CpcPH. Lastly, we are unable to rule out that some of the subjects classified as CpcPH were actually PAH subjects with left HF, since there is currently no practical way to clinically differentiate between these conditions. This highlights the need to reclassify PH based on molecular phenotype due to challenges presented by the current classification system, which have ultimately impacted our ability to identify innovative treatment
strategies for patients with Group 2 PH\(^6\). Further research into the role of the ID signaling pathway in CpcPH development could be one way to contribute towards this unmet need.

**Data availability**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 11 June 2019; Accepted: 25 November 2019;
Published online: 20 December 2019

**References**

1. Barnett, C. F. & De Marco, T. Pulmonary hypertension associated with left-sided heart disease. *Heart Fail Clin* 8, 447–459 (2012).

2. Vachiery, J. L. et al. Pulmonary hypertension due to left heart diseases. *Journal of the American College of Cardiology* 62, D100–108, https://doi.org/10.1016/j.jacc.2013.10.033 (2013).

3. Guazzi, M. & Borlaug, B. A. Pulmonary hypertension due to left heart disease. *Circulation* 126, 975–990, https://doi.org/10.1161/CIRCULATIONAHA.111.085761 (2012).

4. Guazzi, M., Vicenzi, M., Arena, R. & Guazzi, M. D. Pulmonary hypertension in heart failure with preserved ejection fraction: a target of phosphodiesterase-5 inhibition in a 1-year study. *Circulation* 124, 164–174, https://doi.org/10.1161/CIRCULATIONAHA.110.983866 (2011).

5. Delgado, J. F. et al. Pulmonary vascular remodeling in pulmonary hypertension due to chronic heart failure. *European Journal of heart failure* 7, 1011–1016, https://doi.org/10.1016/j.ejhf.2004.10.021 (2005).

6. Guazzi, M. & Arena, R. Pulmonary hypertension with left-sided heart disease. *Nature reviews. Cardiology* 7, 648–659, https://doi.org/10.1038/nrcardio.2010.144 (2010).

7. Bursi, F. et al. Pulmonary pressures and death in heart failure: a community study. *Journal of the American College of Cardiology* 59, 222–231, https://doi.org/10.1016/j.jacc.2011.06.076 (2012).

8. Ghiyo, S. et al. Independent and additive prognostic value of right ventricular systolic function and pulmonary artery pressure in patients with chronic heart failure. *Journal of the American College of Cardiology* 37, 183–188 (2001).

9. Grigioni, F. et al. Prognostic implications of serial assessments of pulmonary hypertension in severe chronic heart failure. *The Journal of heart and lung transplantation: the official publication of the International Society for Heart Transplantation* 25, 1241–1246, https://doi.org/10.1016/j.healun.2006.06.015 (2006).

10. Hoepner, M. M. et al. Diagnosis, assessment, and treatment of non-pulmonary arterial hypertension pulmonary hypertension. *Journal of the American College of Cardiology* 54, 85–96, https://doi.org/10.1016/j.jacc.2009.04.008 (2009).

11. Lam, C. S. et al. Pulmonary hypertension in heart failure with preserved ejection fraction: a community-based study. *Journal of the American College of Cardiology* 53, 1119–1126, https://doi.org/10.1016/j.jacc.2008.11.051 (2009).

12. Leung, C. C., Moendra, V., Catherwood, E. & Andrus, R. B. Prevalence and risk factors of pulmonary hypertension in patients with elevated pulmonary venous pressure and preserved ejection fraction. *The American journal of cardiology* 106, 284–286, https://doi.org/10.1016/j.amjcard.2010.02.039 (2010).

13. Redfield, M. M. et al. Burden of systolic and diastolic ventricular dysfunction in the community: appreciating the scope of the heart failure epidemic. *Jama* 289, 194–202 (2003).

14. Salamon, J. N. et al. Outcomes in World Health Organization group II pulmonary hypertension: mortality and readmission trends with systolic and preserved ejection fraction-induced pulmonary hypertension. *Journal of cardiac failure* 20, 467–475, https://doi.org/10.1016/j.cardfail.2014.05.003 (2014).

15. Guazzi, M. & Naeije, R. Pulmonary Hypertension in Heart Failure: Pathophysiology, Pathobiology, and Emerging Clinical Perspectives. *Journal of the American College of Cardiology* 69, 1718–1734, https://doi.org/10.1016/j.jacc.2017.01.051 (2017).

16. Vachiery, J. L. et al. Pulmonary hypertension due to left heart disease. *Eur Respir J* 53, https://doi.org/10.1183/13993003.01897-2018 (2019).

17. Gerges, M. et al. Pulmonary Hypertension in Heart Failure. Epidemiology, Right Ventricular Function, and Survival. *Am J Respir Crit Care Med* 192, 1234–1246, https://doi.org/10.1164/rccm.201503-0529OC (2015).

18. Rezaee, M. E., Nichols, E. L., Siddhu, M. & Brown, J. R. Combined Post- and Precapillary Pulmonary Hypertension in Patients With Heart Failure. *Clinical cardiology* 39, 658–664, https://doi.org/10.1002/clc2.12727 (2016).

19. McLaughlin, V. V. et al. ACCF/AHA 2009 expert consensus document on pulmonary hypertension: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association: developed in collaboration with the American College of Chest Physicians, American Thoracic Society, Inc., and the Pulmonary Hypertension Association. *Circulation* 119, 2350–2294, https://doi.org/10.1161/CIRCULATIONAHA.109.192230 (2009).

20. Guazzi, M. Pulmonary hypertension in heart failure preserved ejection fraction: prevalence, pathophysiology, and clinical perspectives. *Circulation. Heart failure* 7, 367–377, https://doi.org/10.1161/CIRCHEARTFAILURE.113.000823 (2014).

21. Assal, T. R. et al. Clinical and Biological Insights Into Combined Post- and Pre-Capillary Pulmonary Hypertension. *Journal of the American College of Cardiology* 66, 2525–2536, https://doi.org/10.1016/j.jacc.2016.09.942 (2016).

22. Kelly, N. J. et al. Mouse Genome-Wide Association Study of Preclinical Group II Pulmonary Hypertension Identifies Epidermal Growth Factor Receptor. *American journal of respiratory cell and molecular biology* 56, 468–496, https://doi.org/10.1165/rcmb.2016-0176OC (2017).

23. Duarte, J. et al. Endothelial nitric oxide synthase genotype is associated with pulmonary hypertension severity in left heart failure patients. *Pulmonary circulation* 8, 2045894018773049, https://doi.org/10.1177/2045894018773049 (2018).

24. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21, https://doi.org/10.1093/bioinformatics/btu635 (2013).

25. Robinson, M. D. & Oshlack, A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol* 11, R25, https://doi.org/10.1186/gb-2010-11-3-r25 (2010).

26. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140, https://doi.org/10.1093/bioinformatics/btp616 (2010).

27. Wardle-Farley, D. et al. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res* 38, W214–220, https://doi.org/10.1093/nar/gks537 (2010).

28. Luo, W., Friedman, M. S., Shedden, K., Hankenson, K. D. & Woolf, P. J. GAGE: generally applicable gene set enrichment for pathway analysis. * BMC Bioinformatics* 10, 161, https://doi.org/10.1186/1471-2105-10-161 (2009).

29. Kanehisa, M. & Goto, S. J. N. a r. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28, 27–30 (2000).

30. Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3, 1101–1108 (2008).

31. Meng, Q. et al. Development of a Mouse Model of Metabolic Syndrome, Pulmonary Hypertension, and Heart Failure with Preserved Ejection Fraction. *American journal of respiratory cell and molecular biology* 56, 497–505, https://doi.org/10.1165/rcmb.2016-0177OC (2017).
32. Sharma, R. K. et al. Involvement of Neuroinflammation in the Pathogenesis of Monocrotaline-Induced Pulmonary Hypertension. *Hypertension* **71**, 1156–1163, https://doi.org/10.1161/HYPERTENSIONAHA.118.10934 (2018).

33. Yang, J., Li, X. & Morrell, N. W. Id proteins in the vasculature: from molecular biology to cardiopulmonary medicine. *Cardiovascular research* **104**, 388–398, https://doi.org/10.1093/cvr/cvu215 (2014).

34. Florio, M. et al. ID2 promotes apoptosis by a novel mechanism independent of dimerization to basic helix-loop-helix factors. *Mol Cell Biol* **18**, 5435–5444 (1998).

35. Langlands, K., Yin, X., Anand, G. & Prochownik, E. V. Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. *J Biol Chem* **272**, 19785–19793 (1997).

36. Kumar, M. S., Hendrix, J. A., Johnson, A. D. & Owens, G. K. Smooth muscle alpha-actin gene requires two E-boxes for proper expression in vivo and is a target of class I basic helix-loop-helix proteins. *Circ Res* **92**, 840–847, https://doi.org/10.1161/01. RES.0000086931.55281.7C (2003).

37. Moya, I. M. et al. Stalk cell phenotype depends on integration of Notch and Smad5/9 signaling cascades. *Dev Cell* **22**, 501–514, https://doi.org/10.1016/j.devcel.2012.01.007 (2012).

38. Norton, J. D. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci* **113**(Pt 22), 3897–3905 (2000).

39. Yang, J. et al. Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension. *Circ Res* **102**, 1212–1213, https://doi.org/10.1161/CIRCRESAHA.108.173567 (2008).

40. Massague, J., Blain, S. W. & Lo, R. S. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* **103**, 295–309 (2000).

41. Morty, R. E. et al. Dysregulated bone morphogenetic protein signaling in monocrotaline-induced pulmonary arterial hypertension. *Arterioscler Thromb Vasc Biol* **27**, 1072–1078, https://doi.org/10.1161/ATvbaha.107.141200 (2007).

42. Yang, J. et al. Id proteins are critical downstream effectors of BMP signaling in human pulmonary arterial smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **305**, 1312–321, https://doi.org/10.1152/ajplung.00524.2013 (2013).

43. Kowanetz, M., Valsalva, U., Bergstrom, R., Heldin, C. H. & Moustakas, A. Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. *Mol Cell Biol* **24**, 4241–4245 (2004).

44. Chen, X. et al. Upregulation of ID protein by growth and differentiation factor 5 (GDF5) through a Smad-dependent and MAPK-independent pathway in HUVSMC. *J Mol Cell Cardiol* **41**, 26–33, https://doi.org/10.1016/j.yjmcc.2006.03.421 (2006).

45. Teogker, K. et al. Smooth muscle cell differentiation from human bone marrow; variations in cell type specific markers and Id gene expression in a new model of cell culture. *Cell Biol Int* **33**, 621–631, https://doi.org/10.1002/cbi.2009.02.003 (2009).

46. Lowery, J. W. et al. Id family protein expression and regulation in hypoxic pulmonary hypertension. *Am J Physiol Regul Integr Comp Physiol* **299**, R1463–1477, https://doi.org/10.1152/ajpregu.00866.2009 (2010).

47. Assad, T. R. et al. Hemodynamic evidence of vascular remodeling in combined post- and precapillary pulmonary hypertension. *Pulmonary circulation* **6**, 313–321, https://doi.org/10.1086/668316 (2016).

48. D’Alessandro, A. et al. Hallmarks of Pulmonary Hypertension: Mesenchymal and Inflammatory Cell Metabolic Reprogramming. *Antioxidants & redox signaling* **28**, 230–250, https://doi.org/10.1089/ars.2017.7217 (2018).

49. Wang, H. et al. Inhibitor of DNA binding-1 promotes the migration and proliferation of endothelial progenitor cells in vitro. *Mol Cell Biochem* **335**, 19–27, https://doi.org/10.1007/s11010-009-0226-9 (2010).

50. Michalakis, E. D. Spatio-temporal diversity of apoptosis within the vascular wall in pulmonary arterial hypertension: heterogeneous BMP signaling may have therapeutic implications. *Circ Res* **98**, 172–175, https://doi.org/10.1161/RES.00000000000004572 (2006).

51. Sako, S. et al. Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* **19**, 1178–1180, https://doi.org/10.1096/fj.04-32611f (2005).

52. Xu, W. & Erzuurum, S. C. Endothelial cell energy metabolism, proliferation, and apoptosis in pulmonary hypertension. *Comprehensive Physiology* **1**, 357–372, https://doi.org/10.1002/cphy.090005 (2011).

53. Warbug, O. On the origin of cancer cells. *Science* (New York, N.Y.) **123**, 309–314 (1956).

54. Archer, S. L. Acquired Mitochondrial Abnormalities, Including Epigenetic Inhibition of Superoxide Dismutase 2, in Pulmonary Hypertension and Cancer: Therapeutic Implications. *Advances in experimental medicine and biology* **903**, 29–53, https://doi.org/10.1007/978-1-4899-7678-9_3 (2016).

55. Guignabert, C. et al. Pathogenesis of pulmonary arterial hypertension: lessons from cancer. *European respiratory review: an official publication of the European Respiratory Society* **22**, 543–551, https://doi.org/10.1183/09059180.0007513 (2013).

56. Michalakis, E. D. & Weir, E. K. The metabolic basis of vascular oxygen sensing: diversity, compartmentalization, and lessons from cancer. *American journal of physiology. Heart and circulatory physiology* **295**, H926–H930, https://doi.org/10.1152/ajpheart.00697.2008 (2008).

57. Zhang, Z. et al. ID2 promotes survival of glioblastoma cells during metabolic stress by regulating mitochondrial function. *FASEB journal* **299**, 621–631, https://doi.org/10.1152/ajpheart.00697.2008 (2010).

58. Zhao, J., Li, S. & Wang, X. BMP signaling may have therapeutic implications. *Dev Cell* **18**, 289–300, https://doi.org/10.1016/j.devcel.2010.02.012 (2010).

59. Anselmi, P. et al. Stalk cell phenotype depends on integration of Notch and Smad5/9 signaling cascades. *Dev Cell* **22**, 501–514, https://doi.org/10.1016/j.devcel.2012.01.007 (2012).

60. Norton, J. D. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci* **113**(Pt 22), 3897–3905 (2000).

61. Marshall, J. D., Bazan, I., Zhang, Y., Fares, W. H. & Lee, P. J. Mitochondrial dysfunction and pulmonary hypertension: cause, effect, or both. *Am J Physiol Lung Cell Mol Physiol* **314**, L782–L796, https://doi.org/10.1152/ajplung.00531.2017 (2018).

62. Dweik, R. A. et al. An official American Thoracic Society Statement: pulmonary hypertension phenotypes. *Am J Respir Crit Care Med* **189**, 345–355, https://doi.org/10.1164/rccm.201311-1954ST (2014).

Acknowledgements

The authors would like to thank Stefan Green and Zarema Arbiyeva for their assistance with RNA sequencing and Cheryl Galloway for her assistance with sample processing and gene expression measurements. The authors would also like to thank Mohan K. Raizada for his assistance with the animal studies.

Author contributions

M.J.A., N.V., C.L. and J.D.D. wrote the manuscript; J.D.D. conceived the project; M.J.A., N.V., C.L., R.K.S. and J.D.D. completed the research; R.F.M. and J.D.D. contributed resources; M.J.A., N.V., C.L., R.K.S., R.F.M. and J.D.D. edited the article.

Competing interests

The authors declare no competing interests.
Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-019-55700-y.

Correspondence and requests for materials should be addressed to J.D.D.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019