# Defining the Clinical Validity of Genes Reported to Cause Pulmonary Arterial Hypertension

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**Take home message**

Using the semi-quantitative NIH Clinical Genome Resource model, twelve out of twenty-seven genes curated had definitive evidence, ten have emerging evidence, five were disputed, and one gene had no evidence to support causal PAH gene-disease relationships.


**ABSTRACT**

**PURPOSE:** Pulmonary arterial hypertension (PAH) is a rare, progressive vasculopathy with significant cardiopulmonary morbidity and mortality. Genetic testing is currently recommended for adults diagnosed with heritable, idiopathic, anorexigen-, hereditary hemorrhagic telangiectasia-, and congenital heart disease-associated PAH, PAH with overt features of venous/capillary involvement, and all children diagnosed with PAH. Variants in at least 27 genes have putative evidence for PAH causality. Rigorous assessment of the evidence is needed to inform genetic testing.

**METHODS:** An international panel of experts in PAH applied a semi-quantitative scoring system developed by the NIH Clinical Genome Resource to classify the relative strength of evidence supporting PAH gene-disease relationships based on genetic and experimental evidence.

**RESULTS:** Twelve genes (*BMPR2*, *ACVRL1*, *ATP13A3*, *CAV1*, *EIF2AK4*, *ENG*, *GDF2*, *KCNK3*, *KDR*, *SMAD9*, *SOX17*, and *TBX4*) were classified as having definitive evidence and three genes (*ABCC8*, *GGCX*, and *TET2*) with moderate evidence. Six genes (*AQP1*, *BMP10*, *FBLN2*, *KLF2*, *KLK1*, and *PDGFD*) were classified as having limited evidence for causal effects of variants. *TOPBP1* was classified as having no known PAH relationship. Five genes (*BMPR1A*, *BMPR1B*, *NOTCH3*, *SMAD1*, and *SMAD4*) were disputed due to a paucity of genetic evidence over time.

**CONCLUSIONS:** We recommend that genetic testing includes all genes with definitive evidence and that caution be taken in the interpretation of variants identified in genes with moderate or limited evidence. Genes with no known evidence for PAH or disputed genes should not be included in genetic testing.



**Key Words:** pulmonary arterial hypertension, genetics, molecular diagnosis, genomic medicine

**Points for clinical practice:**

-All genes with definitive evidence for a PAH gene-disease relationship are strongly recommended to be included in genetic testing.

-Caution should be taken in clinical interpretation for genes with less than definitive or strong evidence, and disputed genes or genes with no known genetic evidence for PAH should not be included in genetic testing.

-Four previously reported TGF-β/BMP pathway genes are disputed for a PAH gene-disease relationship.

-For undiagnosed cases, genetic reanalysis is recommended over time as new evidence for PAH gene-disease relationships emerges.

**Nonstandard Abbreviations and Acronyms**

| | |
|---|---|
| **APAH** | pulmonary arterial hypertension associated with other diseases |
| **BOECs** | blood outgrowth endothelial cells |
| **ClinGen** | Clinical Genome Resource |
| **HPAH** | heritable pulmonary arterial hypertension |
| **IPAH** | idiopathic pulmonary arterial hypertension |
| **LOF** | loss of function |
| **PAECs** | pulmonary arterial endothelial cells |
| **PASMCs** | pulmonary arterial smooth muscle cells |
| **PAH** | pulmonary arterial hypertension |
| **pLOF** | predicted loss of function |
| **RV** | right ventricular |
| **RVSP** | right ventricular systolic pressure |
| **SPS** | small patella syndrome |
| **VUS** | variant of uncertain significance |

## INTRODUCTION

Pulmonary arterial hypertension (PAH) (OMIM #178600) is a rare, often lethal, disease with pulmonary artery remodeling leading to increased pulmonary vascular resistance, right ventricular (RV) hypertrophy, and right heart failure [1-3]. PAH can occur in a heritable manner (HPAH), idiopathically (IPAH) or associated with other diseases including congenital heart disease, autoimmune connective tissue diseases or risk-factor exposure (APAH). PAH may be caused by genetic, epigenetic, and environmental factors, as well as gene-environment interactions which may modify genetic risk. Pathogenic *BMPR2* (bone morphogenetic protein receptor 2) variants are the major cause of HPAH, yet twenty-six additional genes have been implicated in IPAH and some HPAH cases primarily driven by the advent of massively-parallel sequencing technologies. Independent validation of PAH gene-disease relationships is critical to avoid over-interpretation of genetic findings. Moreover, reporting of large numbers of variants of uncertain significance (VUS) often negatively influences patient well-being. Variable levels of evidence for gene-disease relationships complicate the clinical interpretation of genetic testing results and the prioritization of research strategies in the field. Currently, patient education about the option of genetic testing is recommended for adult H/IPAH, anorexigen-, hereditary hemorrhagic telangiectasia-, and congenital heart disease-associated PAH, PAH with overt features of venous/capillary involvement (PVOD/PCH), and all pediatric PAH patients [4-7]. Thus, systematic review of the strength of evidence for PAH gene-disease relationships is needed.

An international panel of scientists, with extensive research experience in PAH gene discovery and characterization, was assembled to systematically assess evidence for PAH gene-disease relationships. We applied the National Institutes of Health Clinical Genome Resource (ClinGen) [8] framework for semiquantitative classification [9], as used for six other cardiovascular diseases [10-15]. Here, we report the results of evidence-based gene curation for twenty-seven genes implicated in PAH.

## METHODS

### Study design and criteria

We assembled a ClinGen pulmonary hypertension gene curation expert panel (PH GCEP) (https://clinicalgenome.org/affiliation/40071/) consisting of fifteen members from eight institutions and representing six countries. An overview of the gene classification process and

scoring criteria is provided in Figure 1. The scope of work included genes implicated in isolated H/IPAH: *BMPR2*, *ABCC8*, *AQP1*, *ATP13A3*, *BMP10*, *BMPR1A*, *BMPR1B*, *CAV1*, *FBLN2*, *GDF2*, *GGCX*, *KCNK3*, *KDR*, *KLF2*, *KLK1*, *NOTCH3*, *PDGFD*, *SMAD1*, *SMAD4*, *SMAD9*, *SOX17*, *TET2*, and *TOPBP1*. We curated three syndromic PAH genes: *ACVRL1* (PAH associated with hereditary hemorrhagic telangiectasia, PAH-HHT), *ENG* (PAH-HHT), and *TBX4* (small patella or *TBX4* syndrome) [16]. *EIF2AK4* was curated based on diagnoses of PVOD/PCH, which can be misdiagnosed as IPAH. APAH and persistent pulmonary hypertension of the newborn cases were excluded. Genes were assigned to expert panel members without conflicts of interest. A total of 168 peer-reviewed reports were evaluated for relevant genetic and experimental evidence for the 27 genes. Genome-wide sequencing data from several moderate- to large-sized cohorts were utilized in multiple gene curations. The UK NIHR Bioresource – Rare Diseases Study for PAH (NBR PAH cohort) comprises whole-genome sequencing of 1,038 unrelated, predominantly European adult IPAH patients [17-19]. The US National Biological Sample and Data Repository for PAH case-control study (PAH Biobank) included 2,572 exome-sequenced unrelated pediatric and adult PAH cases (43% IPAH, 48% APAH, 4% HPAH and 5% other PAH) of mixed ancestry [18-21]. Wang *et al* comprises 331 Han Chinese IPAH cases (Han Chinese IPAH cohort) with exome or genome sequencing [22]. The Spanish Registry includes 300 cases with H/IPAH and APAH analyzed by panel or exome sequencing [23-26].

**Genetic and functional evaluations of gene-disease relationships**

Lead curators scored genetic and experimental evidence according to the updated ClinGen framework (Standard Operating Protocol v9.0). The predominant type of genetic evidence scored was case-level variant data as family segregation and case-control association analyses rarely reached ClinGen criteria for inclusion. The threshold for inclusion was allele frequency less than 1/10,000 (gnomAD all, v2.1.1 controls, or relevant genetic ancestry) and variant type predicted loss of function (pLOF; nonsense, frameshift, canonical splice variant, and whole exon deletions) or missense with *in silico* predictions of deleteriousness ((CADD score ≥20 [27] or REVEL score with gene-specific thresholds [20]). Case-level evidence scores were weighted based on variant type, available functional data, and *de novo* inheritance. Experimental evidence included: a) expression in PAH relevant tissues/cells; b) known function including intracellular pathways, cell proliferation, apoptosis; c) functional alteration in variant-positive patient cells; d) PAH-relevant

animal models and rescue. Expression and functional studies referenced in the curations specified whether cell/tissue samples were from affected PAH patients or healthy controls. Where noted, expression data were taken from the Genotype-Expression Project, GTEx. Comparative studies utilized age-matched controls but genetic ancestry was not always available. As defined by SOP v9.0 guidelines individual PAH gene-disease relationships were classified as strong, moderate, limited, no known relationship, or disputed due to a lack of genetic evidence over time. Definitive classifications were assigned to genes with strong evidence for causality plus independent evaluation over ≥3 years post-discovery without contradictory evidence. Provisional gene curations were discussed by the full PH GCEP monthly. Genes curated before the 3-year post-discovery timepoint underwent recuration at the 3-year timepoint. Once a consensus decision was made, final classifications were published to the ClinGen website and made publicly available via our PH GCEP (https://search.clinicalgenome.org/kb/affiliate/10071) or the Hemostasis/Thrombosis GCEP (https://search.clinicalgenome.org/kb/affiliate/10028) for *ACVRL1* and *ENG*.

Curation and classification of the twenty-seven PAH genes occurred over a two-year period, mid 2019-2021. Group meetings were held on a monthly or bi-monthly basis and genes were assigned to distribute individual curator burden from month to month. Approximately 1-4 genes were curated per month, with re-evaluations when additional information was needed (i.e. relatedness of probands or functional experiment details), curation inconsistencies were identified (i.e. case inclusion criteria or variable use of *in silico* predictors of variant deleteriousness), or at the 3-year recuration timepoint.

## RESULTS

### 1. Strength of evidence for genes implicated in isolated H/IPAH

For the twenty-three genes assessed for isolated disease, gene-disease relationships were classified as definitive (n=8), moderate (n=3), limited (n=6), disputed (n=5), or no known relationship (n=1) (Table 1 and Figure 2).

### BMP pathway genes

Definitive

*BMPR2* encodes a type II receptor of the TGF-β superfamily that in complex with type I receptors drives phosphorylation of SMAD signaling molecules and tightly regulates processes related to development, differentiation, and growth [28, 29]. Linkage analysis in autosomal dominant (AD) PAH families led to the identification of *BMPR2* variants that segregated among affected family members with incomplete penetrance [30, 31]. Currently, more than 650 unique PAH-associated *BMPR2* variants have been reported [17, 20, 32], of which the majority are pLOF variants. Missense variants cluster in the conserved ligand-binding and protein kinase domains. *BMPR2* variants cause 70-80% of familial cases and 10-20% of sporadic cases but are rarely found in APAH cases. *BMPR2* is expressed in the pulmonary vasculature with reduced expression in patient-derived cells [33, 34]. Pulmonary arterial endothelial cells (PAECs) derived from PAH patients with *BMPR2* truncating variants demonstrated haploinsufficiency as a pathogenetic mechanism [35], with confirmation in mouse and rat models heterozygous for *Bmpr2* null alleles [36-38] or transgenic for a dominant-negative[39]. The rodent models exhibited increased right ventricular systolic pressure (RVSP) and increased arteriole muscularization with rescue of RV function by wild-type BMPR2 [40] or BMPR2 ligand [41].

*CAV1* encodes caveolin-1, the main structural and signaling protein of caveolae. *CAV1* was first identified as a putative PAH gene by exome sequence analysis in a multi-generational AD PAH family with incomplete penetrance [42]. Screening of 260 unrelated H/IPAH patients identified an additional *de novo* frameshift variant associated with reduced CAV1 protein in small artery endothelial cells compared to a control [42]. Independent studies identified seven additional *CAV1* variants among H/IPAH patients [20, 23, 43, 44]. CAV1 is expressed in lung endothelial and smooth muscle cells, expression is decreased or absent in plexiform lesions [45], and *CAV1* c.474del (transcript NM_001753.5) patient fibroblasts demonstrated reduced caveolae density and caveolar protein levels [45]. Increased SMAD1/5/8 phosphorylation in *CAV1* patient fibroblasts was not rescued by transduction with wild-type CAV1 [46]. Expression of the mutant protein caused reduced wild-type protein [46, 47], consistent with a dominant-negative mechanism. *Cav1* knockout mice exhibited pulmonary vascular remodeling and other pathological features consistent with PAH [48, 49]. Of note, the phenotype was rescued by endothelial re-expression of *CAV1* [50]. Rare variants in *CAV1* cause autosomal dominant and recessive lipodystrophies but the observed variants are different from those associated with H/IPAH and the molecular

mechanisms are likely different. Thus, *CAV1* was curated independently by the PH and monogenic diabetes GCEPs.

*GDF2* encodes BMP9, a circulating ligand member of the BMP signaling pathway. *GDF2* was first identified by burden testing using the NBR PAH cohort [17]. Subsequently, 47 unrelated patients with 45 unique heterozygous variants (pLOF and missense) were identified in three independent cohorts [20, 22, 44]. David *et al*. identified BMP9 as a functional activator of the endothelial-specific BMPR2/ALK1 signaling pathway [51]. BMP9 plasma levels were decreased among *GDF2* variant-positive patients versus controls or IPAH patients without *GDF2* variants, potentially due to impaired secretion[17]. Treatment of PAECs with wild-type or mutant *GDF2* supernatant resulted in mutant-specific attenuation of the anti-apoptotic response [22]. Recuration (March 16$^{th}$, 2022), three years after the initial gene discovery report, identified four additional heterozygous variants, including one nonsense and three likely deleterious missense variants, based on *in vitro* evidence [52, 53]. Combined analysis of the UK/US PAH cohorts identified *GDF2* as one of seven genes that were significantly associated with IPAH on a genome-wide basis [19]. Identification of homozygous *GDF2* pLOF variants in three children with severe PAH raises the possibility of semi-dominant inheritance [54-56]; however, unaffected siblings with biallelic variants in one family suggested variable expressivity [55]. There is also an emerging picture of overlap with HHT-like phenotypes, notably pulmonary arteriovenous malformations [56-58].

The Mothers Against Decapentaplegic Homolog 9 (*SMAD9*) gene encodes SMAD8, a member of the SMAD signaling protein family. Shintani *et al.* [59] first associated a heterozygous nonsense variant in *SMAD9* with PAH using a candidate gene screen of *ENG* and seven *SMAD*s in Japanese IPAH patients without *BMPR2* or *ACVRL1* variants (n=23). A missense [60] and a unique nonsense variant [61] were then identified in two independent candidate gene screens. Fourteen additional H/IPAH cases heterozygous for rare variants were identified in the PAH Biobank [20], NBR PAH cohort [17], and Han Chinese IPAH cohort [22]. The variants included missense (n=9, located in functional domains), nonsense (n=3) and an in-frame indel variant. *SMAD9* is ubiquitously expressed, including abundant expression in lung tissue (GTEx, on May 1$^{st}$, 2022) [62]. SMAD8 undergoes phosphorylation downstream of BMP type I receptors, inducing interaction with SMAD4 and transcriptional activity [63]. SMAD8 p.(Cys202*) (protein NP_001120689.1) failed to bind SMAD4 and displayed reduced transcriptional activity [59]. Transcript levels of the BMP target gene *Id2* were reduced in patient pulmonary arterial smooth

muscle cells (PASMCs) heterozygous for SMAD8 p.Lys43Glu (NP_001120689.1), although response to ligand was largely preserved suggesting redundancy of SMAD1/5/8 function [60]. *Smad9* knockout mice are viable and show evidence of spontaneous, age-related pulmonary vascular remodeling [64]. Further, SMAD8-dependent post-transcriptional up-regulation of a subset of microRNAs exerts anti-proliferative effects in control cells that was abrogated in patient cells with SMAD8 p.Arg294* (NP_001120689.1) variant, comparable to *BMPR2* exon deletion [61].

Limited

*BMP10* encodes the bone morphogenetic protein 10 (BMP10) ligand, a paralogue of BMP9 with 65% amino acid homology and overlapping function [51, 65]. Eyries and colleagues conducted targeted sequencing of nine known PAH genes plus *BMP10* in 263 patients. Heterozygous nonsense and missense variants in *BMP10* were found in two severely affected IPAH patients [66]. Gelinas and colleagues [67] identified a missense variant in *BMP10* by exome sequencing of a pediatric cohort (n=18). Two more studies independently reported heterozygous *BMP10* substitutions in two IPAH patients [68, 69]. *Bmp10* knockout mice die at an early embryonic stage due to retarded cardiac growth and chamber maturation [70]. In contrast, *Bmp10* conditional knockout mice (induced postnatally) were reported to be viable and fertile, exhibiting no PH phenotype under normoxic or hypoxic conditions [71].

Disputed genes

*BMPR1A* and *BMPR1B* encode type I receptors integral to the canonical BMP signaling pathway [72]. A potential relationship between *BMPR1B* and IPAH first emerged in a candidate gene study of 74 Japanese cases wherein two missense variants were described as pathogenic [73]. However, this analysis revealed that both variants are observed at a frequency exceeding the population prevalence of PAH (8.3KJPN, https://jmorp.megabank.tohoku.ac.jp/202109/variants). Additional publications reported variants in *BMPR1A* or *BMPR1B* [20, 22, 43] but only two missense variants per gene met our minimal inclusion criteria. BMPR1A and BMPR1B are expressed at equivalent levels to BMPR2 in human PASMCs but not detected in PAECs [74]. Due to a paucity of genetic evidence over time, both genes are disputed.

*SMAD1* and *SMAD4* encode additional members of the SMAD signaling protein family [75]. Targeted sequencing of *SMAD* genes in 324 PAH cases identified *SMAD4* predicted splice-site and missense variants, and a *SMAD1* missense variant in three IPAH patients [60]. Overexpression of the *SMAD1* variant demonstrated modestly reduced luciferase activity [60]. While additional *SMAD1* and *SMAD4* variants have been reported, the overall number remains small [20, 22, 23]. Both SMAD proteins are expressed in human lung (GTEx on November 24th, 2021) [62] and function as critical mediators of *BMPR2* signaling [76]. SMAD1/4 protein levels were reduced in a rat PH model [77] but there are contradictory reports in animal [78] and human [79] lung tissue studies. Based on the weak human genetic data for both *SMAD1* and *SMAD4* over time, both gene-disease relationships are disputed.

**Transporter and channel genes**

Definitive

*ATP13A3* encodes a transmembrane cation transporter that transports polyamines, small metabolites required for normal cell growth and proliferation [80]. Monoallelic *ATP13A3* variants were identified in the NBR PAH cohort (6 pLOF, 4 missense variants) [17]. Subsequently, four missense and five pLOF/missense variants were identified in the Han Chinese IPAH cohort [22] and PAH Biobank [20], respectively. Recently, biallelic *ATP13A3* variants were identified in three families with severe, early onset PAH and high mortality [81]. *ATP13A3* is highly constrained for loss-of-function variants (pLoF = 1) [82] and most of the PAH-associated missense variants occur in conserved protein domains [83]. These data indicate a dose-dependent, semi-dominant mode of inheritance for *ATP13A3*. ATP13A3 is expressed in PASMCs, PAECs, and blood outgrowth endothelial cells (BOECs) from IPAH patients [17], with decreased proliferation and increased apoptosis of BOECs transfected with *ATP13A3* siRNA [17]. Elevated ATP13A3 plasma concentrations have been reported in multiple cancers and PAH [84, 85]. The protein-truncating variants are predicted to undergo nonsense-mediated decay indicating haploinsufficiency as the likely disease mechanism. For the missense variants, the mechanism is unclear.

*KCNK3* encodes a two-pore domain potassium channel, a regulator of resting membrane potential and pulmonary vascular tone [86]. Exome sequencing identified *KCNK3* as the cause of AD PAH in a multi-generational family wherein a novel heterozygous missense variant co-

segregated with disease, but with incomplete penetrance [87]. Targeted sequencing detected additional missense variants in 5/320 unrelated H/IPAH cases, accounting for 1.9% of the total cohort. Electrophysiological analyses indicated reduced current in mutant channels [87], independently confirmed for two variants identified in an independent Spanish cohort [88, 89], indicating a LOF disease mechanism. To date, more than 20 likely pathogenic missense variants have been reported in H/IPAH [17, 20, 22, 23, 43, 90-93]. Identification of biallelic variants in two families [88, 94], suggests potential semi-dominant inheritance for *KCNK3*. *KCNK3* is expressed in PASMCs [86], with reduced mRNA and protein expression and increased sensitivity to selective KCNK3 channel blockade in PAH patient-derived pulmonary arteries and PASMCs compared to controls [95]. *Kcnk3* mutant rats expressing a truncated channel demonstrated age-related increased RVSP and other pathological features of PAH [96].

Moderate

ATP binding cassette subfamily C member 8 (*ABCC8*) encodes sulfonylurea receptor-1 (SUR1), a regulatory subunit of adenosine triphosphate (ATP)-sensitive potassium channel, Kir6.2. Heterozygous *ABCC8* variants were identified in 12/913 unrelated H/IPAH patients by exome sequencing [97]. Electrophysiological assays demonstrated reduced mutant channel function [97]. pLOF and missense variants were identified in twenty-one additional patients with H/IPAH [20, 24, 67], with incomplete penetrance [24, 97]. SUR1 protein expression was demonstrated in proximal pulmonary arteries and alveolar macrophages of IPAH patients [97]; however, mechanistic interpretation remains dependent on further functional data. Homozygous pathogenic variants in *ABCC8* are known to cause hyperinsulinemic hypoglycemia of infancy [98]. Based on differing modes of inheritance and only a single patient exhibiting overlapping phenotypes, *ABCC8* was curated separately for PAH and monogenic diabetes.

Limited

*AQP1* encodes aquaporin 1, a water transport channel that also promotes endothelial cell migration and angiogenesis [99]. Increased *AQP1* missense variant burden was demonstrated in the NBR PAH cohort [17]. Two missense variants were shown to co-segregate with AD PAH in three families, with incomplete penetrance, and insufficient segregation evidence to count in our scoring [17]. Three additional missense variants were identified in the Han Chinese IPAH cohort

[22]. Expression of *AQP1* was demonstrated in PAH lung endothelium and healthy donor PAECs [17] but no functional effects of patient variants has been reported. In mice, homozygosity for *Aqp1* null alleles resulted in *attenuated* hypoxic PH [100]. Currently, only heterozygous missense variants are considered potentially disease relevant.

**Growth and transcription/translation factor genes**

Definitive

*KDR* encodes the kinase insert domain receptor for vascular endothelial growth factor type 2 (VEGFR2). Ligand activation of VEGFR2 promotes cell proliferation, cell survival, and migration. Protein-truncating *KDR* variants were reported in 4/1048 IPAH cases from the NBR PAH cohort [17]. Subsequently, 2/311 IPAH cases were identified with *KDR* variants associated with a low diffusion capacity for carbon monoxide (DLCO); co-segregation analysis indicated that variant heterozygotes were either affected by PAH or had decreased DLCO [101]. Four additional pLOF [18] and three missense variants, including one recurrent in three unrelated IPAH patients [19], were identified during recuration. *KDR* is highly expressed in PAECs [102]. Mice exposed to chronic hypoxia combined with SU5416-mediated inhibition of VEGFR resulted in vascular remodelling, PAEC proliferation and obliteration, and severe PH; biomarker analysis revealed a signature analogous to human PAH patients [103]. Endothelial-specific *Kdr* deletion resulted in a mild PAH phenotype under normoxia that worsened under hypoxia [104].

*SOX17* is a two-exon gene encoding the SRY-box transcription factor 17. *SOX17* is critical in cardiovascular morphogenesis and postnatal vascular remodeling [105]. Gene burden testing in the NBR PAH cohort revealed enrichment of *SOX17* variants in IPAH cases compared to controls [17]. Subsequently, 21 pLOF and missense variants were identified in H/IPAH patients from diverse populations [20, 106-108]. Most PAH-associated nonsense and frameshift variants occur in the terminal exon, in the conserved β-catenin-binding domain, and are predicted to escape nonsense-mediated decay. Wang *et al.* [108] reported a terminal exon *SOX17* nonsense variant in a multi-generational family associated with a 14-fold reduction of target gene *NOTCH1* reporter activity and de-repression of β-catenin compared to wild-type. Of interest, endothelial-specific deletion of *Notch1* results in worsened PH in mice [109]. Immunolocalization of SOX17

established endothelial specific expression in the pulmonary arterioles of wild-type cells and PAH vascular lesions [17].

Limited

*KLF2* encodes Krüppel-like factor 2, a transcriptional repressor of inflammation, endothelial activation, and proliferation [110]. Eichstaedt and colleagues [111] identified a heterozygous missense variant in two affected siblings with HPAH but not an unaffected brother. Functional analyses indicated loss of *KLF2* nuclear localization in patient-derived lung [112] and PAECs [113], and decreased transcriptional activity in transfected cells [112]. No *KLF2* variants have been reported in other H/IPAH cases. KLF2 is highly expressed in human [114] and rodent [115] lung, and decreased in PAH lung compared to healthy lung [113]. *KLF2* overexpression in pulmonary vascular cells demonstrated decreased apoptosis and cell proliferation [113]. The classification is based on limited genetic evidence to date.

Gene burden testing in a combined analysis of the PAH Biobank and the NBR PAH cohort demonstrated increased burden for five previously reported PAH genes and two new genes, *PDGFD* and *FBLN2* (see 'Other genes' below) [19]. Nine IPAH cases were identified carrying seven unique and two recurrent *PDGFD* missense variants [19]. Gelinas *et al.* [67] reported an additional IPAH case with a novel missense variant. *PDGFD* encodes a member of the platelet-derived growth factor family, a mesenchymal mitogenic factor involved in regulation of embryonic development, cell proliferation, cell migration, survival, and chemotaxis [116-118]. *PDGFD* is expressed in lung (GTEx, on March 11$^{th}$, 2022) [62] and arterial vasculature cells [119], but expression in the pulmonary vasculature has not been assessed. Cardiac-specific *Pdgfd* transgenic mice have increased SMC proliferation, vessel wall thickening, fibrosis, heart failure, and premature death [120],

No evidence

*TOPBP1* encodes a topoisomerase binding protein required for DNA replication. Common variants in *TOPBP1* were identified by exome sequencing in twelve IPAH patients [121] but with similar allele frequency in the control populations and without segregation with PAH [122]. Rare variants have not been reported for PAH cases. *TOPBP1* expression is altered in PAH lung tissues but there is no genetic evidence for a gene-disease relationship [121].

Disputed

The Notch pathway is a highly conserved signalling cascade with important roles in human development and tissue homeostasis [123]. Two *NOTCH3* missense variants were identified in IPAH patients by a targeted candidate gene screen [124]. One variant had an allele frequency (JPN8.3K, MAF: 0.002) that exceeded our threshold for inclusion and the other had functional data contradictory to a presumed gain-of-function mechanism [124]. Targeted sequencing in other cohorts [23, 92, 125-127] identified only two additional *NOTCH3* missense variants in H/IPAH that met our minimal threshold. NOTCH3 is expressed in PASMCs and IPAH patient cells display overexpression of *NOTCH3* [128]. However, the paucity of rare *NOTCH3* variants in PAH patients indicates that the regulation of NOTCH3 signaling is independent of genetic variation.

**Other genes**

Moderate

Gene burden testing in the PAH Biobank [20] identified two PAH candidate genes, gamma-glutamyl carboxylase (*GGCX*) and kallikrein 1 (*KLK1*) (see 'Limited' below). Heterozygous *GGCX* variants (5 pLOF, 9 missense) were identified in 18 H/IPAH cases, with three missense variants recurrent in at least two cases each [20]. *GGCX* plays important roles in blood coagulation, bone formation, vascular integrity, and inflammation [129]. *GGCX* expression was detected in lung and liver (GTEx, on March 8th, 2021) but a potential pathogenetic mechanism cannot be established without experimental data. Biallelic variants in *GGCX* cause vitamin K-dependent coagulation factor deficiency (MIM #277450) [129] but the genetic variants and mode of inheritance are distinct. *GGCX* was curated independently by the PH and hemostasis/thrombosis GCEPs.

*TET2* encodes tet-methylcytosine-dioxygenase-2, an epigenetic regulatory enzyme implicated in cancer [130-132], cardiovascular disease [133, 134] and inflammation [135]. Targeted burden analysis in the PAH Biobank demonstrated increased burden of *TET2* variants in PAH cases compared to controls, largely due to heterozygous pLOF variants (9 pLOF, 3 missense) and IPAH cases (8/12 cases) [21]. Seventy-five percent were predicted germline and 25% predicted somatic. Increases in sequencing depth will likely increase *TET2* variant identification among PAH cases.

*TET2* is expressed in lung (GTEx, on March 23rd, 2022) and decreased circulating TET2 associated with circulating proinflammatory cytokines was reported in IPAH cases compared to controls [21]. Spontaneous PH was demonstrated in hematopoietic-specific mouse models, and treatment of the mice with an IL-1beta inhibitor reversed the pro-inflammatory phenotype and PH [21].

Limited

Fibulin proteins are secreted as glycoproteins into the extracellular matrix and function in developmental processes, tissue remodeling, and maintenance of basement membrane and elastic fibers. In the PAH Biobank/NBR PAH combined analysis [19], seven unique *FBLN2* variants were identified in IPAH, including a recurrent variant carried by four cases, predicted to affect splicing. *FBLN2* is expressed in developing heart and smooth muscle precursor cells, amongst other tissues [117, 136, 137]. *Fbln2* knockout mice are viable, fertile, and have intact elastic fiber formation. They exhibit attenuated angiotensin II-induced, TGF-β mediated, cardiac hypertrophy and myocardial fibrosis [138, 139] but have not been tested for PH.

*KLK1* encodes a kininogenase contributing to the formation of the vasoactive peptide bradykinin. In the PAH Biobank [20], eight unique variants were identified (3 pLOF, 5 missense), with three of the variants recurrent in at least two cases. *KLK1* is expressed in several tissues including lung and vascular tissues [140-142]. Overexpression of *Klk1* resulted in hypotension in transgenic mice whereas *Klk1* knockout mice were normotensive but showed blunted flow dependent vasodilatation [143-145]. While *KLK1* has been implicated in pathogenic processes related to PAH development [146-148], clinical indications of PH have not been assessed.

## 2. Strength of evidence for genes implicated in syndromic forms of PAH

The three genes curated for syndromic forms of PAH have all been classified as having a definitive relationship with PAH (Table 2 and Figure 2).

*ACVRL1* encodes activin A receptor like type 1 involved in BMP signaling [149, 150]. Variants in *ACVRL1* were first identified as causal for HHT, an autosomal dominant vasculopathy characterized by abnormal blood vessel formation in multiple organs [151]. PAH is a rare complication of HHT, with most cases attributable to missense *ACVRL1* variants [152-154]. The majority are harbored in the conserved protein kinase domain, especially in a nonactivating,

nondown-regulating (NANDOR) box subdomain located in the terminal exon [155]. The NANDOR box is required for downstream SMAD signaling [155]; other rare HHT-PAH associated missense variants have been shown to cause subcellular mislocalization to the endoplasmic reticulum [153]. Small deletion and nonsense variants have been identified in some cases [152-154]. *ACVRL1* is predominantly expressed in PAECs and in PAH plexiform lesions [152]. BMP9 and BMP10 were identified as the cognate ligands for ACVRL1 in endothelial cells [51]. Homozygous *Acvrl1* knockout mice demonstrated embryonic lethality with severe vascular malformations [156]. Heterozygous *Acvrl1* mice developed adult-onset spontaneous PH with increased RVSP, RV hypertrophy, and vascular remodeling [157].

*ENG* encodes endoglin, an accessory protein that interacts with ACVRL1 to promote TGF-β/BMP signaling [158, 159]. Like *ACVRL1*, heterozygous pLOF variants in *ENG* are predominantly associated with HHT [160]. Trembath *et al*. reported novel *ENG* nonsense variants in 2/11 HHT-PAH patients [153]. Subsequently, Harrison *et al*. [161] reported an HHT-PAH associated splicing variant with demonstrated exon skipping. Other studies have identified at least ten additional *ENG* variants (4 nonsense, 5 missense, 1 in-frame deletion) [17, 20, 32, 162]. *ENG* variants were rarely reported in the absence of HHT. However, in one case the diagnosis of PAH was made at three months of age, preceding the onset of HHT by eight years [161, 163]. Endoglin is expressed in healthy and PAH lung endothelial cells [164] and plays an important role in angiogenesis [165, 166]. High expression observed in some PAH vascular lesions could be considered contradictory evidence; however, the analysis did not distinguish between L-endoglin and S-endoglin isoforms, which have opposing effects on TGF-β vs BMP signaling [167]. *Eng* heterozygous knockout mice spontaneously develop characteristic hemodynamic features of PAH, with increased reactive oxygen species levels [168].

*TBX4* encodes T-box transcription factor 4 [169], which plays a major role in lung branching morphogenesis and skeletal system development [170]. Heterozygous *TBX4* variants were first reported in families with small patella syndrome (SPS, OMIM #147891), an AD skeletal dysplasia [171]. *TBX4*-containing microdeletions were implicated in PAH [172], followed by identification of two intragenic *TBX4* frameshifts and one missense variant in SPS/pediatric-onset PAH cases [173]. Other studies have reported numerous protein-truncating and missense variants clustering in the T-box domain [17, 20, 25, 43, 174]. Luciferase reporter assays demonstrated variant-specific LOF and gain-of-function effects for the missense variants [175]. *TBX4* variants are more

prevalent in pediatric PAH than adult-onset cases [43], and are often associated with a syndrome involving PAH, other lung and cardiac anomalies, and SPS [176-178]. *TBX4* is strongly expressed in developing lung [179], but its role in PAH pathogenesis is currently unclear. *Tbx4* knockout mice showed reduced phospho-SMAD1/5 levels in fetal lung fibroblasts [180]. However, PH *per se* has not yet been demonstrated in the mouse model.

### 3. Strength of evidence for *EIF2AK4* implicated in PVOD/PCH

*EIF2AK4* encodes eukaryotic translation initiation factor 2 alpha kinase 4, a serine-threonine kinase inducer of nutrient-mediated changes in gene expression. Biallelic *EIF2AK4* variants were identified independently in 13 PVOD families [181] and one family and two sporadic PCH cases [90], suggesting autosomal recessive inheritance. Subsequent studies reported at least ten additional probands with biallelic variants and clinical diagnoses of PVOD/PCH or early-onset IPAH with poor survival[182, 183]. EIF2AK4 was detected in lung tissue from an unaffected control and a PVOD patient without *EIF2AK4* variants but was not detected in a PVOD patient with pathogenic variants in *EIF2AK4* [181]. Despite the paucity of experimental data, the genetic evidence is strong and remains uncontradicted, yielding a classification of definitive.

## DISCUSSION

Of twenty-four genes curated for isolated H/IPAH (or PVOD/PCH for *EIF2AK4*), nine were classified as definitive (*ATP13A3*, *BMPR2*, *CAV1*, *EIF2AK4*, *GDF2*, *KCNK3*, *KDR*, *SMAD9*, *SOX17*), three as moderate (*ABCC8*, *GGCX*, *TET2*), and six as limited (*AQP1*, *BMP10*, *FBLN2*, *KLF2*, *KLK1*, *PDGFD*). One gene was determined to have no known relationship (*TOPBP1*) and five were disputed (*BMPR1A*, *BMPR1B*, *NOTCH3*, *SMAD1*, *SMAD4*). Three genes curated for syndromic PAH (*ACVRL1*, *ENG*, *TBX4*) were classified as definitive. Four of the disputed genes are from the TGF-β/BMP pathway, originally implicated through candidate gene screens but not confirmed in larger, rigorous next generation sequencing studies. Moderate and limited genes may change classification with new evidence, and recurations can be tracked on the ClinGen website. These results offer guidance to clinicians and genetic testing laboratories.

The inclusion or classification of some genes in this report differ from the 6$^{th}$ World Symposium on Pulmonary Hypertension report [7] due to new genetic and experimental evidence.

For example, *KDR* was not included in the Symposium report but is now classified as definitive, and *AQP1* moved from "higher level of evidence" to "limited" in this report.

Identification of a genetic cause of PAH in individual cases can have implications for clinical management including treatment (mono- vs multimodal therapy), surgical intervention and transplantation decisions, and screening for associated conditions [184]. A genetic diagnosis can lead to early treatment of associated medical conditions, cascade genetic testing of family members to identify those at risk for developing PAH, and clarification of reproductive risks to inform family planning decisions.

Based on our analyses, we recommend a tiered genetic diagnostic testing approach. Tiered testing can simplify clinical interpretation of results and decrease the reporting of VUSs. Tier 1 should include definitive and strong genes. For cases without a genetic diagnosis from tier 1 testing, moderate (tier 2) and limited (tier 3) genes could be screened. Lack of genetic evidence over time for *TOPBP1* and the five disputed genes indicates that these genes should no longer be included in routine PAH genetic testing. Given the potential reclassification of limited evidence genes and new gene discovery over time, we encourage regular review of testing panels for gene inclusion and adjustment of tiered analyses for both testing panels and exome/genome sequence data as appropriate. Thus, routine reanalysis of case-level data for undiagnosed cases by genetic testing laboratories is highly recommended.

The use of exome (or genome) sequencing for molecular diagnosis has become more cost-effective. Benefits of exome/genome sequencing over panel testing include gene inclusion/exclusion flexibility, copy-number variant detection, and reanalysis of stored data from undiagnosed cases following evidence of new PAH gene-disease relationships.

## Conclusions

Twelve genes have definitive evidence for causal effects of variants on PAH using a standardized evidence-based classification system. Our continued efforts to recurate known genes and assess evidence for newly identified genes will provide continuity of expert review.

## Author contributions

Conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing original draft, review, editing: C.L.W., M.A.A., S.B., D.D., C.A.E., S.G., A.C.H., R.D.M., D.P., M.P., M.S., L.S., J.T-C., W.K.C. Project administration: C.L.W., W.K.C.

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**Disclosures**

None.

**Data Availability**

All gene curations reported herein are publicly available at https://search.clinicalgenome.org/kb/affiliate/10071 or https://search.clinicalgenome.org/kb/affiliate/10028 (*ACVRL1* and *ENG*).

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**Table 1. Strength of PAH-gene relationships for genes implicated in isolated H/IPAH.**

| Gene | Gene name | MOI[a] | Genetic evidence | Variant type score[b] | Experimental evidence | Evidence type scored[c] | Total score | >3 yrs? | Classification | Tissue/cell expression[d] | Molecular mechanism[e] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *ATP13A3* | ATPase 13A3 | AD/AR | 12 | pLOF | 1 | F/expression; FA/non-patient | 13 | Y 2018 | Definitive | PASMC, PAEC, BOEC | Unknown |
| *BMPR2* | Bone morphogenetic protein receptor 2 | AD | 12 | pLOF | 6 | F/expression, biochemical, interaction; FA/patient; M/non-human; R/non-human | 18 | Y 2000 | Definitive | PASMC, PAEC | Haploinsufficiency |
| *CAV1* | Caveolin 1 | AD | 6 | pLOF, missense | 6 | F/biochemical; FA/patient; M/non-human; R/non-human | 12 | Y 2012 | Definitive | Lung EC | Dominant negative |
| *GDF2* | Growth differentiation factor 2 | AD | 12 | pLOF, missense | 6 | F/expression, biochemical, interaction; FA/patient, non-patient; M/cell culture | 18 | Y 2016 | Definitive | HMVEC/PAEC | Haploinsufficiency |
| *KCNK3* | Potassium two pore domain channel subfamily K member 3 | AD | 7 | missense | 5 | F/expression; FA/patient; M/non-human | 12 | Y 2013 | Definitive | Lung, PA, PASMC | LOF |
| *KDR* | Kinase insert domain receptor | AD | 6.5 | pLOF | 6 | F/expression; M/non-human | 12.5 | Y 2018 | Definitive | PAEC | Haploinsufficiency |
| *SMAD9* | Smad family member 9 | AD | 9.6 | pLOF, missense | 4.5 | F/biochemical, interaction; FA/patient, non-patient; R/patient cells | 14.1 | Y 2009 | Definitive | PAEC, PASMC | LOF |
| *SOX17* | SRY-box transcription factor 17 | AD | 11.8 | pLOF, missense | 1.5 | F/expression; FA/non-patient | 13.3 | Y 2018 | Definitive | PAEC, PAH plexiform lesions | Haploinsufficiency |
| *ABCC8* | ATP binding cassette subfamily C member 8 | AD | 9.0 | pLOF, missense | 1.0 | F/expression | 10 | Y 2018 | Moderate | Lung, PA | LOF |
| *GGCX* | Gamma glutamyl carboxylase | AD | 8.8 | pLOF, missense | 0.5 | F/expression | 9.3 | Y 2019 | Moderate | Lung | Unknown |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *TET2* | Tet-methylcytosine-dioxygenase-2 | AD | 4.6 | pLOF, missense | 3.5 | F/expression, biochemical; M/non-human | 8.1 | N 2020 | Moderate | Lung | LOF |
| *AQP1* | Aquaporin 1 | AD | 3.3 | missense | 0.5 | F/expression | 3.8 | Y 2018 | Limited | PASMC, PAEC, BOEC | N/A |
| *BMP10* | Bone morphogenetic protein 10 | AD | 1.9 | pLOF, missense | 1.1 | F/expression, biochemical, interaction | 3.0 | Y 2019 | Limited | Plasma, right atrium | Haploinsufficiency |
| *FBLN2* | Fibulin 2 | AD | 2.0 | missense | 0.5 | F/expression | 2.5 | N 2021 | Limited | Heart, aorta coronaries; basement membrane | Unknown (GOF?) |
| *KLF2* | Krüppel-like factor 2 | AD | 0.5 | missense | 3.0 | F/expression, interaction; FA/patient | 3.5 | Y 2017 | Limited | Lung, vasculature | N/A |
| *KLK1* | Tissue Kallikrein | AD | 5.2 | pLOF, missense | 0.5 | F/expression | 5.7 | Y 2019 | Limited | Lung, vasculature | Unknown (haploinsufficiency and/or LOF?) |
| *PDGFD* | Platelet Derived Growth Factor D | AD | 2.1 | 2.0 case-CTL data + 0.1 missense | 0.5 | F/expression | 2.6 | N 2021 | Limited | Lung, vasculature, mesenchyme | Unknown (GOF?) |
| *TOPBP1* | DNA topoisomerase II binding protein 1 | N/A | 0 | none | 1.0 | F/expression; FA/non-patient | 1.0 | N/A | No known disease relationship | Lung, PAEC | N/A |
| *BMPR1A* | Bone morphogenetic protein receptor 1A | N/A | 0 | missense | 2 | F/expression, biochemical, interaction | 2.0 | Y 2018 | Disputed | PASMC | N/A |
| *BMPR1B* | Bone morphogenetic protein receptor 1B | N/A | 0 | missense | 2 | F/expression, biochemical, interaction | 2.0 | Y 2012 | Disputed | PASMC | N/A |
| *NOTCH3* | Notch receptor 3 | N/A | 0 | missense | 2.0 | F/expression, biochemical; FA/non-patient | 2.0 | Y 2014 | Disputed | Lung, PASMC | N/A |
| *SMAD1* | Smad family member 1 | N/A | 0 | missense | 3.0 | F/biochemical; M/non-human | 3.0 | Y 2011 | Disputed | PAEC, PASMC | N/A |
| *SMAD4* | Smad family member 4 | N/A | 0 | missense, other | 1.0 | F/biochemical | 1.0 | Y 2011 | Disputed | PAEC, PASMC | N/A |

[a]MOI, mode of inheritance; AD, autosomal dominant; AR, autosomal recessive; N/A, not applicable.
[b]pLOF, predicted loss of function, including nonsense, frameshift, and canonical splice variants.
[c]F, function (relevant expression, biochemical function, protein interaction); FA, functional alteration (in patient or non-patient cells); M, model (human or non-human, cell culture/human or non-human); R, rescue (human or non-human, cell culture/human or non-human).

[d]BOEC, blood outgrowth endothelial cell; HMVEC, human lung microvascular endothelial cell; PA, pulmonary artery; PAEC, pulmonary artery endothelial cell; PASMC, pulmonary artery smooth muscle cell.
[e]LOF, loss of function; GOF, gain of function; N/A, not applicable.

## Table 2. Strength of PAH-gene relationships for genes implicated in syndromes including PAH.

| Gene | Gene name | Syndrome[a] | Original ClinGen curation GCEP | Original classification | Genetic evidence for PAH | Variant type scored[b] | Experimental evidence for PAH | Evidence type scored[c] | Total score for PAH | >3 yrs | Classification for PAH |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *ACVRL1* | Activin receptor like 1 | HHT | Hemostasis/ thrombosis | Definitive | 12 | pLOF, missense | 6 | F/expression, biochemical, interaction; M/non-human | 16 | Y 2001 | Definitive |
| *ENG* | Endoglin | HHT | Hemostasis/ thrombosis | Definitive | 10.1 | pLOF, missense, other | 3.5 | F/expression, interaction; M/nonhuman | 13.6 | Y 2003 | Definitive |
| *TBX4* | T-box transcription factor 4 | *TBX4* syndrome | --- | --- | 12 | pLOF | 1 | F/expression; FA/patient, non-patient | 13 | Y 2013 | Definitive |

[a]HHT, hereditary hemorrhagic telangiectasia
[b]pLOF, predicted loss of function, including nonsense, frameshift, and canonical splice variants..
[c]F, function (relevant expression, biochemical function, protein interaction); FA, functional alteration (in patient or non-patient cells); M, model (human or non-human, cell culture/human or non-human); R, rescue. (human or non-human, cell culture/human or non-human)

## Table 3. Strength of PVOD/PCH-*EIF2AK4* relationship.

| Gene | Gene name | MOI[a] | Genetic evidence | Variant type scored[b] | Experimental evidence | Evidence type scored[c] | Total score | >3 yrs? | Classification | Tissue/cell expression[d] | Molecular mechanism[e] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *EIF2AK4* | Eukaryotic translation initiation factor 2 alpha kinase 4 | AR | 12 | pLOF, missense | 0.5 | F/expression | 12.5 | Y 2014 | Definitive | Lung, PASMCs | LOF |

PVOD/PCH, pulmonary veno-occlusive disease/pulmonary capillary hemangiomatosis.
[a]AR, autosomal recessive
[b]pLOF, predicted loss of function, including nonsense, frameshift, and canonical splice variants.
[c]F, function (relevant expression).
[d]PASMCs, pulmonary artery smooth muscle cells.
[e]LOF, loss of function.

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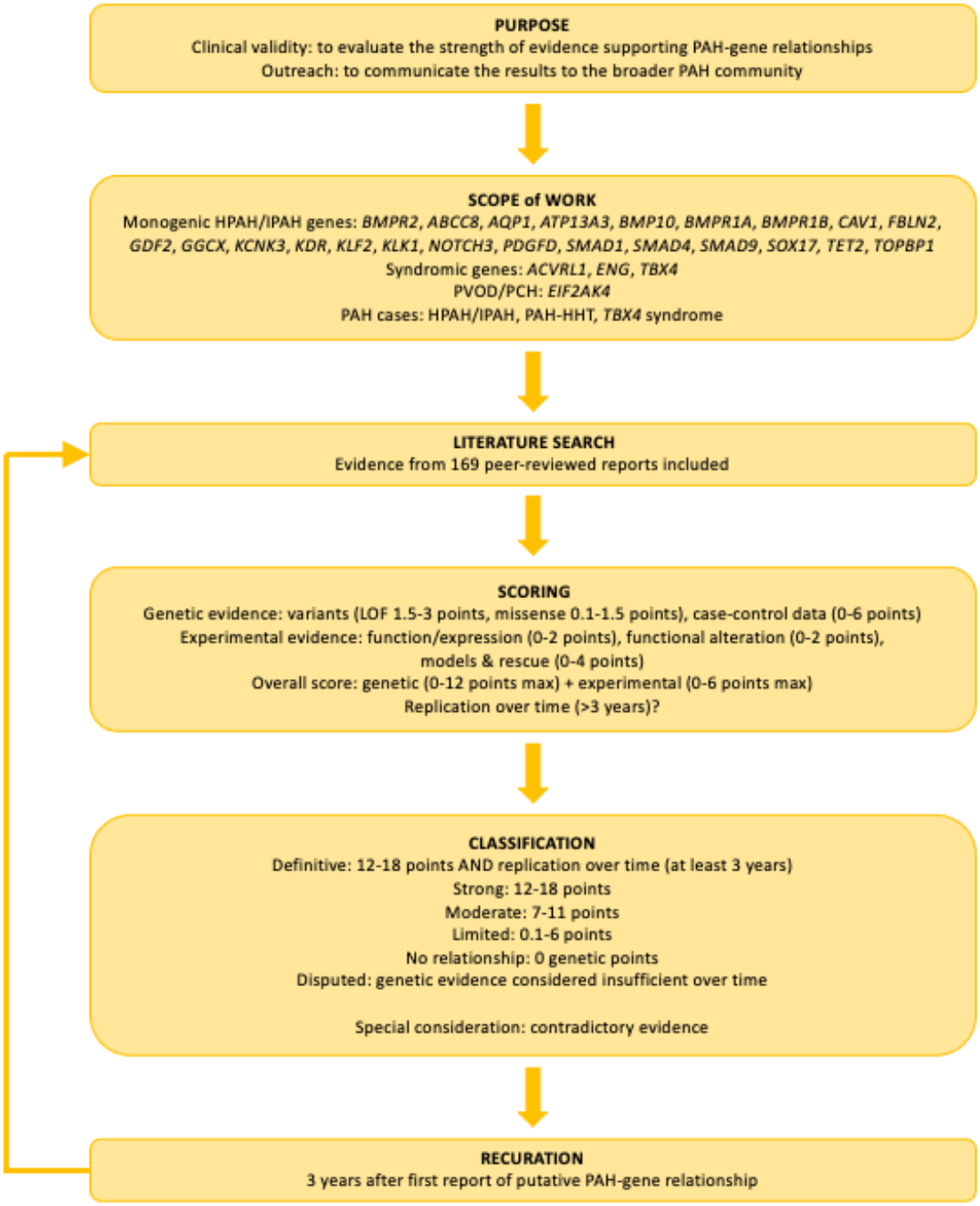
PURPOSE
Clinical validity: to evaluate the strength of evidence supporting PAH-gene relationships
Outreach: to communicate the results to the broader PAH community
SCOPE of WORK
Monogenic HPAH/IPAH genes: BMPR2, ABCC8, AQP1, ATP13A3, BMP10, BMPR1A, BMPR1B, CAV1, FBLN2, GDF2, GGCX, KCNK3, KDR, KLF2, KLK1, NOTCH3, PDGFD, SMAD1, SMAD4, SMAD9, SOX17, TET2, TOPBP1
Syndromic genes: ACVRL1, ENG, TBX4
PVOD/PCH: EIF2AK4
PAH cases: HPAH/IPAH, PAH-HHT, TBX4 syndrome
LITERATURE SEARCH
Evidence from 169 peer-reviewed reports included
SCORING
Genetic evidence: variants (LOF 1.5-3 points, missense 0.1-1.5 points), case-control data (0-6 points)
Experimental evidence: function/expression (0-2 points), functional alteration (0-2 points), models & rescue (0-4 points)
Overall score: genetic (0-12 points max) + experimental (0-6 points max)
Replication over time (>3 years)?
CLASSIFICATION
Definitive: 12-18 points AND replication over time (at least 3 years)
Strong: 12-18 points
Moderate: 7-11 points
Limited: 0.1-6 points
No relationship: 0 genetic points
Disputed: genetic evidence considered insufficient over time
Special consideration: contradictory evidence
RECURATION
3 years after first report of putative PAH-gene relationship

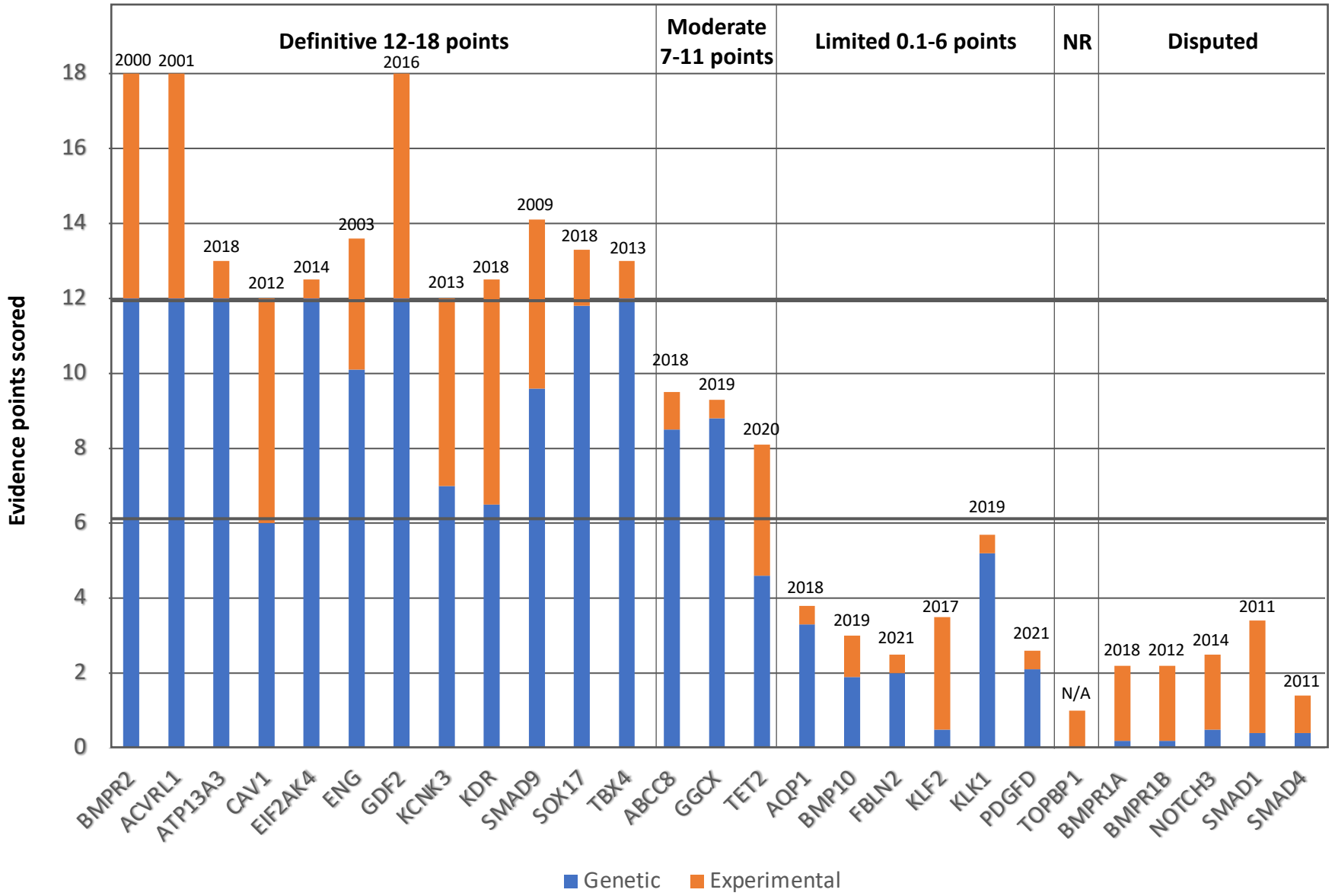


**Figure 2. Quantitative contributions of genetic and experimental evidence to the clinical validity classifications of genes curated for PAH.** The sums of genetic (blue) and experimental (orange) evidence scores are shown for genes classified as having definitive, moderate, or limited evidence of a monogenic relationship, no relationship (NR) or disputed relationship for H/IPAH, PVOD/PCH (*EIF2AK4*), or syndromic PAH (*ACVRL1*, *ENG*, *TBX4*). Dates above the bars indicate date of first report of a gene variant identified in a PAH case.

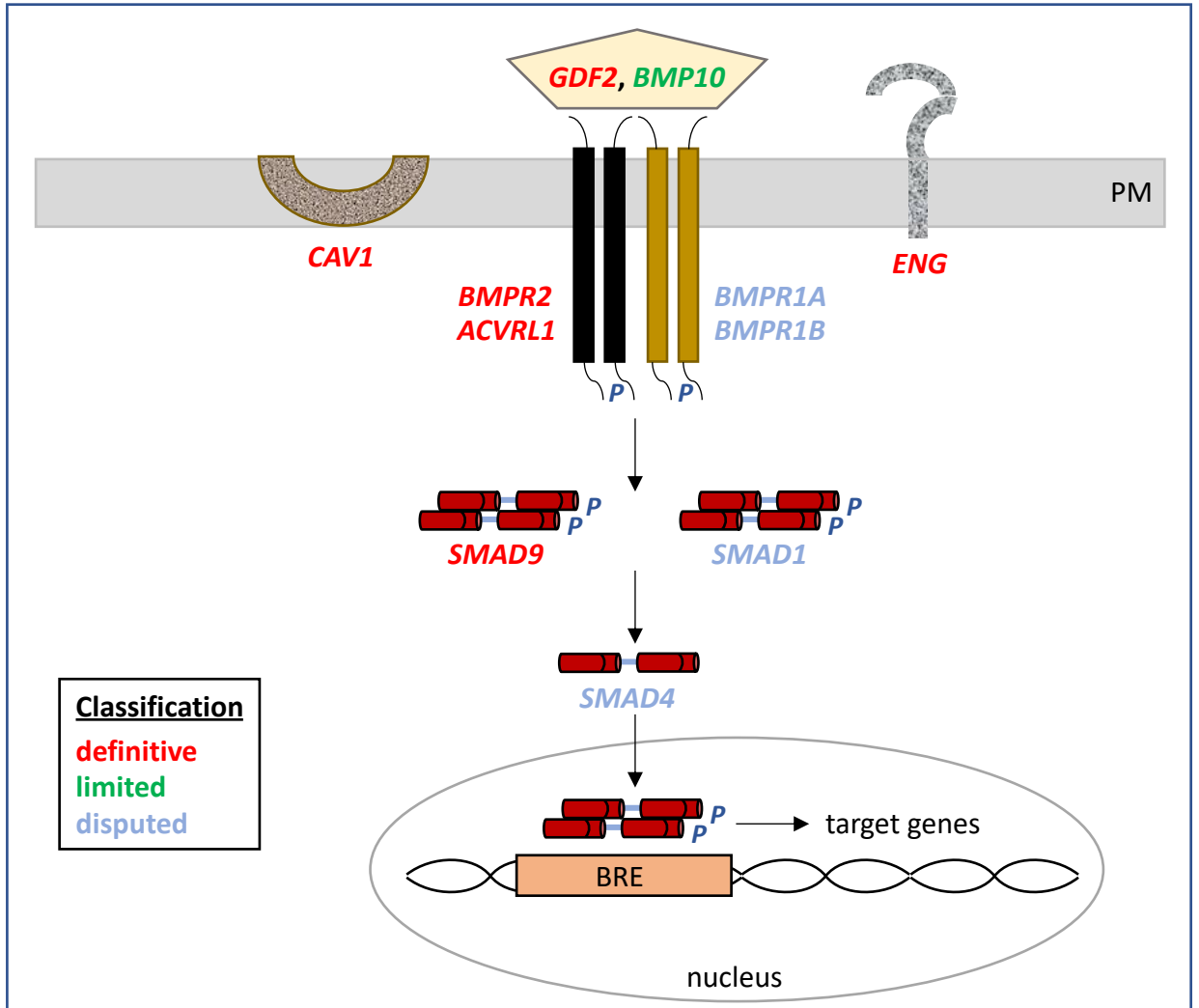


**Figure 3. Updated classifications of *BMPR2* pathway genes implicated in PAH.** The relative strength of evidence of curated genes is indicated by color-coded classifications. PM, plasma membrane; *P*, phosphate; BRE, BMPR2 response element.