Exome data clouds the pathogenicity of genetic variants in Pulmonary Arterial Hypertension
Abbasi, Yeganeh; Jabbari, Javad; Jabbari, Reza; Glinge, Charlotte; Izadyar, Seyed Bahador; Spiekerkoetter, Edda; Zamanian, Roham T.; Carlsen, Jørn; Tfelt-Hansen, Jacob

Published in:
Molecular Genetics and Genomic Medicine

DOI:
10.1002/mgg3.452

Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
Abbasi, Y., Jabbari, J., Jabbari, R., Glinge, C., Izadyar, SB., Spiekerkoetter, E., ... Tfelt-Hansen, J. (2018). Exome data clouds the pathogenicity of genetic variants in Pulmonary Arterial Hypertension. Molecular Genetics and Genomic Medicine, 6(5), 835-844. https://doi.org/10.1002/mgg3.452
Exome data clouds the pathogenicity of genetic variants in Pulmonary Arterial Hypertension

Yeganeh Abbasi1,2 | Javad Jabbari3 | Reza Jabbari1,2 | Charlotte Glinge1 | Seyed Bahador Izadyar1 | Edda Spiekerkoetter4 | Roham T. Zamanian4 | Jørn Carlsen1,2 | Jacob Tfelt-Hansen1,2,5

1Heart Centre, Department of Cardiology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark
2Department of Cardiology, Section for Pulmonary Hypertension and Right Heart Failure, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark
3LEO Pharma A/S, Ballerup, Denmark
4Division of Pulmonary and Critical Care, Stanford University School of Medicine, California
5Department of Forensic Medicine, Faculty of Medical Sciences, University of Copenhagen, Denmark

Correspondence
Yeganeh Abbasi, The Heart Centre, Copenhagen University Hospital, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen Ø, Denmark.
Email: yeganehabasi@gmail.com

Funding information
Novo Nordisk

Abstract

Background: We aimed to provide a set of previously reported PAH-associated missense and nonsense variants, and evaluate the pathogenicity of those variants.

Methods: The Human Gene Mutation Database, PubMed, and Google Scholar were searched for previously reported PAH-associated genes and variants. Thereafter, both exome sequencing project and exome aggregation consortium as background population searched for previously reported PAH-associated missense and nonsense variants. The pathogenicity of previously reported PAH-associated missense variants evaluated by using four in silico prediction tools.

Results: In total, 14 PAH-associated genes and 180 missense and nonsense variants were gathered. The BMPR2, the most frequent reported gene, encompasses 135 of 180 missense and nonsense variants. The exome sequencing project comprised 9, and the exome aggregation consortium counted 25 of 180 PAH-associated missense and nonsense variants. The TOPBP1 and ENG genes are unlikely to be the monogenic cause of PAH pathogenesis based on allele frequency in background population and prediction analysis.

Conclusion: This is the first evaluation of previously reported PAH-associated missense and nonsense variants. The BMPR2 identified as the major gene out of 14 PAH-associated genes. Based on findings, the ENG and TOPBP1 gene are not likely to be the monogenic cause of PAH.

KEYWORDS
ESP, exome sequencing project, HGMD, PAH-associated gene, pulmonary arterial hypertension

1 | INTRODUCTION

Pulmonary arterial hypertension (PAH) is a rare, progressive, and deadly disease (Hoeper et al., 2014; Machado, 2012; Simonneau et al., 2013). Despite advances in treatment of the PAH, the mortality rate is still high (Ling et al., 2012). The incidence of PAH estimated in a wide range from 2.4 to 25 cases per million per year (Gaine & Rubin, 1998; Humbert et al., 2006; Ling et al., 2012).

PAH is subclassified into four groups: idiopathic PAH, heritable PAH, drug, and toxin induced, and lastly associated with certain other diseases (e.g. connective tissue...
disease, HIV infection, portal hypertension, congenital heart diseases, and schistosomiasis) (Simonneau et al., 2013).

The pathogenesis of PAH is complex, and, albeit the extensive effort, is still not completely understood (Badesch et al., 2009; Hoeper et al., 2013; Simonneau et al., 2013). Genetic studies have identified association of several genetic loci to PAH (Austin & Loyd, 2014; Rabinovitch, et al., 2009; Hoeper et al., 2013; Simonneau et al., 2013). The bone morphogenetic protein receptor type II (BMPR2) plays a major role in the etiology of idiopathic and heritable PAH (Ma & Chung, 2014; Simonneau et al., 2013). Other reported PAH-associated genes includes activin A receptor like type 1 (ACVRL1), endoglin (ENG), SMAD family 1, 4, 9, caveolin 1 (CAV1), bone morphogenetic protein receptor type 1b (BMPR1B), potassium voltage-gated channel subfamily a member 5 (KCNAS), potassium channel subfamily K member 3 (KCNK3), T-box transcription factor 4 (TBX4), DNA topoisomerase 2-binding protein 1 (TOPBP1), growth differentiation factor 2 (GDF2) and eukaryotic translation initiation factor 2-alpha kinase 4 (EIF2AK4) at a lower frequency (Machado, 2012; Soubrier et al., 2013). The genes that were classified as PAH-associated genes in the guideline were BMPR2, ALK-1, ENG, SMAD9, CAV1, and KCNK3 (Simonneau et al., 2013). Genetic testing has become an important tool in the clinical evaluation of PAH patients, especially for patients with a positive family history of PAH. Therefore, it is important to determine whether PAH associated genes and variants are truly disease causing.

We aimed to provide an encyclopedia by gathering all previously published PAH-associated genes and variants, and further evaluate the pathogenicity of each variant by performing comprehensive in silico prediction analysis, together with investigating the frequency of each PAH-associated variant in two large online exome databases.

2 | MATERIALS AND METHODS

The Human Gene Mutation Database (HGMD), PubMed, and Google Scholar were searched for previously published PAH-associated genes and variants until October 2016. The following queries were used: (‘pulmonary arterial hypertension’ (MeSH)) or (pulmonary arterial hypertension)), (‘genetics’ (MeSH)) or (genetic), (‘mutation’ (MeSH)) or (mutation)) and (‘variants’ (MeSH)) or (variants)). We revisited all identified genetic variants searching for published data on functional and familial cosegregation studies. In order to have a solid baseline, familial cosegregation was defined as at least two genotype positive family members having the same phenotype. The Hugo Genome Organisation and Gene Nomenclature Committee was used for standard nomenclature of human genes (HGNC database of human gene names | HUGO Gene Nomenclature Committee). The publicly available Ensembl genome database was used to find the location of variants in the genome and determine the amino acid changes in the protein coding regions of genes (Ensembl Genome Browser 85).

2.1 | Exome sequencing project

In the Exome Sequencing Project (ESP), next-generation sequencing of all protein coding regions in 6,503 individuals of African American (n = 2,203) and European American (n = 4,300) from different population studies have been carried out (Exome Variant Server). Clinical data were not available. The ESP was searched for previously published PAH-associated variants. The ESP lacks the data regarding variants positioned in promotors, introns, and untranslated regions, therefore variants found in these regions were not included in present study.

All previously identified PAH-associated variants in our investigation were subdivided into two groups; those that were identified in the ESP (ESP-positive) and those that were not identified in the ESP (ESP-negative).

2.2 | The exome aggregation consortium

In the exome aggregation consortium (ExAC) comprehend exome sequencing data from 60,706 unrelated individuals (ExAC Browser). African/African American (n = 5,203), Latino (n = 5,789), East Asian (n = 4,327), Finnish (n = 3,307), Non-Finnish European (n = 33,370), South Asian (n = 8,256), and other (n = 454) nationalities are presented in the ExAC database (ExAC Browser). Like the ESP, this browser only encompasses human genome data that encodes proteins as part of several various exome-studies on populations with specific diseases (ExAC Browser). The PAH-associated variants were subdivided into those that were identified in the ExAC (ExAC-positive) and those that were not identified in the ExAC (ExAC-negative).

The ESP and ExAC databases are considered as back ground population in this study.

2.3 | In silico prediction analysis

The functional effects of all missense variants were assessed by using the four prediction tools including conservation across species, Grantham Score, PolyPhen-2 (Polymorphism Phenotyping v2), and SIFT (Sorting Intolerant from Tolerant, v5.1.1). Data for conservation across species were obtained from Ensembl, and classified as occurring at a position with no substitutions (conserved/pathogenic) or ≥1 substitutions (not conserved/benign). Grantham physicochemical values were calculated using
the Grantham amino acid difference matrix. We defined a value above 100 as radical (pathogenic), and value under 100 as conservative (benign). Using PolyPhen-2, each variant were labeled “probably damaging”, “possibly damaging”, or “benign”. Variants labeled “probably damaging” and “possibly damaging” considered “damaging” (pathogenic) in our analysis. Finally, SIFT prediction classified variants as “tolerant” (benign) or “damaging” (pathologic). In a final analysis using all prediction tools, a variant was considered pathogenic if ≥3 in silico prediction tools determined the variant to be pathogenic, as previously described (Giudicessi et al., 2012). Variants that predicted pathogenic by only 1 or 2 tools was considered to be variants of uncertain significance (VUS).

3 | RESULTS

To date, 14 genes and 180 missense/nonsense variants have been identified as PAH-associated genes and variants (Table 1).

The BMPR2 was the most frequent reported gene and encompasses 135 (missense=86 and nonsense=49) of the 180 identified missense/nonsense PAH-associated variants (Figure). The other previously identified PAH-associated genes: ACVRL1, BMPR1B, CAV1, KCNA5, KCNK3, SMAD1, SMAD4, SMAD9, TBX4, TOPBP1, ENG, GDF2, and EIF2AK4 included 45 (missense=40 and nonsense=5) of the 180 missense/nonsense PAH-associated variants (Table 1).

We performed a prediction analysis of only the missense PAH-associated variants (n = 126), because the nonsense variants are classified to be damaging by nature of the uncompleted translation. By doing so, 76 of 126 missense PAH-associated variants were predicted as pathogenic (Table 2). Accordingly, 52 of 86 PAH-associated nonsense variants in BMPR2 gene were predicted pathogenic. Prediction analyses for all PAH-associated genes are available in the Table 2.

By investigating the frequency of the PAH-associated variants in the two large population databases, we found that the ESP comprised 9 of 180 variants (Table 3), while the ExAC in counted 25 PAH-associated missense/nonsense variants (Tables 3 and 4).

In the most frequent reported PAH-associated gene, BMPR2, we found 2 ESP-positive and 12 ExAC-positive variants of 135 variants (Tables 3 and 4).

In the literature, functional studies had been performed in 29 of 180 PAH-associated variants (Table 5), assessing the functional properties of the resulted protein using in vivo and/or in vitro studies. All functional studies showed that mutated proteins, except 2 variants (p.Ser160-Asn (rs149589961) and p.Phe392Leu) in BMPR1B, displayed a loss of function phenotype (Table 5).

4 | DISCUSSION

In this novel study, we provide the clinicians the first comprehensive evaluation tool for genetic diagnostic of PAH, by evaluating the allele frequency of previously reported PAH-associated variants in the two large background

| TABLE 1 Overview of PAH-associated genes and variants |
|----------------|----------------|----------------|
| Gene           | Missense variants | Nonsense variants |
| BMPR2          | 86               | 49             |
| ACVRL1         | 16               | 1              |
| BMPR1B         | 2                | –              |
| CAV1           | 1                | –              |
| KCNA5          | 4                | –              |
| KCNK3          | 6                | –              |
| SMAD1          | 1                | –              |
| SMAD4          | 1                | –              |
| SMAD9          | 1                | 2              |
| TBX4           | 3                | –              |
| TOPBP1         | 3                | –              |
| ENG            | 2                | –              |
| GDF2           | 1                | –              |
| EIF2AK4        | 1                | –              |
| 14 genes       | 126 missense     | 54 nonsense    |

| TABLE 2 Prediction analysis of missense variants with ≥ 3 in silico prediction tools agreement |
|-----------------------------------------------|----------------|----------------|
| Gene             | Damaging | VUS | Benign |
|------------------|----------|-----|--------|
| BMPR2            | 52       | 24  | 10     |
| ACVRL1 (ALK1)    |          | 2   | 1      |
| BMPR1B (ALK6)    | 0        | 2   | 0      |
| CAV1             | 0        | 0   | 1      |
| KCNA5            | 2        | 2   | 0      |
| KCNK3            | 5        | 1   | 0      |
| SMAD1            | 0        | 1   | 0      |
| SMAD4            | 0        | 1   | 0      |
| SMAD9            | 1        | 0   | 0      |
| TBX4             | 2        | 0   | 1      |
| TOPBP1           | 1        | 1   | 0      |
| ENG              | 0        | 2   | 0      |
| 14 genes         | 76       | 36  | 14     |
### TABLE 3 Exome Sequencing Project-positive PAH-associated variants

| Gene | Variant | dbSNP ID | All allele (EA + AA) | MAF (%) (EA/AA/All) | All genotype (EA + AA) | Grantham score | PolyPhen | SIFT | Conservation | Agreement of ≥3 tools | References |
|------|---------|----------|----------------------|---------------------|-----------------------|-------------------|-----------|------|--------------|-----------------------|------------|
| **BMPR2** | p.R266T  | rs374694591 | C = 1/G = 13005 (0.00007) | 0.0116/0.0/0.0077 | CC = 0/C/G = 1/G/G = 6502 (0.01%) | 71 | Damaging | Nottolerated | Conserved | D | Machado et al. (2006) |
| | p.N903S  | rs373725296 | G = 1/A = 13005 (0.00007) | 0.0116/0.0/0.0077 | GG = 0/G/G = 1/A/A = 6502 | 46 | Damaging | Tolerated | Conserved | VUS | Thomas et al. (2009) |
| **CAV1** | p.V1551  | rs150368249 | A = 6/G = 13000 (0.00046) | 0.0349/0.0681/0.0461 | AA = 0/A/G = 6/G/G = 6497 | 29 | Benign | Tolerated | Notconserved | B | Austin et al. (2012) |
| **KCN5** | p.E211D  | rs35853292 | C = 15/G = 12991 (0.00115) | 0.1512/0.0454/0.1153 | CC = 0/C/G = 15/G/G = 6488 | 45 | Benign | Tolerated | Conserved | VUS | Remillard et al. (2007) |
| **TOPBP1** | p.S817L  | rs17301766 | A = 1921/G = 10445 (0.18391) | 19.4923/7.2995/15.5345 | AA = 182/AG = 1557/G/G = 4444 | 145 | Benign | Tolerated | Notconserved | VUS | de Jesus Perez et al. (2014) |
| | p.N1042S | rs10935070 | C = 2869/T = 8855 (0.32399) | 32.7297/5.8791/24.4712 | CC = 438/CT = 1993/TT = 3431 | 46 | Benign | Tolerated | Notconserved | B | |
| | p.R309C  | rs55633281 | A = 779/G = 10999 (0.07082) | 7.629/4.343/6.614 | AA = 22/AG = 735/G/G = 5132 | 180 | Damaging | Tolerated | Conserved | D | |
| **ENG** | p.G214S  | rs150932144 | T = 3/c = 12821 (0.00023) | 0.0118/0.046/0.0234 | TT = 0/TC = 3/CC = 6409 | 56 | Damaging | Tolerated | Notconserved | VUS | Pfarr et al. (2013) |
| | p.G545S  | rs142896669 | T = 8/c = 12998 (0.00061) | 0.0814/0.0227/0.0615 | TT = 0/TC = 8/CC = 6495 | 56 | Damaging | Tolerated | Conserved | VUS | |

**Note.** B: benign; D: damaging; VUS: variants with uncertain significance.
population databases (ESP and ExAC), and also by adding in silico prediction analysis using an established conservative method (Abbasi et al., 2016; Jabbari et al., 2013; Risgaard et al., 2013). Surprisingly, in the literature we identified very limited data on familial cosegregation, thus, unfortunately, the familial cosegregation in our evaluation was very limited. This, however, goes hand in hand and support our findings that the identified ESP- and ExAC-positive variants may not be the monogenic cause of the PAH. The pathogenic PAH-associated variants in BMPR2 gene have reduced penetrance and gender dependant (Austin, Loyd, & Phillips, 1993). Therefore, ESP and ExAC databases most likely include unaffected heterozygote parents. The penetrance information for pathogenic PAH-associated variants in ACVR1L, KCNK3, CAV1, SMAD9, and BMPR1B genes is unknown (Austin et al., 1993).

Our investigation supports that the BMPR2 gene is of major importance in the development of the heritable and idiopathic PAH (Simonneau et al., 2013; Soubrier et al., 2013). According to our findings, BMPR2 included 75% (135 of 180) of the previously reported missense/nonsense PAH-associated variants. Familial cosegregation was only identified for three variants (p.W13*, p.E386V and p.K512T) in BMPR2 gene (Fu et al., 2008; Hamid et al., 2010; Machado et al., 2006). Prediction analysis of BMPR2 missense variants (n = 86), using agreement of ≥ 3 of 4 in

### TABLE 4: Exome aggregation consortium-positive variant

| Gene    | Variant | rs ID     | Allele frequency | Grantham score | PolyPhen | SIFT      | Conservation | Agreement ≥3 tools | References                      |
|---------|---------|-----------|------------------|----------------|----------|-----------|--------------|-------------------|---------------------------------|
| BMPR2   | p.Q92H  | rs140683387| 0.000107         | 24             | Benign   | Tolerated | Not conserved | B                 | Kabata et al. (2013)            |
|         | p.W508* | X         | 0.000008         | –              | –        | –         | –            | –                 | Pfarr et al. (2010)             |
|         | p.R591* | X         | 0.000016         | 58             | Benign   | Tolerated | Not conserved | B                 | Sztrymf et al. (2008)           |
|         | p.T766A | X         | 0.000008         | 43             | Damaging | Tolerated | Conserved    | VUS               | Sztrymf et al. (2008)           |
|         | p.R873Q | X         | 0.0000115        | 43             | Damaging | Not        | Conserved    | VUS               |                                 |
|         | p.R266T | rs374694591| 0.000041         | 71             | Damaging | Not        | Conserved    | D                 | Machado et al. (2006)           |
|         | p.R303H | rs200948870| 0.000033         | 29             | Damaging | Tolerated | Conserved    | VUS               | Machado et al. (2006)           |
|         | p.V563M | X         | 0.000008         | 21             | Damaging | Tolerated | Conserved    | VUS               | Machado et al. (2006)           |
|         | p.R899P | rs137852752| 0.000008         | 103            | Damaging | Tolerated | Not conserved | VUS               | Vattulainen et al. (2015)       |
|         | p.A24E  | X         | 0.000008         | 107            | Benign   | Tolerated | Not conserved | VUS               | Machado et al. (2009)           |
|         | p.N903S | rs373725296| 0.000016         | 46             | Damaging | Tolerated | Conserved    | VUS               | Thomas et al. (2009)            |
|         | p.E427D | X         | 0.000008         | 45             | Benign   | Tolerated | Conserved    | VUS               | van der Bruggen et al. (2016)   |
| CAV1    | p.V155I | rs150368249| 0.000585         | 29             | Benign   | Tolerated | Not conserved | B                 | Austin et al., 2012;            |
| KCNA5   | p.E211D | rs35853292 | 0.000833         | 45             | Benign   | Tolerated | Conserved    | VUS               | Remillard et al. (2007)         |
|         | p.G182R | X         | 0.000141         | 125            | Damaging | Not        | Conserved    | D                 |                                 |
| SMAD4   | p.N13S  | rs281875323| 0.000024         | 46             | Damaging | Tolerated | Not conserved | VUS               | Nasim et al. (2011)             |
| SMAD9   | p.K43E  | X         | 0.000107         | 56             | Damaging | Not        | Not tolerated | D                 | Nasim et al. (2011)             |
|         | p.R294* | X         | 0.000008         | –              | –        | –         | –            | –                 | Drake et al. (2011)             |
| TBX4    | p.A35V  | rs148424252| 0.007833         | 64             | Benign   | Tolerated | Not conserved | B                 | Kerstjens-Frederikse et al., 2013; (p4) |
|         | p.Y382S | X         | 0.000041         | 144            | Benign   | Tolerated | Not conserved | D                 |                                 |
| TOPBP1  | p.S817L | rs17301766 | 0.1411           | 145            | Benign   | Tolerated | Not conserved | VUS               | de Jesus Perez et al. (2014)    |
|         | p.R309C | rs55633281 | 0.0533           | 180            | Damaging | Tolerated | Conserved    | D                 |                                 |
|         | p.N1042S| rs10935070 | 0.2898           | 46             | Benign   | Tolerated | Not conserved | B                 |                                 |
| ENG     | p.G214S | rs150932144| 0.000155         | 56             | Damaging | Tolerated | Not conserved | VUS               | Pfarr et al. (2013)             |
|         | p.G545S | rs142896669| 0.000520         | 56             | Damaging | Tolerated | Conserved    | VUS               |                                 |

Note: B: benign; D: damaging; VUS: variants with uncertain significance.
silico prediction tools indicated that only 60.4% variants ($n = 52$) were predicted pathogenic.

The annual incidence of PAH is estimated from 2.4 to 25 cases per million per year in the general population (Gaine & Rubin, 1998; Humbert et al., 2006; Ling et al., 2012). In total, 12 variants in $\textit{BMPR2}$ were identified in the ESP and ExAC databases (Tables 3 and 4). This means ~9% of previously identified PAH-associated variants in $\textit{BMPR2}$ were found in the background population. According to the incidence of PAH in background population, this is an expected frequency of PAH-associated variants in $\textit{BMPR2}$ in the background population. The 12 identified functional studies on variants in $\textit{BMPR2}$ gene revealed that all mutated proteins had a loss of function phenotype (Table 5). Taken together, these findings point to a pivotal role of $\textit{BMPR2}$ in pathogenesis of PAH.

In contrast, we found all the three PAH-associated missense variants (p.S817L [rs17301766], p.N1042S [rs10935070] and p.R309C [rs55633281]) in $\textit{TOPBP1}$ gene in the ESP and ExAC databases (de Jesus Perez et al., 2014). The allele frequency of p.S817L (in ESP = 0.1839 and in ExAC = 0.1411), p.N1042S (in ESP = 0.3239 and in ExAC = 0.2898), and p.R309C (in ESP = 0.0708 and in ExAC = 0.0533) in ESP and ExAC is very high.

### Table 5: Functional studies

| Gene       | Amino acid substitution | Type of cell/Animal                                      | Result                        | References                        |
|------------|-------------------------|-----------------------------------------------------------|-------------------------------|-----------------------------------|
| $\textit{ACVRL1 (ALK1)}$ | R484W                   | NIH-3T3 fibroblasts and COS-7 cells                      | Loss of function              | Ricard et al. (2010)             |
|            | R484Q                   | NIH-3T3 fibroblasts and COS-7 cells                      | Loss of function              |                                   |
|            | L381P                   | NIH-3T3 fibroblasts and COS-7 cells                      | Loss of Function              |                                   |
| $\textit{BMPR1B (ALK6)}$ | S160N                   | COS1 cells (in vitro)                                    | Gain of function              | Chida et al. (2012)              |
|            | F392L                   | COS1 Cells (in vitro)                                    | Gain of function              |                                   |
| $\textit{BMPR2}$ | W16*                   | PASMCs and microvascular endothelial cells               | Loss of Function              | Dewachter et al. (2009)          |
|            | R491W                   | PASMCs and microvascular endothelial cells               | Loss of Function              |                                   |
|            | Q495*                   | PASMCs and microvascular endothelial cells               | Loss of Function              |                                   |
|            | S301P                   | PASMCs and microvascular endothelial cells               | Loss of Function              |                                   |
|            | E195*                   | PASMCs and microvascular endothelial cells               | Loss of Function              |                                   |
|            | S107*                   | PASMCs and microvascular endothelial cells               | Loss of Function              |                                   |
|            | R321*                   | Blood outgrowth endothelial cells (BOECs)                | Loss of Function              | Drake et al. (2011), Dunmore et al. (2013) |
|            |                         | Pulmonary artery endothelial (PAEC) & Pulmonary artery smooth muscle cells (PASMC) |                   |                                   |
|            |                         | W9* PASMCs                                               | Loss of Function              | Thomas et al. (2009)             |
|            |                         | C347R PASMCs                                             | Loss of Function              |                                   |
|            |                         | C347Y PASMCs                                             | Loss of Function              |                                   |
|            |                         | N903S PASMCs                                             | Loss of Function              |                                   |
|            |                         | R899* A mouse model (In vivo)                            | Loss of Function              | Long et al. (2015)               |
| $\textit{KCNA5}$ | E211D                   | COS-1(mammalian) cells & HEK-293*(human) cells          | Loss of Function              | Burg et al. (2010)               |
|            | G182R                   | COS-1(mammalian) cells & HEK-293*(human) cells          | Loss of Function              |                                   |
| $\textit{KCNK3}$ | T8K                     | COS-7cells                                               | Loss of Function              | Ma et al. (2013)                 |
|            | G97R                    | COS-7cells                                               | Loss of Function              |                                   |
|            | E182K                   | COS-7cells                                               | Loss of Function              |                                   |
|            | Y192C                   | COS-7cells                                               | Loss of Function              |                                   |
|            | G203D                   | COS-7cells                                               | Loss of Function              |                                   |
|            | V221L                   | COS-7cells                                               | Loss of Function              |                                   |
| $\textit{SMAD1}$ | V3A                     | PASMCs                                                   | Loss of Function              | Nasim et al. (2011)              |
| $\textit{SMAD4}$ | N13S                    | PASMCs                                                   | Loss of Function              | Nasim et al. (2011)              |
| $\textit{SMAD9}$ | K43E                    | PASMCs                                                   | Loss of Function              | Nasim et al. (2011), Suo et al. (2013) |
|            | C202*                   | COS1 Cells                                               | Loss of Function              | Shintani et al. (2009)           |
(Tables 3 and 4; de Jesus Perez et al., 2014). Our prediction analysis showed that only p.R309C was predicted pathogenic. Since the PAH-associated variants in the **TOPBP1** have high allele frequency in the background population (n = 3 variants) is not likely to be the monogenic cause of PAH. No functional studies have been reported on these variants and these are indeed needed to clarify the effect of variants in **TOPBP1** as a modifier gene in the pathogenesis of PAH. Furthermore, no familial cosegregation are reported in order to support **TOPBP1** monogenic cause of PAH (de Jesus Perez et al., 2014).

In 2013, the 5th World Symposium on Pulmonary Hypertension established the ENG gene to be a PAH-associated gene, since two missense PAH-associated variants (p.G214S [rs150932144] and p.G545S [rs142896669]) were reported (Simonneau et al., 2013). In our analysis, these two variants were predicted VUS (Table 2), questioning the pathogenicity of these variants in the PAH-etioloxy. Furthermore, both the p.G214S and p.G545S were present in the ESP and ExAC databases (Tables 3 and 4). The allele frequency of p.G214S in the ESP was 0.0002 and 0.0001 in the ExAC database. The p.G545S variant found in the ESP with allele frequency 0.0006 and 0.0005 in the ExAC browser (Tables 3 and 4). Although ENG gene known as a PAH-associated gene in the development of PAH, our data and analysis do not support that ENG variants are likely to be a monogenic or one of the major causes in the pathogenesis of PAH. It is important to perform a comprehensive functional study to determine the exact effect of the reported amino acid changes in the ENG and the effect of p.G214S and p.G545S in expression level of the protein.

In the literature we found five PAH-associated variants in **SMAD** genes: **SMAD1** (p.V3A), **SMAD4** (p.N13S) and **SMAD9** (p.K43E, p.C202* and p.R294*). None of the five were found in the ESP, but we found three variants (p.N13S, p.K43E and p.R294*) in the ExAC (Table 4). Using pulmonary artery smooth muscle cells (PASMCs), Nasim M.T. et al. demonstrated that the p.V3A in **SMAD1** gene, p.N13S in **SMAD4** gene, and p.K43E in **SMAD9** gene resulted in reduced signaling activity in vitro of amino acid substitutions (Nasim et al., 2011). Another functional study analyzed the function of p.C202* in **SMAD9** (aliases: **SMAD8**) by using COS1 cells (a fibroblast-like cell). This study revealed that the mutated protein was not able to have interaction with **SMAD4** gene (Tables 2 and 5; Shintani, Yagi, Nakayama, Saji, & Matsuoka, 2009). Although the p.V3A and p.N13S were predicted as VUS, the results of functional studies (loss of function) support the effect of these variants in the pathogenesis of PAH.

In the **ACVRL1** gene 16 missenses and one nonsense variants were reported (Table 1). Thirteen variants (81.25%) were predicted as pathogenic (Table 2). None of these variants were identified in ESP or ExAC databases.

One in vitro functional study used NIH 3T3 fibroblasts and COS-7 cells analyzing the protein expression of three PAH-associated variants (p.L381P, p.R484Q and p.R484W) (Ricard et al., 2010). The study reported that p.R484Q and p.R484W were inactive in the transactivation step (Ricard et al., 2010). The mutated protein of p.L381P did not respond to the bone morphogenetic protein 9 (BMP9) stimulation (loss of function) (Ricard et al., 2010). These findings support the hypothesis of the role of mutated proteins in **ACVRL1** in pathogenesis of PAH, despite the lack of data of familial cosegregation.

The two PAH-associated variants in **BMPR1B** gene were investigated in a functional study by using COS1 cells (Chida et al., 2012). They showed that amino acid changes in p.F392L and p.S160N increased the activation of proteins above wild-type (gain of function) (Chida et al., 2012). The p.S160N and p.F392L identified are unlikely to be an important cause of development of PAH based on results of the functional study. Furthermore, the p.S160N and p.F392L were predicted VUS, which supports the result of the functional study (Table 2; Chida et al., 2012).

To describe the function of all six variants in **KCNK3**, Lijiang Ma et al. performed a functional analysis by using COS-7 cells (Ma et al., 2013). The mutated proteins showed the loss of ion-channel function (Ma et al., 2013). Supporting these results, our *in silico* prediction analysis predicted that all PAH-associated variants in **KCNK3** except p.V221L were pathogenic (Table 2).

Burg ED et al. analyzed the mutated protein of p.E211D and p.G182R in **KCNA5** gene (Burg, Platoshyn, Tsigelny, Lozano-Ruiz, & Rana, 2010). In an in vitro study, they compared the function of mutated proteins with wild type using human embryonic kidney cells (HEK-293) and COS-1 (Burg et al., 2010). They found that mutated proteins accelerated the inactivity of the voltage-gated K+ (K(V)) channels, which have an important role in regulating PASMCs (Burg et al., 2010). These findings support the role of p.E211D and p.G182R in **KCNA5** gene as uncommon cause of the etiology of PAH, although these two variants predicted as VUS (Tables 2, 3 and 4).

Song et al. (2016) identified the p.Y311* as a heterozygote mutation in **EIF2AK4** gene in an heritable or idiopathic PAH patient. The p.Y311*/EIF2AK4* was not present in the ESP and ExAC. A functional characterization of p.Y311* by a protein-expression study and cosegregation analysis in a pedigree will support the role of p.Y311*/EIF2AK4* in pathogenesis of PAH.

### Conclusion

To our knowledge, this is the first evaluation of previously reported rare PAH-associated genes and variants. In the
literature, we found 14 genes and 180 missense/nonsense variants. **BMPR2** were identified to be the most important and common reported cause of PAH.

By using prediction analysis and the allele frequency of PAH-associated variants in **TOPBP1** and **ENG** genes in the background population, suggests that these variants are unlikely to be the monogenic cause of the PAH pathogenesis. Further functional studies are required to clarify the function of mutated proteins.

**ACKNOWLEDGMENTS**

The authors thank the NHLBIGO Exome Sequencing Project and its ongoing studies that produced and provided exome variant calls for comparison.

We thank the Exome Aggregation Consortium (ExAC) to provide the largest collection of variation in human protein-coding regions in a publicly accessible database.

We to thank the Human Gene Mutation Database for collecting data over the years.

This study was supported by research grants from the Novo Nordisk to Pr. Tfelt-Hansen and the Research Foundation of the Heart Centre at Rigshospitalet. We acknowledge the support from the different institutions.

**CONFLICT OF INTEREST**

Javad Jabbari is employed at LEO Pharma A/S. There are no financial interests to report. This study was supported by research grants from the Research Foundation at the Heart Center, Rigshospitalet, Copenhagen and the Novo Nordisk Foundation to Pr. Tfelt-Hansen.

**ORCID**

Yeganeh Abbasi [http://orcid.org/0000-0002-9157-2594](http://orcid.org/0000-0002-9157-2594)

**REFERENCES**

Abbasi, Y., Jabbari, J., Jabbari, R., Yang, R. Q., Risgaard, B., Køber, L., ... Tfelt-Hansen, J. (2016). The pathogenicity of genetic variants previously associated with left ventricular non-compaction. *Molecular Genetics and Genomic Medicine, 4*(2), 135–142. https://doi.org/10.1002/mgg3.182

Austin, E. D., & Loyd, J. E. (2014). The genetics of pulmonary arterial hypertension. *Circulation Research, 115*(1), 189–202. https://doi.org/10.1161/CIRCRESAHA.115.303404

Austin, E. D., Loyd, J. E., & Phillips, J. A. (1993). Heritable pulmonary arterial hypertension. In M. P. Adam, H. H. Ardinger & R. A. Pagon, et al. (Eds.) *GeneReviews®* Seattle, WA: University of Washington. Seattle. http://www.ncbi.nlm.nih.gov/books/NBK1485/. Accessed April 16, 2018.

Austin, E. D., Ma, L., LeDuc, C., Berman Rosenzweig, E., Borczuk, A., Phillips, J. A. 3rd, ... Chung, W. K. (2012). Whole exome sequencing to identify a novel gene (cenexin-1) associated with human pulmonary arterial hypertension. *Circulation: Cardiovascular Genetics, 5*(3), 336–343. https://doi.org/10.1161/CIRCGENETICS.111.961888

Badesch, D. B., Champion, H. C., Sanchez, M. A. G., Hoeper, M. M., Loyd, J. E., Manes, A., ... Torbicki, A. (2009). Diagnosis and assessment of pulmonary arterial hypertension. *Journal of the American College of Cardiology, 54*(1 Suppl), S55–S66. https://doi.org/10.1016/j.jacc.2009.04.011

van der Bruggen, C. E., Happe, C. M., Dorfmüller, P., Trip, S., Sprijit, O. A., Rol, N., ... de Man, F. S. (2016). Bone morphogenetic protein receptor type 2 mutation in pulmonary arterial hypertension: A view on the right ventricle. *Circulation, 133*(18), 1747–1760. https://doi.org/10.1161/CIRCULATIONAHA.115.020696

Burg, E. D., Platoshyn, O., Tsigelny, I. F., Lozano-Ruiz, B., Rana, B., K., ... Yuam, JX.-J. (2010). Tetramerization domain mutations in KCNA5 affect channel kinetics and cause abnormal trafficking patterns. *American Journal of Physiology. Cell Physiology, 298*(3), C496–C509. https://doi.org/10.1152/ajpcell.00464.2009

Chida, A., Shintani, M., Nakayama, T., Furutani, Y., Hayama, E., Inai, K., ... Nakanishi, T. (2012). Missense mutations of the BMPR1B (ALK6) gene in childhood idiopathic pulmonary arterial hypertension. *Circ J Off J Jpn Circ Soc, 76*(6), 1501–1508.

Dewachter, L., Adnot, S., Guignabert, C., Tu, L., Marcos, E., Fadel, E., ... Eddahibi, S. (2009). Bone morphogenetic protein signalling in heritable versus idiopathic pulmonary hypertension. *European Respiratory Journal, 34*(5), 1100–1110. https://doi.org/10.1183/09031936.00183008

Drake, K. M., Zygmun, D., Mavrikas, L., Harbor, P., Wang, L., Comhair, S. A., ... Aldred, M. A. (2011). Altered MicroRNA processing in heritable pulmonary arterial hypertension: An important role for Smad8. *American Journal of Respiratory and Critical Care Medicine, 184*(12), 1400–1408. https://doi.org/10.1016/j.rccm.201106-1130OC

Dunmore, B. J., Drake, K. M., Upton, P. D., Toshner, M. R., Aldred, M. A., & Morrell, N. W. (2013). The lysosomal inhibitor, chloroquine, increases cell surface BMPR-II levels and restores BMP9 signalling in endothelial cells harbouring BMPR-II mutations. *Human Molecular Genetics, 22*(18), 3667–3679. https://doi.org/10.1093/hmg/ddt216

Ensembl genome browser. http://www.ensembl.org/index.html. Accessed August 10, 2016.

ExAC Browser. http://exac.broadinstitute.org/. Accessed August 10, 2016.

Exome Variant Server. http://evs.gs.washington.edu/EVS/. Accessed August 10, 2016.

Fu, L., Zhou, A., Huang, M., Shen, S. H., Shen, J., Zhang, Z. F., Li, F. (2008). A novel mutation in the BMPR2 gene in familial pulmonary arterial hypertension. *Chin Med J (Engl), 121*(5), 399–404.

Gaine, S. P., & Rubin, L. J. (1998). Primary pulmonary hypertension. *Lancet Lond Engl, 352*(9129), 719–725. https://doi.org/10.1016/S0140-6736(98)02111-4

Giudicessi, J. R., Kapplinger, J. D., Tester, D. J., Alders, M., Salisbury, B. A., Wilde, A. A., & Ackerman, M. J. (2012). Phylogenetic and physicochemical analyses enhance the classification of rare nonsynonymous single nucleotide variants in type 1 and 2 long-QT syndrome. *Circulation: Cardiovascular Genetics, 5*(5), 519–528. https://doi.org/10.1161/CIRCGENETICS.112.963785

Hamid, R., Hedges, L. K., Austin, E., Phillips, J. A., Loyd, J. E., ... Cogan, J. D. (2010). Transcripts from a novel BMPR2 termination variant.
mutation escape nonsense mediated decay by downstream translation re-initiation: Implications for treating pulmonary hypertension. *Clinical Genetics*, 77(3), 280–286. https://doi.org/10.1111/j.1399-0004.2009.01311.x

HGNC database of human gene names | HUGO Gene Nomenclature Committee. http://www.genenames.org/. Accessed August 10, 2016.

Hoepfer, M. M., Bogaard, H. J., Condliffe, R., Frantz, R., Khanna, D., Kurzyna, M., ... Badesch, D. B. (2013). Definitions and diagnosis of pulmonary hypertension. *Journal of the American College of Cardiology*, 62(Suppl), D42–D50. https://doi.org/10.1016/j.jacc.2013.10.032

Hoepfer, M. M., Bogaard, H. J., Condliffe, R., Frantz, R., Khanna, D., Kurzyna, M., ... Badesch, D. B. (2014). Definitions and diagnosis of pulmonary hypertension. *Journal of the American College of Cardiology*, 62(Suppl 1), 55–66.

Humbert, M., Sitbon, O., Chaouat, A., Bertocchi, M., Habib, G., Gressin, V., ... Simonneau, G. (2006). Pulmonary arterial hypertension in France: Results from a national registry. *American Journal of Respiratory and Critical Care Medicine*, 173(9), 1023–1030. https://doi.org/10.1164/rccm.200510-1668OC

Jabbari, J., Jabbari, R., Nielsen, M. W., Holst, A. G., Nielsen, J. B., Haunso, S., ... Olesen, M. S. (2013). New exome data question the pathogenicity of genetic variants previously associated with catecholaminergic polymorphic ventricular tachycardia. *Circulation: Cardiovascular Genetics*, 6(5), 481–489. https://doi.org/10.1161/CIRCGENETICS.113.000118

de Jesus Perez, V. A., Yuan, K., Lyuksyutova, M. A., Dewey, F., Orcholski, M. E., Shuffle, E. M., ... Zamanian, R. T. (2014). Whole-exome sequencing reveals TopBP1 as a novel gene in idiopathic pulmonary arterial hypertension. *American Journal of Respiratory and Critical Care Medicine*, 189(10), 1260–1272. https://doi.org/10.1164/rccm.201310-1749OC

Kabata, H., Satoh, T., Kataoka, M., Tamura, Y., Ono, T., Yamamoto, M., ... Asano, K. (2013). Bone morphogenetic protein receptor type 2 mutations, clinical phenotypes and outcomes of Japanese patients with sporadic or familial pulmonary hypertension. *Respirology*, 18(7), 1076–1082. https://doi.org/10.1111/resp.12117

Kerstjens-Frederikse, W. S., Bongers, E. M. H. F., Roofthooft, M. T., Leter, E. M., Douwes, J. M., Van Dijk, A., ... Berger, R. M. (2013). TBX4 mutations (small patella syndrome) are associated with childhood-onset pulmonary arterial hypertension. *Journal of Medical Genetics*, 50(8), 500–506. https://doi.org/10.1136/jmedgenet-2012-101152

Ling, Y., Johnson, M. K., Kiely, D. G., Condliffe, R., Elliot, C. A., Gibbs, J. S., ... Peacock, A. J. (2012). Changing demographics, epidemiology, and survival of incident pulmonary arterial hypertension: Results from the pulmonary hypertension registry of the United Kingdom and Ireland. *American Journal of Respiratory and Critical Care Medicine*, 186(8), 790–796. https://doi.org/10.1164/rccm.2012-0338OC

Liu, D., Liu, Q.-Q., Eyries, M., Wu, W. H., Yuan, P., Zhang, R., ... Jing, Z. C. (2012). Molecular genetics and clinical features of Chinese idiopathic and heritable pulmonary arterial hypertension patients. *European Respiratory Journal*, 39(3), 597–603. https://doi.org/10.1183/09031936.00072911

Long, L., Ormiston, M. L., Yang, X., Southwood, M., Gräf, S., Machado, R. D., ... Morrell, N. W. (2015). Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. *Nature Medicine*, 21(7), 777–785. https://doi.org/10.1038/nm.3877

Ma, L., & Chung, W. K. (2014). The genetic basis of pulmonary arterial hypertension. *Human Genetics*, 133(5), 471–479. https://doi.org/10.1007/s00439-014-1419-3

Ma, L., Roman-Campos, D., Austin, E. D., Eyries, M., Sampson, K. S., Soubrier, F., ... Chung, W. K. (2013). A novel channelopathy in pulmonary arterial hypertension. *New England Journal of Medicine*, 369(4), 351–361. https://doi.org/10.1056/NEJMoa1211097

Machado, R. D. (2012). The molecular genetics and cellular mechanisms underlying pulmonary arterial hypertension. *Scientifica*, 2012, 106576. https://doi.org/10.6064/2012/106576

Machado, R. D., Aldred, M. A., James, V., Harrison, R. E., Patel, B., Schwalbe, E. C., ... Trembath, R. C. (2006). Mutations of the TGF-beta type II receptor BMPR2 in pulmonary arterial hypertension. *Human Mutation*, 27(2), 121–132. https://doi.org/10.1002/humu.20285

Machado, R. D., Eickelberg, O., Elliott, C. G., Geraci, M. W., Hanaoka, M., Loyd, J. E., ... Chung, W. K. (2009). Genomics and genetics of pulmonary arterial hypertension. *Journal of the American College of Cardiology*, 54(1 Suppl), S32–S42. https://doi.org/10.1016/j.jacc.2009.04.015

Nasim, M. T., Ogo, T., Ahmed, M., Randall, R., Chowdhury, H. M., Snape, K. M., ... Machado, R. D. (2011). Molecular genetic characterization of SMAD signaling molecules in pulmonary arterial hypertension. *Human Mutation*, 32(12), 1385–1389. https://doi.org/10.1002/humu.21605

Pfarr, N., Fischer, C., Ehiken, N., Becker-Grüning, T., López-González, V., Gorenflo, M., ... Grünig, E. (2013). Hemodynamic and genetic analysis in children with idiopathic, heritable, and congenital heart disease associated pulmonary arterial hypertension. *Respir Res.*, 14, 3. https://doi.org/10.1186/1465-9921-14-3

Pfarr, N., Szamalek-Hoegel, J., Fischer, C., Hinderhofer, K., Nagel, C., Ehiken, N., ... Grünig, E. (2011). Hemodynamic and clinical onset in patients with hereditary pulmonary arterial hypertension and BMPR2 mutations. *Respiratory Research*, 12, 99. https://doi.org/10.1186/1465-9921-12-99

Rabinovitch, M. (2012). Molecular pathogenesis of pulmonary arterial hypertension. *Journal of Clinical Investigation*, 122(12), 4306–4313. https://doi.org/10.1172/JCI60658

Remillard, C. V., Tigno, D. D., Platsdyn, O., Burg, E. D., Brevnova, E. E., Conger, D., ... Yuan, J. X. (2007). Function of Kv1.5 channels and genetic variations of KCNA5 in patients with idiopathic pulmonary arterial hypertension. *American Journal of Physiology. Cell Physiology*, 292(5), C1837–C1853. https://doi.org/10.1152/ajpcell.00405.2006

Ricard, N., Bidart, M., Mallet, C., Lesca, G., Giraud, S., Prudent, R., ... Bailly, S. (2010). Functional analysis of the BMP9 response of ALK1 mutants from HHT2 patients; A diagnostic tool for novel ACVRL1 mutations. *Blood*, 116(9), 1604–1612. https://doi.org/10.1182/blood-2010-03-276881

Risgaard, B., Jabbari, R., Refsgaard, L., Holst, A. G., Haunsø, S., ... Tielt-Hansen, J. (2013). High prevalence of genetic variants previously associated with Brugada syndrome in new exome data. *Clinical Genetics*, 84(5), 489–495. https://doi.org/10.1111/cge.12126

Shintani, M., Yagi, H., Nakayama, T., Saji, T., & Matsuoka, R. (2009). A new nonsense mutation of SMAD8 associated with...
pulmonary arterial hypertension. *Journal of Medical Genetics*, 46 (5), 331–337. https://doi.org/10.1136/jmg.2008.062703

Simonneau, G., Gatzoulis, M. A., Adatia, I., Celermajer, D., Denton, C., Ghofrani, A., … Souza, R. (2013). Updated clinical classification of pulmonary hypertension. *Journal of the American College of Cardiology*, 62(25 Suppl), D34–D41. https://doi.org/10.1016/j.jacc.2013.10.029

Song, J., Eichstaedt, C. A., Viales, R. R., Benjamin, N., Harutyunova, S., Fischer, C., … Hinderhofer, K. (2016). Identification of genetic defects in pulmonary arterial hypertension by a new gene panel diagnostic tool. *Clinical Science (London, England: 1979)* 130(22), 2043–2052. https://doi.org/10.1042/cs20160531

Soubrier, F., Chung, W. K., Machado, R., Grünig, E., Aldred, M., Geraci, M., … Humbert, M. (2013). Genetics and genomics of pulmonary arterial hypertension. *Journal of the American College of Cardiology*, 62(25 Suppl), D13–D21. https://doi.org/10.1016/j.jacc.2013.10.035

Suo, S.-B., Qiu, J.-D., Shi, S.-P., Chen, X., Huang, S.-Y., & Liang, R.-P. (2013). Proteome-wide analysis of amino acid variations that influence protein lysine acetylation. *Journal of Proteome Research*, 12(2), 949–958. https://doi.org/10.1021/pr301007j

Sztrymf, B., Coulet, F., Giret, B., Yaici, A., Jais, X., Sibton, O., … Humbert, M. (2008). Clinical outcomes of pulmonary arterial hypertension in carriers of BMPR2 mutation. *American Journal of Respiratory and Critical Care Medicine*, 177(12), 1377–1383. https://doi.org/10.1164/rccm.200712-1807OC

Thomas, M., Docx, C., Holmes, A. M., Beach, S., Duggan, N., England, K., … Budd, D. C. (2009). Activin-like kinase 5 (ALK5) mediates abnormal proliferation of vascular smooth muscle cells from patients with familial pulmonary arterial hypertension and is involved in the progression of experimental pulmonary arterial hypertension induced by monocrotaline. *American Journal of Pathology*, 174(2), 380–389. https://doi.org/10.2353/ajpath.2009.080565

Vattulainen, S., Aho, J., Salmenperä, P., Bruce, S., Tallila, J., Gentile, M., … Myllykangas, S. (2015). Accurate genetic diagnosis of Finnish pulmonary arterial hypertension patients using oligonucleotide-selective sequencing. *Molecular Genetics and Genomic Medicine*, 3(4), 354–362. https://doi.org/10.1002/mgg3.147

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Abbasi Y, Jabbari J, Jabbari R, et al. Exome data clouds the pathogenicity of genetic variants in Pulmonary Arterial Hypertension. *Mol Genet Genomic Med*. 2018;6:835–844. https://doi.org/10.1002/mgg3.452