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Phenotypic Characterisation of *EIF2AK4* Mutation Carriers in a Large Cohort of Patients Diagnosed Clinically with Pulmonary Arterial Hypertension

Running Title: *Hadinnapola et al.; Phenotypes of EIF2AK4 Mutation Carriers*

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Abstract

***Background*—**Pulmonary arterial hypertension (PAH) is a rare disease with an emerging genetic basis. Heterozygous mutations in the gene encoding the bone morphogenetic protein receptor type 2 (*BMPR2*) are the commonest genetic cause of PAH, whereas biallelic mutations in the eukaryotic translation initiation factor 2 alpha kinase 4 gene (*EIF2AK4*) are described in pulmonary veno-occlusive disease and pulmonary capillary haemangiomatosis (PVOD/PCH). Here, we determined the frequency of these mutations and define the genotype-phenotype characteristics in a large cohort of patients diagnosed clinically with PAH.

***Methods*—**Whole genome sequencing was performed on DNA from patients with idiopathic and heritable PAH, as well as PVOD/PCH recruited to the NIHR BioResource - Rare Diseases Study. Heterozygous variants in *BMPR2* and biallelic *EIF2AK4* variants with a minor allele frequency of < 1:10,000 in control data sets and predicted to be deleterious (by CADD, PolyPhen-2 and SIFT predictions) were identified as potentially causal. Phenotype data from the time of diagnosis were also captured.

***Results*—**Eight hundred and sixty-four patients with idiopathic or heritable PAH and 16 with PVOD/PCH were recruited. Mutations in *BMPR2* were identified in 130 patients (14.8%). Biallelic mutations in *EIF2AK4* were identified in 5 patients with a clinical diagnosis of PVOD/PCH. Furthermore, 9 patients with a clinical diagnosis of PAH carried biallelic *EIF2AK4* mutations. These patients had a reduced transfer coefficient for carbon monoxide (KCO: 33 [IQR: 30 - 35] % predicted) and younger age at diagnosis (29 [23 - 38] years) as well as more interlobular septal thickening and mediastinal lymphadenopathy on computed tomography of the chest, compared to PAH patients without *EIF2AK4* mutations. However, radiological assessment alone could not accurately identify biallelic *EIF2AK4* mutation carriers. PAH patients with biallelic *EIF2AK4* mutations had a shorter survival.

***Conclusions*—**Biallelic *EIF2AK4* mutations are found in patients classified clinically as idiopathic and heritable PAH. These patients cannot be identified reliably by CT, but a low KCO and a young age of diagnosis suggests the underlying molecular diagnosis. Genetic testing can identify these misclassified patients, allowing appropriate management and early referral for lung transplantation.

Key Words: genetics, human; pulmonary hypertension; prognosis; EIF2AK4, pulmonary veno-occlusive disease

Clinical Perspective

What is new?

- 1% of patients with a clinical diagnosis of PAH carry biallelic *EIF2AK4* mutations.
- Patients diagnosed clinically with PAH who had a KCO < 50% predicted and age of diagnosis < 50 years were more likely to carry biallelic *EIF2AK4* mutations. The diagnostic yield for genetic testing in this group was 53%.
- Radiological assessment was unable to distinguish reliably between these patients and idiopathic PAH patients.
- Histology from these patients may show predominately pulmonary arteriopathy, with subtle involvement of the pulmonary veins and capillaries.
- PAH patients with biallelic *EIF2AK4* mutations had a worse prognosis compared to other PAH patients.

What are the clinical implications?

- Younger patients diagnosed with idiopathic PAH, but with a low KCO, have a high frequency of biallelic *EIF2AK4* mutations.
- Such patients should be reclassified as pulmonary veno-occlusive disease/pulmonary capillary haemangiomatosis (PVOD/PCH).
- Similar to patients with PVOD/PCH these patients have a poor prognosis compared to other PAH patients.
- The spectrum of radiological and histological changes associated with biallelic *EIF2AK4* mutations is wider than previously assumed. The presence of only subtle or infrequent features associated with PVOD may lead to misclassification of these patients as PAH.
- Genetic testing allows early identification of these patients, facilitating appropriate management.

Pulmonary arterial hypertension (PAH) is a heterogeneous and rare disorder that can be classified into idiopathic and heritable forms, associated with an underlying condition, such as connective tissue disease or congenital heart disease, or related to specific drugs and toxins [1, 2]. In addition, pulmonary veno-occlusive disease (PVOD) and pulmonary capillary haemangiomatosis (PCH) are even rarer forms of pulmonary hypertension that are grouped together with PAH under the current classification system [2].

Clinical features described in patients with PVOD/PCH include a low transfer coefficient for carbon monoxide (KCO) and oxygen desaturation on exertion, as well as the presence of centrilobular ground glass opacification, interlobular septal thickening and mediastinal lymphadenopathy on high resolution computed tomography (HRCT) of the lung parenchyma [3, 4]. However, these clinical and radiological features have also been reported in idiopathic PAH [5-7]. Consequently, the clinical distinction between PVOD/PCH and idiopathic PAH can be challenging. It has been estimated that 10% of patients with PVOD/PCH are misdiagnosed as idiopathic PAH [8, 9]. The diagnosis of PVOD/PCH is often only confirmed post mortem, or from explanted lungs, by histology.

The histological features of PVOD/PCH typically include pulmonary venous obstructions and pulmonary capillary proliferation, although the distribution of these changes within the lung can be heterogeneous [10, 11]. Pulmonary artery smooth muscle hypertrophy and intimal hyperplasia, similar to the changes observed in other forms of PAH, may also be present. Furthermore, pulmonary venous changes have been reported in cases of idiopathic PAH, scleroderma-associated PAH and those with *BMPR2* mutations, to varying extents [12, 13].

A major advance in the molecular diagnosis of PVOD/PCH was the finding of biallelic mutations in the gene encoding the eukaryotic translation initiation factor 2 alpha kinase 4

(EIF2AK4) in both familial (100%) and sporadic (20-25%) cases of PVOD/PCH [14, 15]. EIF2AK4 is an activator of the integrated stress response (ISR) pathway, and responds to environmental stresses, including amino acid deprivation, by phosphorylating the alpha subunit of eukaryotic translation initiation factor 2 [11, 16, 17]. These discoveries suggest that *EIF2AK4* mutations are specific to PVOD/PCH and that finding biallelic *EIF2AK4* mutations in a patient with pulmonary hypertension would be diagnostic of PVOD/PCH. Patients with PVOD/PCH have a poor prognosis and risk fatal pulmonary oedema with the use of pulmonary artery vasodilator therapies [4, 18-20]. Consequently, early and accurate diagnosis is vital to guide clinical management.

Heterozygous mutations in the gene encoding the bone morphogenetic protein type 2 receptor (*BMPR2*) are the most common genetic cause of PAH. They are found in approximately 17% of individuals with idiopathic PAH and 82% with a family history of the disease [21]. However, mutations in *BMPR2* have also been reported in patients with histologically proven PVOD [4, 22-24]. Thus, there remains considerable uncertainty to what extent the finding of *EIF2AK4* or *BMPR2* mutations reliably predict the clinical phenotype and response to therapy in a population of patients with PAH.

Here we report the genetic and phenotypic characteristics of patients assessed for *BMPR2* and *EIF2AK4* mutations, through whole genome sequencing, within a large cohort (n=880) of PAH patients recruited to the National Institute of Health Research (NIHR) BioResource – Rare Diseases (BRIDGE) Study (Supplementary Table 1). The frequency of mutations in other previously reported genes associated with PAH will be reported in a future publication. In this study, we identified and characterised patients with a clinical and radiological diagnosis of idiopathic PAH who were found to possess biallelic *EIF2AK4* mutations. These patients had a

low KCO and were diagnosed at a younger age compared with idiopathic PAH patients without mutations in these genes. We show that, in common with patients diagnosed clinically with PVOD/PCH, PAH patients with biallelic *EIF2AK4* mutations have a shorter survival. We conclude that clinical assessment alone is inadequate for the accurate diagnosis of PVOD/PCH. Clinical genetic testing in younger patients presenting clinically with PAH but with a low KCO, will allow appropriate classification, leading to better risk stratification and management of these patients.

Methods

Ethical approval and consent

UK patients (621 [70.6%]) were recruited prospectively to the BRIDGE Study and provided written informed consent for genetic analysis and the capture of clinical data (NIHR BioResource - Rare Diseases Study 13/EE/0325). Additionally, the study included patients recruited retrospectively from non-UK centres (191 [21.7%]), and deceased UK patients (68 [7.7%]), if they had signed local tissue bank consent forms allowing genetic sequencing. Explanted lung tissue from an individual undergoing lung transplantation for end stage PAH was collected under Papworth Hospital Research Tissue Bank ethics (08/H0304/56).

Recruitment and patients

The BRIDGE Study is a prospective study recruiting both prevalent and incident patients with selected rare diseases. Recruitment to the BRIDGE PAH Study started in January 2013 and the last patient included in this analysis was recruited on 15/06/2016. Patients with idiopathic PAH, heritable PAH, PVOD and PCH, diagnosed according to international guidelines at specialist pulmonary hypertension centres in the United Kingdom, Netherlands and France, were recruited

(Figure 1 and Supplementary Table 2) [2]. This included 14 patients with confirmed mutations in *BMPR2*.

Throughout the manuscript, we classify patients recruited to the study as idiopathic PAH or familial PAH based on the absence or presence of a family history of the disease. The term heritable PAH does not distinguish between sporadic PAH patients with a mutation, and patients with a mutation where there is a family history. Therefore, the term "heritable PAH" is only used when referring to previous publications and guidelines.

Patients with other rare diseases and their unaffected relatives recruited to the BRIDGE Study (Supplementary Table 3) acted as non-PAH controls for the genetic analysis.

Whole genome sequencing and variant calling

Next generation sequencing using 100-150 base pair paired-end sequencing was performed on DNA libraries created from genomic DNA using Illumina HiSeq 2500 and HiSeq X (Illumina Inc, San Diego, USA).

Reads were aligned against the Genome Reference Consortium human genome (build 37) (GRCh37) and variants were called using the Issac Aligner and Variant Caller respectively (version 2, Illumina Inc.). Variants in *BMPR2* and *EIF2AK4* were extracted and annotated using Ensembl's Variant Effect Predictor (VEP) v84 [25]. Deletions (resulting in the loss of more than 50bp) were identified by applying Isaac Copy Number Variant Caller (Canvas, Illumina) and Isaac Structural Variant Caller (Manta, Illumina). Further information is provided in the supplemental materials.

Likely causal variants were identified based on minor allele frequency (MAF) and predicted deleteriousness. Variants were considered further if they had a MAF of less than 1 in 10,000 in unrelated non-PAH BRIDGE controls and the ExAC database [26]. The rare variants that

passed the MAF filtering were then assessed for deleteriousness. Variants were considered pathogenic based on a combined annotation dependent depletion (CADD) score of 15 or higher and PolyPhen-2 *or* SIFT predictions not classified as "benign" or "tolerated" respectively [27-29].

Over-representation analyses

For comparison of variant frequencies between disease and control groups only variants from unrelated individuals were used. The PRIMUS software package was used to identify non-related individuals amongst both non-PAH BRIDGE controls and PAH patients [30]. The number of unrelated control subjects was maximised by including either patients with other rare diseases or their unaffected relatives. The frequency of rare and predicted deleterious heterozygous *EIF2AK4* variants in PAH index cases was also compared to publically available information in the ExAC database (http://exac.broadinstitute.org) [26]. This analysis provides the maximum estimate of the frequency of heterozygous *EIF2AK4* variants in the ExAC database as variants in ExAC were assumed not to be in a compound heterozygous state.

Phenotypic data capture and CT assessment

Paper and electronic patient records of PAH patients were reviewed to capture demographic and phenotypic variables from the time of diagnosis and follow up. Survival data for UK patients were obtained from recruiting centres through the NHS National Spine and local databases. Anonymised information was captured securely online using the free OpenClinica® software, adapted for data capture specific to PAH.

CT images of the chest, where available, were reviewed independently by 2 cardiothoracic radiologists (AS and NS), with specialist imaging experience in pulmonary hypertension, blinded to the underlying diagnoses using a customised proforma. Further

information is provided in the supplemental materials, Supplementary Table 4 and Supplementary Table 5.

Statistical analysis

Statistical analysis was performed in R (www.r-project.org). Further information is provided in the supplemental materials.

Semi-parametric Cox-proportional hazard models were used to assess survival between groups using the "survival" package in R. Time from diagnosis to both death and death or transplantation was assessed. Age at diagnosis and gender were used as covariates in the models. To avoid immortal time bias arising from the inclusion of retrospectively recruited patients and prevalent patients, a sensitivity analysis was conducted. In this analysis only prospectively recruited patients from the UK were included and patients entered the risk set only from the time they consented to the study. Further information is provided in the supplemental materials.

Results

Study patients

Whole genome sequencing was performed on 932 patients recruited to the NIHR BRIDGE PAH Study and 7134 non-PAH control subjects recruited to other NIHR BRIDGE Study cohorts. Fifty-two patients were excluded from further analysis because they did not have a clinical diagnosis of idiopathic PAH, heritable PAH, PVOD or PCH (Figure 1). The remaining 880 patients (of which 872 were defined as unrelated index cases) consisted of 16 patients (1.8%) with a clinical diagnosis of PVOD/PCH, 56 (6.4%) with PAH and a family history of the disease (referred to as familial PAH) and 808 (91.8%) with idiopathic PAH and no known family

history. One of the 16 patients with a clinical diagnosis of PVOD/PCH had an affected sister, whereas the remainder had the sporadic form of the disease.

BMPR2 mutations in the PAH cohort

Rare and predicted deleterious *BMPR2* mutations (single nucleotide variants, indels and larger deletions) were found in 41 patients (73.2%) with familial PAH and 89 patients (11.0%) with idiopathic PAH. No *BMPR2* mutations were found in patients with a clinical diagnosis of PVOD/PCH.

Rare and predicted deleterious EIF2AK4 variants in the PAH cohort

Sixty-nine rare and predicted deleterious *EIF2AK4* single nucleotide variants and indels were present in the NIHR BRIDGE Study. No large deletions were found that affected the *EIF2AK4* gene locus. The variants are summarised in Supplementary Table 6. Five of the 16 patients (31.3%) with clinically diagnosed PVOD/PCH carried biallelic *EIF2AK4* mutations (2 homozygotes and 3 compound heterozygotes).

Twenty-five *EIF2AK4* variants were also found in 19 patients (2.2%) diagnosed clinically with PAH, in whom there was no clinical diagnosis of PVOD/PCH (5 homozygotes, 4 compound heterozygotes and 10 heterozygotes; Supplementary Table 7). One of these patients with a homozygous *EIF2AK4* mutation (c.3097C>T creating a premature stop codon) had a sister who had died of PAH. There was no reported family history of PVOD/PCH.

The remaining rare *EIF2AK4* variants were found in a heterozygous state in 36 control subjects (0.5%). Four of these variants appeared in more than 1 non-PAH control subject and none were shared with PAH patients.

Over-representation of rare heterozygous EIF2AK4 variants in idiopathic PAH patients compared to control subjects

The proportion of patients with a clinical diagnosis of idiopathic PAH carrying heterozygous rare *EIF2AK4* variants (1.2%) was significantly greater than the non-PAH control subjects (0.5%; p = 0.030). A similar over-representation in idiopathic PAH patients was observed when compared to allele frequencies in the ExAC database (0.6%; p = 0.042). Two idiopathic PAH patients with heterozygous rare *EIF2AK4* variants also carried a rare and predicted deleterious *BMPR2* mutation.

Phenotype of patients with a clinical diagnosis of PAH and biallelic EIF2AK4 mutations

Patients with a clinical diagnosis of PAH and biallelic *EIF2AK4* mutations presented at a younger age (median [IQR]: 29 [23 - 38] years) compared to patients without these variants (51 [37 - 65] years; p = 0.024) (Table 1). Mean pulmonary artery pressure, cardiac output and pulmonary vascular resistance were not significantly different between PAH patients with biallelic *EIF2AK4* mutations and the other groups. As previously reported, haemodynamic variables were significantly more severe in patients with *BMPR2* mutations compared to those without any mutations in these genes.

The PAH patients with biallelic *EIF2AK4* mutations exhibited a reduced KCO (33 [30 - 35] % predicted) compared to *BMPR2* mutation carriers (81 [73 - 92] % predicted, p < 0.001) and PAH patients with no identified mutation (71 [51 - 85] % predicted, p = 0.001). PAH patients with biallelic *EIF2AK4* mutations had no obstructive or restrictive deficit on spirometry. These differences remained after exclusion of patients with abnormal spirometry in the other groups (FEV_1 < 80% or FVC < 80%) (Supplementary Table 8).

Digital clubbing was over-represented amongst patients with biallelic *EIF2AK4* mutations diagnosed clinically with PAH (42%; p=0.002). Eleven percent of patients with a clinical diagnosis of PVOD were clubbed.

Only one patient with a heterozygous rare and predicted deleterious *EIF2AK4* variant (c.2516T>C) had a reduced KCO (54% predicted) with normal spirometry (FEV_1 102% predicted, FVC 98% predicted and TLC 100% predicted). Although, there was mild paraseptal emphysema on thoracic CT (< 5% of the lung parenchyma affected). This patient, a 44-year-old Caucasian male diagnosed with idiopathic PAH, also carried a rare and deleterious *BMPR2* splice acceptor mutation (c.853-2A>G).

We questioned whether KCO was a predictor of biallelic *EIF2AK4* mutations in the wider cohort. However, amongst PAH patients with no mutations and normal spirometry (n=255), a reduced KCO (< 50% predicted) was present in 65 patients (25.5%). In these patients with a reduced KCO and preserved spirometry, 90.8% were aged over 50 years at diagnosis and 69.2% had a history of coronary artery disease, left ventricular dysfunction or cardiovascular risk factors (diabetes mellitus, systemic hypertension or hyperlipidaemia).

Given the high prevalence of a low KCO with preserved spirometry in the wider cohort, we restricted an analysis to patients under the age of 50 years, who at the time of diagnosis had normal spirometry (n=164). Even, in this group a significant proportion (15, 9.1%) had a KCO < 50% predicted (Figure 2). Eight of these 15 patients carried biallelic *EIF2AK4* mutations. One patient with biallelic *EIF2AK4* mutations was aged 70 years at diagnosis and subsequently did not meet this cut-off.

Amongst patients with normal spirometry, the presence of a KCO < 50% predicted and age at diagnosis < 50 years had a high sensitivity (0.889) and specificity (0.977) for identifying

patients who carry biallelic *EIF2AK4* mutations, the positive predictive value was low (0.533). Nevertheless, in terms of the diagnostic yield, while genetic testing for biallelic *EIF2AK4* mutations in the entire cohort of patients diagnosed clinically with PAH yielded a 1% detection rate, the presence of biallelic *EIF2AK4* mutations in PAH patients with a KCO < 50% with normal spirometry and aged under 50 at diagnosis was 53%.

CT features of EIF2AK4 mutation carriers

Centrilobular ground glass opacification extent, mediastinal lymphadenopathy and interlobular septal thickening are considered suggestive of PVOD/PCH. However, we found subtle or gross centrilobular ground glass opacification in 38% of patients diagnosed clinically with PAH and carrying no mutations (n=21) and 67% of PAH patients with *BMPR2* mutations (n=21). This was not significantly different compared to patients with a clinical diagnosis of PAH and biallelic *EIF2AK4* mutations (86%, n=7) and patients with a clinical diagnosis of PVOD (50%, n=14). Gross interlobular septal thickening and mediastinal lymphadenopathy was significantly more frequent amongst patients with PAH and biallelic *EIF2AK4* mutations (29% and 57% respectively) and those with PVOD (64% and 79%) compared to patients with PAH and no mutation (5% and 0%) or *BMPR2* mutations (5% and 10%). A radiological suspicion of PVOD/PCH was raised in 71% of those with PVOD, 57% of patients with a clinical diagnosis of PAH and biallelic *EIF2AK4* mutations, 14% of PAH patients with no mutation, and 5% of those with *BMPR2* mutations (Table 2).

A further CT analysis comparing patients with biallelic *EIF2AK4* mutations (with a clinical diagnosis of PVOD/PCH or PAH; n=11) and those with a clinical diagnosis of PVOD but not carrying biallelic *EIF2AK4* mutations (n=10) was made (Supplementary Table 9). Patients with biallelic *EIF2AK4* mutations were younger at diagnosis (27 [IQR: 23 - 34] years)

compared to those with PVOD and no *EIF2AK4* mutations (68 [64 - 72] years, p=0.001). The patients with biallelic *EIF2AK4* mutations also had a lower KCO (32 [29 – 33] % predicted) compared to patients with PVOD and no *EIF2AK4* mutations (41.4 [37 – 54] % predicted, p=0.013). Centrilobular ground glass opacification appeared more extensive in those with biallelic *EIF2AK4* mutations (82%) compared to those without a mutation (10%; p=0.012). However, pleural effusions were more common amongst those without a mutation (40%) compared to patients with biallelic *EIF2AK4* mutations (0%, p=0.035). This may suggest that patients with biallelic *EIF2AK4* mutations have a distinct radiological phenotype compared to patients with PVOD and no biallelic *EIF2AK4* mutations.

Response to pulmonary artery vasodilator therapies

The response to pulmonary artery vasodilator therapies at 1 and 3 years was assessed for patients with a clinical diagnosis of PAH and biallelic *EIF2AK4* mutations as well as the other PAH patients included in the CT analysis. Patients with a clinical diagnosis of PAH and biallelic *EIF2AK4* mutations did not improve their functional class at either 1 year or 3 years post diagnosis unlike the other PAH groups (Supplementary Table 10).

Histological features of biallelic EIF2AK4 mutation carrier

The explanted lungs of one patient diagnosed with idiopathic PAH but found to have a homozygous *EIF2AK4* missense mutation (c.1795G>C, p.G599R) were assessed. The predominant histological feature was pulmonary arterial vasculopathy. The pulmonary arteries predominantly showed concentric and eccentric intimal fibrosis. No plexiform lesions were observed. Although infrequent, there was some fibrosis of the septal veins and venules, some of which were nearly completely occluded. Although there was evidence of capillary congestion, no capillary hemangiomatosis was observed (Figure 3). The missense variant carried by this patient

was not reported in the ExAC database, occurs in a conserved area of the genome (GERP score 5.5) and was predicted to be deleterious (CADD score 32, PolyPhen-2 prediction of "probably damaging [1]", SIFT prediction of "deleterious [0]"). The same homozygous mutation was also found in a second unrelated patient with a clinical diagnosis of idiopathic PAH.

Impact of genotype on survival

Eight hundred and fifty-eight patients were included in the Cox proportional hazards model (Supplementary Table 11, Supplemental Figure 1). Patients diagnosed clinically as PAH with biallelic *EIF2AK4* mutations had a shorter survival time from diagnosis compared to the *BMPR2* mutation carriers ($p < 0.001$) and those without any variants in PAH associated genes ($p < 0.001$). Age ($p < 0.001$) and gender ($p = 0.001$) also had a significant effect on survival, with male sex and an older age at diagnosis associated with shorter survival in the model. Similar results were obtained when assessing the time to death or transplantation (Supplementary Tables 12). In the sensitivity analysis, including only prospectively recruited UK patients, only 2 events occurred in the biallelic *EIF2AK4* group. Thus no significant difference was observed in mortality between patients diagnosed clinically as PAH with biallelic *EIF2AK4* mutations and patients with *BMPR2* mutations ($p = 0.215$), or patients without any variants in PAH associated genes ($p = 0.282$; Supplementary Table 13).

Discussion

This is the first study to analyse the frequency of *EIF2AK4* rare variation in a large cohort of PAH patients and make detailed phenotypic and radiological assessments. Previously the presence of biallelic *EIF2AK4* mutations were reported in patients with a clear clinical diagnosis of PVOD/PCH as well as a large kindred and a single family with a possible diagnosis of PAH [20,

[31, 32]. As expected, we identified a high frequency of biallelic *EIF2AK4* mutations in patients with a clear clinical presentation of PVOD/PCH. However, we also found biallelic *EIF2AK4* mutations in patients with a clinical diagnosis of PAH.

The discovery of biallelic *EIF2AK4* mutations in PVOD/PCH raised the possibility of rapid molecular diagnosis in the majority of patients with familial, and up to 25% of patients with sporadic PVOD/PCH [14, 15]. In the present study, the presence of biallelic *EIF2AK4* mutations was associated with a poor prognosis, even in patients who have a clinical diagnosis of PAH, and who did not develop pulmonary oedema in response to pulmonary artery vasodilator therapies. Therefore, early identification of these patients through genetic testing may prompt early referral for lung transplantation similar to patients with clinically diagnosed PVOD/PCH [18].

The presence of biallelic *EIF2AK4* mutations in patients with a clinical diagnosis of PAH raises the question whether *EIF2AK4* mutations can cause classical idiopathic PAH, or whether there are cases of PVOD/PCH caused by *EIF2AK4* mutations that are wrongly classified even by expert centres. We further show that phenotypic, radiological and histological assessments can be difficult to interpret. The presence of subtle or infrequent features may lead to an incorrect diagnosis of PAH in patients with biallelic *EIF2AK4* mutations. This study suggests that patients with pathogenic biallelic *EIF2AK4* mutations may present with a spectrum of phenotypic, radiological and histological features that can overlap with PAH.

PAH patients with biallelic *EIF2AK4* mutations demonstrated a reduced KCO despite normal spirometry, which is characteristic of patients with PVOD/PCH. The reduced KCO likely reflects widespread reduction in alveolar gas exchange due to endothelial proliferation and patchy thickening of the blood gas barrier by the process of capillary haemangiomatosis. Ultrastructural thickening of the capillary basement membrane may also play a role [33]. In

keeping with previous reports in PVOD/PCH we also show that PAH patients with biallelic mutations in *EIF2AK4* are younger at diagnosis than patients with either *BMPR2* mutations or no known mutation [14, 20]. However, the presence of these characteristic features has a low positive predictive value for the identification of patients with biallelic *EIF2AK4* mutations.

In contrast to previous descriptions of patients with PVOD, none of the patients with clinically diagnosed PAH and biallelic *EIF2AK4* mutations developed pulmonary oedema in response to pulmonary artery vasodilator therapies. For example, intravenous prostanoids were used in 50% of these patients. In classical PVOD patients, pulmonary oedema with intravenous prostanoids has been reported in up to 44% of patients after a median treatment duration of just 9 days [4]. Presumably the extent and severity of the pulmonary venous involvement in these patients might underlie the differing responses to prostanoids.

It is generally considered that HRCT imaging is a useful non-invasive test to assist in the diagnosis of suspected PVOD/PCH [11]. Although there was an increased prevalence of mediastinal lymphadenopathy and interlobular septal thickening in PAH patients with biallelic *EIF2AK4* mutations, we found that radiological features at the time of diagnosis could not accurately determine the underlying genotype [6]. The differing radiological features of all patients with biallelic *EIF2AK4* mutations compared with PVOD cases without mutations is of interest. This may reflect differences between the younger onset genetic cases of PVOD, compared with the predominantly older group of patients without *EIF2AK4* mutations in whom other non-genetic factors, such as exposure to inorganic solvents, may play an important role [34].

Histological examination (usually post mortem or from explanted lungs) is often considered essential for diagnostic confirmation of PVOD/PCH but may be confounded by the heterogeneous nature of vascular pathology [35]. Surgical biopsy of the lung in patients with severe

PAH is contraindicated and a limitation of this study is that lung tissue from only one patient with biallelic *EIF2AK4* mutations was available for analysis. This patient had a rare and predicted deleterious homozygous missense mutation in *EIF2AK4.* The predominant feature on assessment of the explanted lung tissue was of pulmonary arteriopathy, as usually seen in PAH. Although only infrequent, fibrosis of the septal venules and the possible presence of siderophages in the alveolar space were observed. These features are found in patients with PVOD/PCH. This case supports the hypothesis that patients with biallelic *EIF2AK4* mutations may present with a spectrum of venous and arterial involvement.

There are increasing reports of phenotypic, radiological and histological similarities between PAH and PVOD/PCH [6, 12, 13]. Tenorio et al. reported a homozygous missense mutation in *EIF2AK4* in a large kindred of Iberian Romani with apparent heritable PAH [31]. This kindred is likely to have PVOD/PCH as these diagnoses were not confirmed histologically and PVOD was suspected in half the patients. More recently, Best et al. also report two sisters with apparent heritable PAH carrying biallelic *EIF2AK4* mutations [32]. These patients also had a reduced KCO but had not had HRCT assessment of their lung parenchyma which may have altered their clinical diagnosis. Taken together, these previous reports are compatible with the findings in this larger cohort, that patients with a clinical presentation of idiopathic or heritable PAH may in fact have underlying PVOD/PCH as determined by genetic analysis.

A strength of this study is the centralised reporting of radiographic features. However, the data collection was retrospective and incomplete in some cases. Assessing rare diseases, such as PAH and PVOD/PCH, with a prospective study recruiting incident cases would take a prohibitively long time. This is especially true when assessing survival and response to therapy. In this study including prevalent and retrospectively recruited patients, we demonstrated a worse

prognosis in patients with a clinical diagnosis of PAH and biallelic *EIF2AK4*. However, the inclusion of prevalent and retrospectively recruited patients can introduce bias such as immortal time bias, when there are long periods between diagnosis and enrolment in the study. The effect of immortal time bias and other confouders such as the inclusion of prevalent and incident cases can be difficult to predict. In all groups there are likely to be patients who died prior to study enrolment, and thus would not feature in any analysis. When we attempted to eliminate these sources of bias in a sensitivity analysis restricted to prospectively recruited patients from the UK, the study did not have sufficient power to show a difference in survival between different genotypes. Further studies of survival and response to therapy will be needed to definitively show whether "misclassified" PAH patients with biallelic *EIF2AK4* mutations have a similarly poor prognosis as classical PVOD patients with these mutations.

The genetic architecture of idiopathic and heritable PAH remains to be fully elucidated. Ongoing analysis of whole genome sequence data in our cohort is likely to reveal novel rare variation underlying this condition. Mutations in *BMPR2* account for approximately 17% of idiopathic PAH patients and other known PAH genes account for approximately 1-2% of all cases [21, 36]. In the present study *BMPR2* mutations were found in 11% of patients without a family history of PAH. It is worth noting that patients with the sporadic form of the disease with no reported family history represent a higher burden of *BMPR2* mutations (n=89) compared to those with a family history (n=49). This has important implications for clinical genetic testing in patients with sporadic as well as familial disease.

In previous studies mutations in both *EIF2AK4* alleles are required to cause PVOD and PCH [14, 15]. In autosomal recessive disorders, it is unusual for the heterozygous state to manifest the disease phenotype and thus heterozygous *EIF2AK4* variants would not be expected to be

pathogenic. In this study, we found a significant over-representation of heterozygous rare and predicted deleterious *EIF2AK4* variants in PAH compared to control subjects and report 2 patients with rare variants in both *BMPR2* and *EIF2AK4*. Recently, the possibility that heterozygous *EIF2AK4* variants influence the penetrance of *BMPR2* mutations has been raised in a single family with PAH [37]. Further studies are required to determine whether heterozygous *EIF2AK4* variants contribute to aetiology in PAH.

In summary, we demonstrate that biallelic *EIF2AK4* mutations are found in patients diagnosed clinically with idiopathic and familial PAH. These patients may have subtle features suggestive of PVOD/PCH on close inspection and are likely to have underlying PVOD/PCH. The spectrum of phenotypic, radiological and histological features found in patients with biallelic *EIF2AK4* mutations made by current clinical assessments is wider and less clear cut than previously recognised. This may lead to misclassification of patients as PAH rather than PVOD and hinders accurate risk stratification. Ascertaining the *EIF2AK4* mutation status of patients through clinical genetic testing provides additional information to aid risk stratification and guide management. In a young patient presenting with apparent PAH, the presence of a low KCO with normal spirometry strongly suggests the presence of underlying biallelic *EIF2AK4* mutations. Patients with an apparent clinical diagnosis of PAH and biallelic *EIF2AK4* mutations have a worse prognosis compared to patients with *BMPR2* mutations and those without these mutations. Clinical genetic testing should aid identification of this high-risk group and facilitate early referral for lung transplantation and appropriate management.

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Table 1. Phenotypic summary of *EIF2AK4* variant carriers. Patients with a clinical diagnosis of PAH and biallelic *EIF2AK4* mutations are younger at diagnosis and have a significantly reduced KCO compared to other groups.

	PAH patients with *BMPR2* mutations *	**PAH patients with no mutations in PAH associated genes**	**PAH patients with *EIF2AK4* heterozygous variants**	**PAH patients with biallelic *EIF2AK4* mutations**	**PVOD/PCH patients**	**p**
n	130	704	8	9	16	
Age (years)	39 [31 - 52]	51 [37 - 65]	49 [36 - 67]	29 [23 - 38]	57 [41 - 69]	<0.001
Gender (n female [%])	85 [65.4%]	494 [70.2%]	7 [87.5%]	4 [44.4%]	9 [56.2%]	0.18
Ethnicity (n white Caucasian [%])	108 [83.1%]	551 [78.5%]	5 [62.5%]	2 [22.2%]	13 [81.2%]	0.002
Digital clubbing (n [%])	6 [9.7%]	10 [3.4%]	0 [0%]	3 [42.9%]	1 [11.1%]	0.002
BMI	28 [24 - 33]	28 [24 - 33]	26 [23 - 28]	24 [20 - 27]	27 [24 - 31]	0.216
mPAP (mmHg)	57 [51 - 69]	52 [44 - 61]	44 [42 - 52]	52 [46 - 65]	48 [40 - 58]	<0.001
CO (L/min)	3 [3 - 4]	4 [3 - 5]	3 [3 - 5]	5 [3 - 6]	4 [3 - 4]	<0.001
PVR (WU)	15 [11 - 20]	10 [7 - 14]	9 [6 - 10]	9 [8 - 13]	10 [9 - 12]	<0.001
Vasoresponders (n [%])	0 [0%]	28 [17.5%]	0 [0%]	0 [0%]	0 [0%]	0.011
FEV_1 (%pred)	90 [78 - 99]	84 [72 - 95]	83 [71 - 94]	94 [85 - 100]	85 [70 - 95]	0.031
FVC (%pred)	97 [86 - 109]	95 [82 - 106]	96 [75 - 98]	100 [86 - 119]	97 [81 - 103]	0.310
KCO (%pred)	81 [73 - 92]	71 [51 - 85]	81 [72 - 95]	33 [30 - 35]	37 [32 - 47]	<0.001
Resting S_AO_2 (%)	96 [94 - 97]	96 [93 - 97]	98 [98 - 98]	91 [90 - 94]	94 [91 - 95]	0.010
S_AO_2 post walk test (%)	94 [90 - 97]	92 [85 - 96]	94 [84 - 96]	78 [75 - 82]	88 [85 - 89]	<0.001

BMI - body mass index, mPAP - mean pulmonary artery pressure, PVR - pulmonary vascular resistance, FEV1 - forced expiratory volume in 1 second, FVC - forced vital capacity, KCO - transfer coefficient for carbon monoxide. * Also includes the 2 patients with a heterozygous EIF2AK4 variant and a BMPR2 variant. Data presented as median [IQR] unless indicated. Percentages were calculated using the number of patients for whom data were available as the denominator.

Table 2. Radiological features and consensus radiological diagnosis of PAH patients in the CT substudy

	Group	PAH patients with *BMPR2* mutations	PAH patients with no mutations in the previously reported PAH genes	PAH patients with heterozygous *EIF2AK4* variants	PAH patients with biallelic *EIF2AK4* mutations	PVOD	p
	n	21	21	4	7	14	
Centrilobular ground glass opacification density	None	7 [33.3%]	13 [61.9%]	2 [50.0%]	1 [14.3%]	7 [50.0%]	0.122
	Subtle	12 [57.1%]	5 [23.8%]	0 [0.0%]	2 [28.6%]	3 [21.4%]	
	Present	2 [9.5%]	3 [14.3%]	2 [50.0%]	4 [57.1%]	4 [28.6%]	
Centrilobular ground glass opacification extent	None	8 [38.1%]	13 [61.9%]	2 [50.0%]	1 [4.3%]	8 [57.1%]	0.077
	<5%	0 [0.0%]	3 [14.3%]	0 [0.0%]	1 [14.3%]	1 [7.1%]	
	5-25%	2 [9.5%]	0 [0.0%]	1 [25.0%]	2 [28.6%]	1 [7.1%]	
	25-50%	2 [9.5%]	4 [19.0%]	0 [0.0%]	0 [0.0%]	2 [14.3%]	
	50-75%	5 [23.8%]	1 [4.8%]	0 [0.0%]	2 [28.6%]	0 [0.0%]	
	75-100%	4 [19.0%]	0 [0.0%]	1 [25.0%]	1 [14.3%]	2 [14.3%]	
Interlobular septal thickening	None	17 [81.0%]	18 [85.7%]	4 [100.0%]	5 [71.4%]	4 [28.6%]	0.001
	Subtle	3 [14.3%]	2 [9.5%]	0 [0.0%]	0 [0.0%]	1 [7.1%]	
	Present	1 [4.8%]	1 [4.8%]	0 [0.0%]	2 [28.6%]	9 [64.3%]	
Mediastinal lymphadenopathy	None	19 [90.5%]	21 [100.0%]	4 [100.0%]	3 [42.9%]	3 [21.4%]	<0.001
	Present	2 [9.5%]	0 [0.0%]	0 [0.0%]	4 [57.1%]	11 [78.6%]	
Pleural effusion	None	17 [81.0%]	21 [100.0%]	3 [75.0%]	7 [100.0%]	10 [71.4%]	0.048
	Small	4 [19.0%]	0 [0.0%]	1 [25.0%]	9 [0.0%]	4 [28.6%]	
Neovascularity	None	12 [57.1%]	18 [85.7%]	4 [100.0%]	6 [85.7%]	13 [92.9%]	0.077
	Present	9 [42.9%]	3 [14.3%]	0 [0.0%]	1 [14.3%]	1 [7.1%]	
CT diagnosis	PAH	20 [95.2%]	18 [85.7%]	3 [75.0%]	3 [42.9%]	4 [28.6%]	
	Possible PVOD/PCH	1 [4.8%]	3 [14.3%]	1 [25.0%]	4 [57.1%]	10 [71.4%]	

Data presented as n [%].

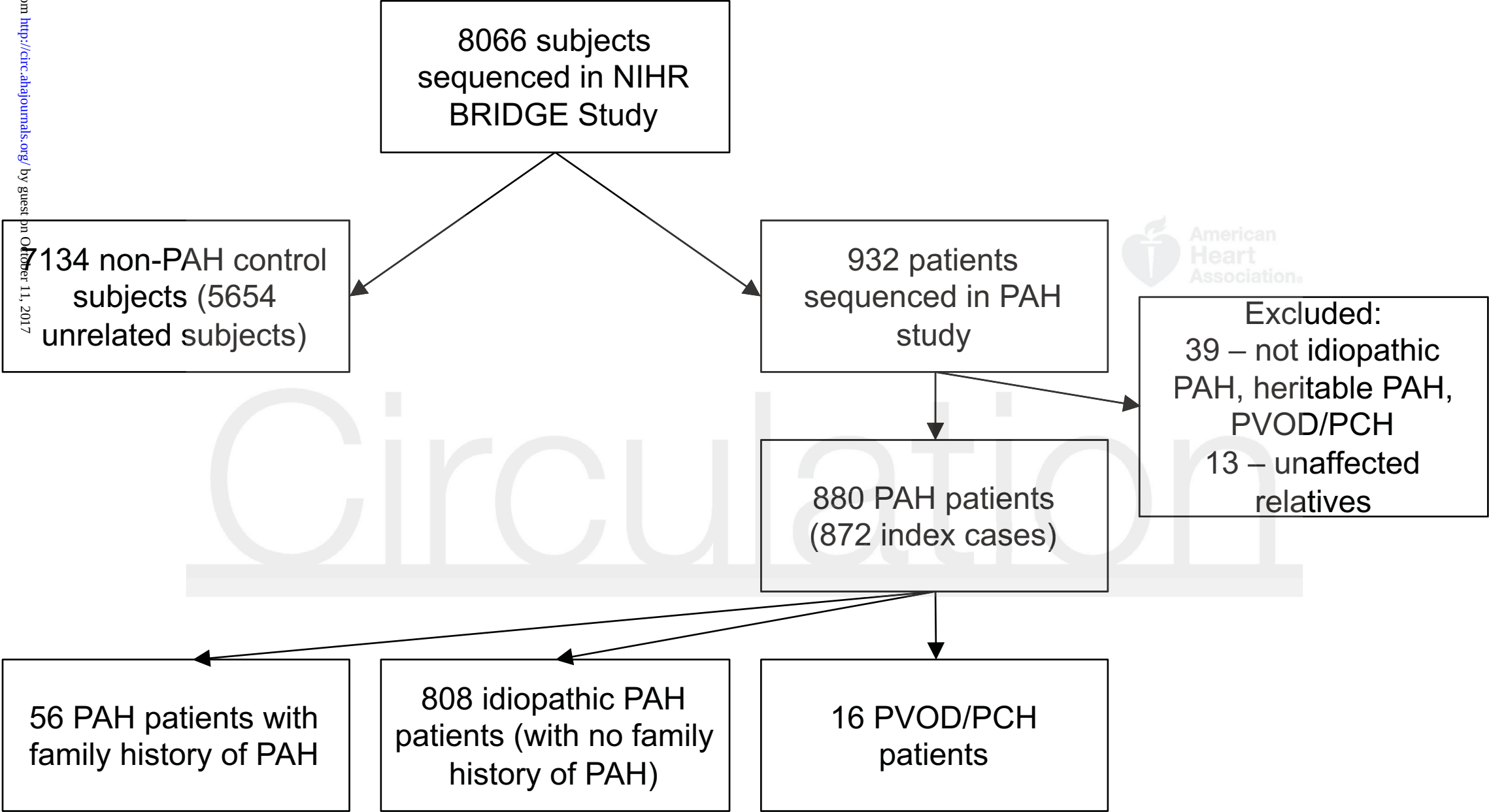
Figure Legends

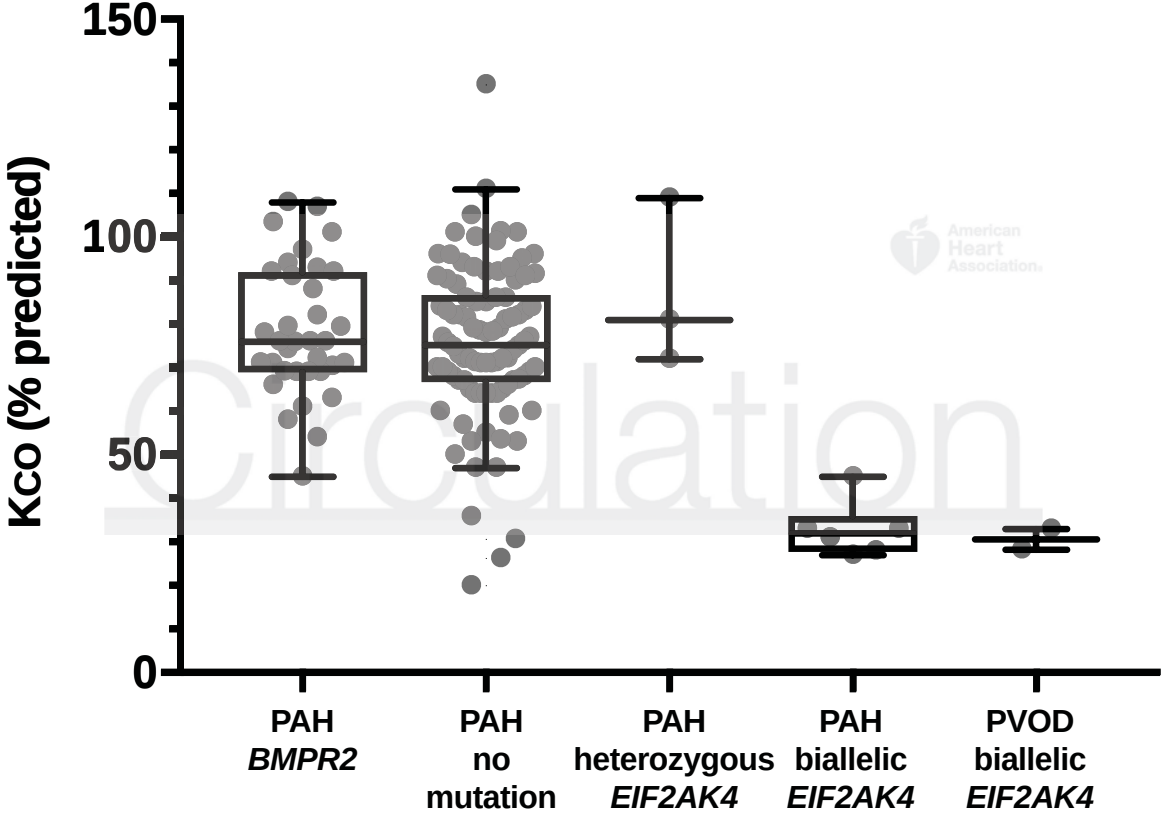
Figure 1. Subjects recruited to the NIHR BioResource – Rare Diseases Study and the clinical diagnostic categories of PAH patients included in this study.

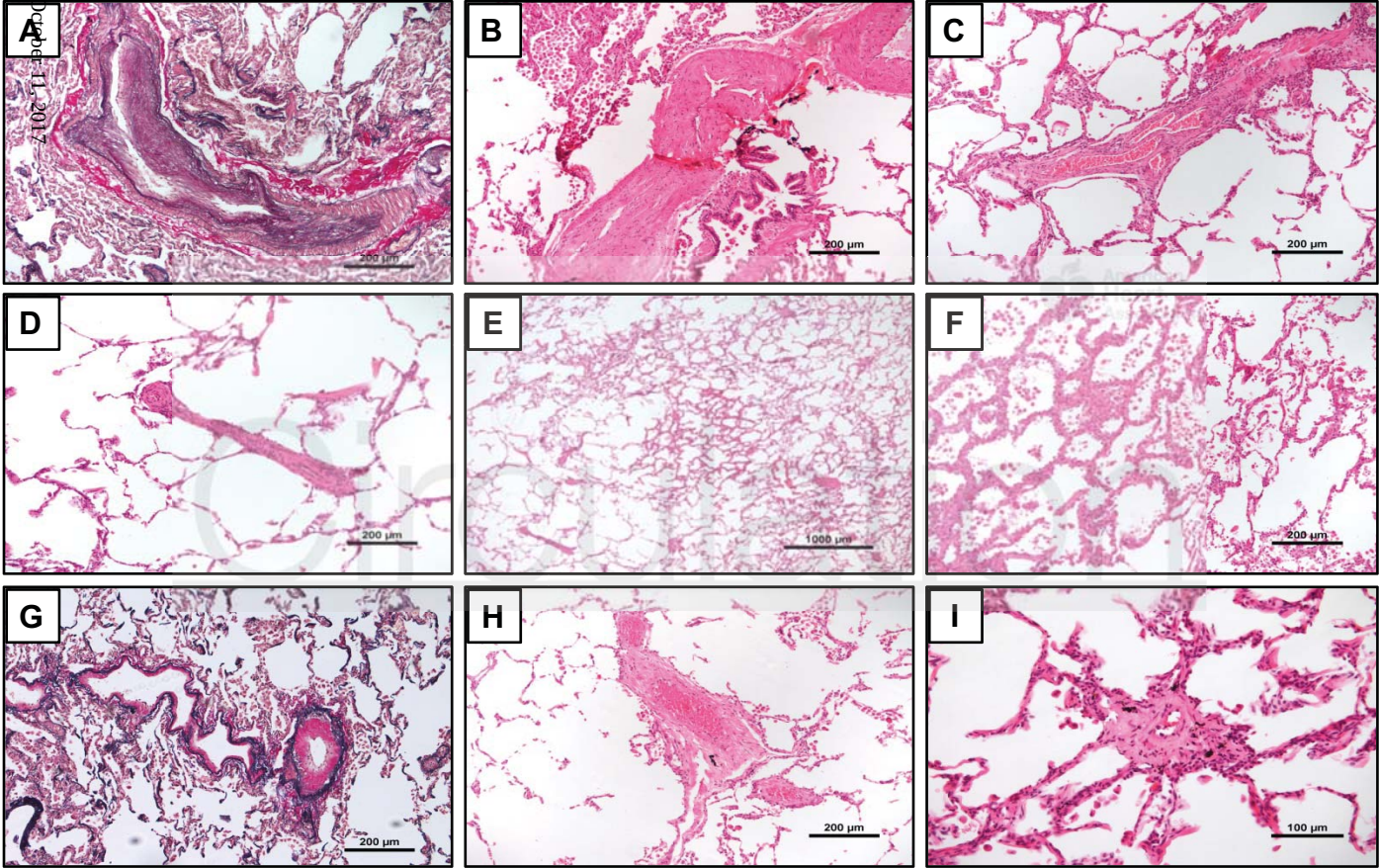
Figure 2. The transfer coefficient for carbon monoxide (KCO) is influenced by genotype in pulmonary arterial hypertension. Patients with FEV_1 < 80 % predicted and FVC < 80 % predicted and diagnosed with PAH or PVOD/PCH after 50 years of age excluded from the plot.

Figure 3. Representative histopathological images from one patient with clinically diagnosed idiopathic PAH but found to have a rare (not reported in the ExAC database) and predicted deleterious (CADD score 32) homozygous *EIF2AK4* missense variant (c.1795G>C). The patient was of Pakistani origin and did not have a family history of PAH or PVOD. At presentation, he was 22 years old and had a reduced KCO (31% predicted) despite preserved spirometry. HRCT of his chest showed subtle but extensive (50-75% involvement) ground glass opacification. No interlobular septal thickening or mediastinal lymphadenopathy was observed. No suspicion of PVOD/PCH was raised based on radiological appearances. Histopathology was reviewed by two independent pathologists each confirming the predominant histological pattern to be one of pulmonary arterial vasculopathy. The pulmonary arteries showed eccentric and concentric intimal fibrosis and medial hypertrophy (A, B) as well as some lesions with features of recanalised thrombus (C). Several concentrically muscularised arterioles were also observed (D). No complex plexiform lesions were present. There was patchy thickening of the alveolar septa with capillary congestion and pigmented intra-alveolar

macrophages similar to PCH (E, F). Venous remodelling was difficult to trace and infrequent, but present. Fibrous thickening of the intima in septal veins (G, I) and a micro-vessel (H).



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Phenotypic Characterisation of EIF2AK4 Mutation Carriers in a Large Cohort of Patients Diagnosed Clinically with Pulmonary Arterial Hypertension

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The NIHR BioResource - Rare Diseases Consortium & UK National Cohort Study of Idiopathic and Heritable PAH

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APPENDIX

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SUPPLEMENTAL MATERIAL:

Phenotypic characterisation of *EIF2AK4* mutation carriers in a large cohort of patients diagnosed clinically with pulmonary arterial hypertension

Hadinnapola et al.

Supplemental Methods:

Whole genome sequencing

Genomic DNA was extracted from whole blood samples prior to assessment of concentration by Qubit, and quality by gel electrophoresis. After fragmentation of DNA into 200bp fragments (Covaris E220, Covaris Inc, Woburn, USA) DNA libraries were created using Tru SeqDNA LT Prep kit (Illumina Inc, San Diego, USA). The libraries underwent next generation sequencing using 100-150 base pair paired-end sequencing using Illumina HiSeq 2500 and HiSeq X (Illumina Inc, San Diego, USA).

Variant calling

Reads were aligned against the Genome Reference Consortium human genome (build 37) (GRCh37) and variants were called using the Issac Aligner and Variant Caller respectively (version 2, Illumina Inc.). Genebuilds for *BMPR2* and *EIF2AK4* genes were based on Ensembl v75. Variants from these genes were extracted and annotated using Ensembl's Variant Effect Predictor (VEP) v84 [1]. VEP was also used to annotate data from the Exome Aggregation Consortium's (ExAC) database [2].

Deletions (resulting in the loss of more than 50bp) were identified by applying Isaac copy number variant caller (Canvas, Illumina) and Isaac Structural Variant Caller (Manta, Illumina).

To be called by both Canvas and Manta deletions required a reciprocal overlap of ≥ 20%. Overlapping deletions represented in the Zarrei dataset with a reciprocal overlap of ≥ 50% and deletions with a non-PAH BRIDGE control frequency of more than 1 in 1,000 were excluded [3].

Analysis of computed tomographic images of the chest

CT images of the chest, where available, were reviewed independently by 2 cardiothoracic radiologists (AS and NS), with specialist imaging experience in pulmonary hypertension, blinded to the underlying diagnoses using a customised proforma (Supplemental Table 4). In addition to CT scans of patients with *EIF2AK4* mutations or with a clinical diagnosis of PVOD in the cohort, CT scans of patients from Papworth Hospital and the Royal Hallamshire Hospital with normal spirometry (FEV_1 > 80% predicted and FVC > 80% predicted) and either *BMPR2* mutations (n=21) or no variants in the known PAH genes (n=21) were analysed (Supplemental Table 5). A consensus read was undertaken for individual CT features and a mutually agreed overall radiological diagnosis was recorded.

Histology

The explanted lung tissue of one patient with a clinical diagnosis of idiopathic PAH and biallelic *EIF2AK4* mutations was available for further analysis. Four micrometre (µm) tissue sections were cut from formalin-fixed paraffin wax embedded blocks from the explanted lung tissue. Representative sections from each lobe of both lungs were stained with Elastic-Van Gieson and Haemotoxylin and Eosin stains. Two expert histopathologists examined the sections independently by light microscopy.

Statistical analysis

Statistical analysis was performed in R (www.r-project.org).

Differences between groups of categorical variables were assessed using the Fisher Exact test. Where one of the variables was an ordinal the Cochran-Armitage test was applied using the chisq_test function from the "coin" package [4]. Differences in continuous variables were assessed using the Mann–Whitney U test (2 comparator groups) and the Kruskal-Wallis test (3 or more comparator groups). Post-hoc pairwise comparisons were performed using Dunn's Test for multiple testing.

Semi-parametric Cox-proportional hazards models were used to assess survival between groups using the "survival" package in R [5]. Survival time from diagnosis to death and diagnosis to death or transplantation was assessed. Patients were censored at the date of transplantation for the primary survival analysis. Age at diagnosis and gender were used as covariates in the models.

The proportional hazards assumptions were tested by assessing Schoenfeld residuals over log time [6]. The goodness of fit of the model was assessed by plotting the log of cumulative hazard of Cox-Snell residuals against the log of time and confirming the simple regression has 0 intercept and slope of 1 [7].

The inclusion of retrospectively recruited and prevalent patients in a survival analysis assessing time from diagnosis to death/transplantation can cause immortal time bias. The immortal time is the period between diagnosis and enrolment in the study and so patients

had to have survived till this point. Patients with worse prognosis diagnosed at a similar time may not have survived long enough to enrol in the study. To further explore this potential bias, a sensitivity analysis was performed including only on UK patients recruited prospectively to the study. In this multivariate Cox-proportional hazards model, the survival period was defined as the time period from date of diagnosis to date of death and patients only entered the risk set after enrolment into the study (consent date).

Supplemental Tables

Supplemental Table 1. NIHR BioResource – Rare Diseases Collaboration. See spreadsheet.

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Supplemental Table 2. Specialist pulmonary hypertension centres participating in the study

Recruiting cohorts	n
Genomics England	1965
Specialist Pathology: Evaluating Exomes in Diagnostics	1356
Primary Immune Disorders	1299
Bleeding and Platelet Disorders	1004
Pulmonary Arterial Hypertension	932
Multiple Primary Malignant Tumours	376
Hypertrophic Cardiomyopathy	187
Cerebral Small Vessel Diseases	183
Steroid Resistant Nephrotic Syndrome	161
Intrahepatic Cholestasis of Pregnancy	140
Stem Cell & Myeloid Disorders	132
Primary Membranoproliferative Glomerulonephritis	128
Neuropathic Pain Disorder	114
Leber Hereditary Optic Neuropathy	59
Control	15
Ehlers-Danlos Syndromes	15

Supplemental Table 3. NIHR BioResource - Rare Diseases Study recruiting cohorts and GEL

Parameter	Response
ID	
Date of birth	
Unenhanced CT	(Y/N)
CTPA	(Y/N)
HRCT	(Y/N)
Expiratory CT	(Y/N)
Pulmonary artery diameter (cm)	
Aorta diameter (cm)	
Ground glass opacification centrilobular pattern DENSITY	(None / Subtle / Present)
Ground glass centrilobular pattern EXTENT	(0, <5%, 5-25, 25-50, >50)
Ground glass DISTRIBUTION	(central (C)/peripheral (P)/zonal (Z) or diffuse (D))
Non-specific mosaic pattern / GGO	
Neovascularity vessels	(Y/N)
Arterio-venous malformations	(Y/N)
Bronchial arteries	(Y/N)
Largest bronchial artery size	
Interlobular septal thickening	(None, Subtle, Present)
Mediastinal lymphadenopathy	(Y/N)
Emphysema	(Y/N) and % of parenchyma involved
Fibrosis	(Y/N) and % of parenchyma involved
Pleural effusion	(Y/N)
Air trapping	(Y/N)
Comments	
Likely diagnosis	Any suspicion of PVOD or PCH / PAH

Supplemental Table 4. Proforma used in analysis of CT scans

Group	n
PAH patients with *BMPR2* variants	21
PAH patients with biallelic *EIF2AK4* variants	7
PVOD patients	14
PAH patients with heterozygous *EIF2AK4* variants	4
PAH patients with no variants in the previously reported PAH genes	21

Supplemental Table 5. CT scans of patients with PVOD and patients with PAH carrying biallelic *EIF2AK4* mutations were reassessed by radiologists blinded to the diagnosis. For comparison CT scans of PAH patients with normal spirometry (FEV_1 > 80 % predicted and FVC > 80 % predicted) who either had no mutations in the previously reported PAH genes or carried *BMPR2* mutations were assessed.

Supplemental Table 6. Page 1/9

Project	HGVSc	Consequence	HGVSp	Allele count PAH patients	Allele count non-PAH BRIDGE controls	ExAC MAF	PolyPhen-2	SIFT	CADD Phred Score	*EIF2AK4* genotype
BRIDGE control	c.292C>G	missense variant	p.L98V	0	1	0.00001656	probably damaging (0.999)	deleterious (0)	25.7	Heterozygous variant
BRIDGE control	c.354_355delTG	frameshift variant	p.C118Wfs*7	0	2	Not found in ExAC			35	Heterozygous variant
BRIDGE control	c.745C>T	stop gained & splice region variant	p.R249*	0	1	0.00007451			39	Heterozygous variant
BRIDGE control	c.746G>A	missense variant & splice region variant	p.R249Q	0	1	2.48E-05	probably damaging (0.999)	deleterious (0.02)	34	Heterozygous variant
BRIDGE control	c.767G>T	missense variant	p.C256F	0	1	1.66E-05	possibly damaging (0.904)	deleterious (0.02)	28.4	Heterozygous variant
BRIDGE control	c.985G>A	missense variant	p.E329K	0	1	Not found in ExAC	probably damaging (0.981)	deleterious (0.01)	34	Heterozygous variant
BRIDGE control	c.1153dupG	frameshift variant	p.V385Gfs*30	0	1	0.00003308			32	Heterozygous variant
BRIDGE control	c.1190T>A	missense variant	p.I397N	0	1	Not found in ExAC	possibly damaging (0.67)	deleterious (0)	32	Heterozygous variant

Supplemental Table 6. Summary of rare (MAF < 0.0001) and predicted deleterious (CADD score > 15 and not benign by both PolyPhen-2 and SIFT) *EIF2AK4* variants in NIHR BRIDGE Study. Transcript: ENST00000263791.5. *EIF2AK4* variants are not shared between PAH patients and controls. Biallelic *EIF2AK4* variants are seen only in PAH cases.

Bold - variants identified in more than one patient in the PAH Cohort. MAF - minor allele frequency

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Project	HGVSc	Consequence	HGVSp	Allele count PAH patients	Allele count non-PAH BRIDGE controls	ExAC MAF	PolyPhen-2	SIFT	CADD Phred Score	*EIF2AK4* genotype
BRIDGE control	c.1215C>G	stop gained	p.Y405*	0	2	Not found in ExAC			29.4	Heterozygous variant
BRIDGE control	c.1331A>G	missense variant	p.Y444C	0	1	Not found in ExAC	probably damaging (1)	deleterious (0)	28.7	Heterozygous variant
BRIDGE control	c.1345C>T	missense variant	p.R449C	0	1	0.00001654	probably damaging (1)	deleterious (0)	35	Heterozygous variant
BRIDGE control	c.2249T>A	missense variant & splice region variant	p.L750Q	0	1	Not found in ExAC	probably damaging (1)	deleterious (0)	28	Heterozygous variant
BRIDGE control	c.2298delG	frameshift variant	p.N767Tfs*24	0	1	Not found in ExAC			28.3	Heterozygous variant
BRIDGE control	c.2720A>T	missense variant	p.Y907F	0	4	1.66E-05	probably damaging (1)	deleterious (0)	31	Heterozygous variant
BRIDGE control	c.2828C>T	missense variant	p.T943M	0	1	0.00003311	probably damaging (1)	deleterious (0)	34	Heterozygous variant
BRIDGE control	c.3104_3106delTCT	inframe deletion	p.F1035del	0	1	Not found in ExAC			22	Heterozygous variant

Supplemental Table 6. Summary of rare (ExAC MAF <0.0001) and predicted deleterious (CADD score >15 and not benign by both PolyPhen-2 and SIFT) *EIF2AK4* variants in NIHR BRIDGE Study. Transcript: ENST00000263791.5. *EIF2AK4* variants are not shared between PAH patients and controls. Biallelic *EIF2AK4* variants are seen only in PAH cases.

Bold - variants identified in more than one patient in the PAH Cohort. MAF - minor allele frequency

Supplemental Table 6. Page 3/9										
Project	**HGVSc**	**Consequence**	**HGVSp**	**Allele count PAH patients**	**Allele count non-PAH BRIDGE controls**	**ExAC MAF**	**PolyPhen-2**	**SIFT**	**CADD Phred Score**	***EIF2AK4* genotype**
BRIDGE control	c.3217C>T	missense variant	p.R1073C	0	1	0.0000166	probably damaging (1)	deleterious (0)	35	Heterozygous variant
BRIDGE control	c.3223T>G	missense variant	p.F1075V	0	1	0.0000083	probably damaging (0.997)	deleterious (0)	32	Heterozygous variant
BRIDGE control	c.3344C>T	missense variant	p.P1115L	0	1	8.26E-06	probably damaging (1)	deleterious (0)	35	Heterozygous variant
BRIDGE control	c.3358-3C>T	splice region variant & intron variant	p.NA	0	1	Not found in ExAC			17.15	Heterozygous variant
BRIDGE control	c.3406C>T	stop gained & splice region variant	p.R1136*	0	1	Not found in ExAC			40	Heterozygous variant
BRIDGE control	c.3430A>T	missense variant	p.R1144W	0	1	0.0000248	probably damaging (1)	deleterious (0)	33	Heterozygous variant
BRIDGE control	c.3986T>C	missense variant	p.F1329S	0	1	Not found in ExAC	probably damaging (1)	deleterious (0)	33	Heterozygous variant

Supplemental Table 6. Summary of rare (ExAC MAF <0.0001) and predicted deleterious (CADD score >15 and not benign by both PolyPhen-2 and SIFT) *EIF2AK4* variants in NIHR BRIDGE Study. Transcript: ENST00000263791.5. *EIF2AK4* variants are not shared between PAH patients and controls. Biallelic *EIF2AK4* variants are seen only in PAH cases.

Bold - variants identified in more than one patient in the PAH Cohort. MAF - minor allele frequency

Supplemental Table 6. Page 4/9										
Project	**HGVSc**	**Consequence**	**HGVSp**	**Allele count PAH patients**	**Allele count non-PAH BRIDGE controls**	**ExAC MAF**	**PolyPhen-2**	**SIFT**	**CADD Phred Score**	***EIF2AK4* genotype**
BRIDGE control	c.3992T>C	missense variant	p.F1331S	0	1	8.28E-06	possibly damaging (0.872)	deleterious (0.01)	28.4	Heterozygous variant
BRIDGE control	c.4039G>A	missense variant	p.A1347T	0	1	8.28E-05	probably damaging (1)	deleterious (0)	34	Heterozygous variant
BRIDGE control	c.4388_4389+12 delAGGTAAAGAC GTCA	splice donor variant & coding sequence variant & intron variant	p.NA	0	1	Not found in ExAC			36	Heterozygous variant
BRIDGE control	c.4397C>A	missense variant	p.S1466Y	0	2	Not found in ExAC	probably damaging (0.988)	deleterious (0)	33	Heterozygous variant
BRIDGE control	c.4729G>A	missense variant & splice region variant	p.V1577M	0	1	Not found in ExAC	probably damaging (0.999)	deleterious (0)	29.6	Heterozygous variant
BRIDGE control	c.4751dupT	frameshift variant	p.L1585Ifs*11	0	1	Not found in ExAC			34	Heterozygous variant
BRIDGE control	c.4920_4931delT AGAGATGACTA	inframe deletion	p.R1641_Y1644 del	0	1	Not found in ExAC			23	Heterozygous variant

Supplemental Table 6. Summary of rare (ExAC MAF <0.0001) and predicted deleterious (CADD score >15 and not benign by both PolyPhen-2 and SIFT) *EIF2AK4* variants in NIHR BRIDGE Study. Transcript: ENST00000263791.5. *EIF2AK4* variants are not shared between PAH patients and controls. Biallelic *EIF2AK4* variants are seen only in PAH cases.

Bold - variants identified in more than one patient in the PAH Cohort. MAF - minor allele frequency

Supplemental Table 6. Page 5/9										
Project	**HGVSc**	**Consequence**	**HGVSp**	**Allele count PAH patients**	**Allele count non-PAH BRIDGE controls**	**ExAC MAF**	**PolyPhen-2**	**SIFT**	**CADD Phred Score**	***EIF2AK4* genotype**
PAH	c.44C>T	missense variant	p.P15L	1	0	8.32E-06	unknown (0)	deleterious low confidence (0.03)	23.5	Heterozygous variant
PAH	c.220G>A	missense variant	p.D74N	1	0	1.66E-05	possibly damaging (0.954)	deleterious (0)	32	Heterozygous variant
PAH	c.1072_1073dupGT	frameshift variant	p.V359*	1	0	Not found in ExAC			32	Heterozygous variant
PAH	c.1660G>T	missense variant & splice region variant	p.D554Y	1	0	Not found in ExAC	probably damaging (0.966)	deleterious (0)	28	Heterozygous variant
PAH	c.2446C>T	stop gained	p.Q816*	1	0	Not found in ExAC			41	Heterozygous variant
PAH	c.2516T>C	missense variant	p.I839T	1	0	Not found in ExAC	probably damaging (1)	deleterious (0)	28.9	Heterozygous variant
PAH	c.3218G>T	missense variant	p.R1073L	1	0	Not found in ExAC	probably damaging (0.995)	deleterious (0.01)	35	Heterozygous variant
PAH	c.3604C>T	missense variant	p.H1202Y	1	0	Not found in ExAC	probably damaging (1)	deleterious (0)	29.7	Heterozygous variant

Supplemental Table 6. Summary of rare (ExAC MAF <0.0001) and predicted deleterious (CADD score >15 and not benign by both PolyPhen-2 and SIFT) *EIF2AK4* variants in NIHR BRIDGE Study. Transcript: ENST00000263791.5. *EIF2AK4* variants are not shared between PAH patients and controls. Biallelic *EIF2AK4* variants are seen only in PAH cases.

Bold - variants identified in more than one patient in the PAH Cohort. MAF - minor allele frequency

Supplemental Table 6. Page 6/9

Project	HGVSc	Consequence	HGVSp	Allele count PAH patients	Allele count non-PAH BRIDGE controls	ExAC MAF	PolyPhen-2	SIFT	CADD Phred Score	*EIF2AK4* genotype
PAH	c.3711_3713delGAG	inframe deletion	p.R1238del	1	0	0.0000083			21.6	Heterozygous variant
PAH	c.3722A>G	missense variant	p.E1241G	1	0	Not found in ExAC	probably damaging (0.971)	deleterious (0)	27.2	Heterozygous variant
PAH	c.4646G>A	missense variant	p.R1549H	1	0	0.0000910	probably damaging (0.998)	deleterious (0.01)	35	Heterozygous variant
PAH	c.145-2A>G	splice acceptor variant	p.NA	1	0	Not found in ExAC			23.9	Additional second (likely trans) variant identified
PAH	c.257+4A>C	splice region variant & intron variant	p.NA	1	0	8.28E-06			15.5	Additional second (likely trans) variant identified
PAH	c.1392delT	frameshift variant	p.R465Vfs*38	1	0	2.48E-05			35	Additional second (likely trans) variant identified
PAH	c.1739dupA	frameshift variant	p.R581Efs*9	1	0	Not found in ExAC			35	Additional second (likely trans) variant identified

Supplemental Table 6. Summary of rare (ExAC MAF <0.0001) and predicted deleterious (CADD score >15 and not benign by both PolyPhen-2 and SIFT) *EIF2AK4* variants in NIHR BRIDGE Study. Transcript: ENST00000263791.5. *EIF2AK4* variants are not shared between PAH patients and controls. Biallelic *EIF2AK4* variants are seen only in PAH cases.

Bold - variants identified in more than one patient in the PAH Cohort. MAF - minor allele frequency

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Project	HGVSc	Consequence	HGVSp	Allele count PAH patients	Allele count non-PAH BRIDGE controls	ExAC MAF	PolyPhen-2	SIFT	CADD Phred Score	*EIF2AK4* genotype
PAH	c.1820T>G	missense variant & splice region variant	p.V607G	1	0	Not found in ExAC	probably damaging (1)	deleterious (0)	27.3	Additional second (likely trans) variant identified
PAH	c.2727C>G	missense variant	p.S909R	1	0	Not found in ExAC	probably damaging (1)	deleterious (0)	33	Additional second (likely trans) variant identified
PAH	c.2827A>G	missense variant	p.T943A	1	0	Not found in ExAC	probably damaging (1)	deleterious (0)	26.4	Additional second (likely trans) variant identified
PAH	c.2841delG	frameshift variant	p.I948Sfs*35	1	0	Not found in ExAC			35	Additional second (likely trans) variant identified
PAH	c.3055_3064delCTGACCAACG	frameshift variant	p.L1019Wfs*9	1	0	Not found in ExAC			36	Additional second (likely trans) variant identified
PAH	**c.3097C>T**	**stop gained**	**p.Q1033***	**3**	**0**	**8.24E-06**			**45**	**Additional second (likely trans) variant identified**

Supplemental Table 6. Summary of rare (ExAC MAF <0.0001) and predicted deleterious (CADD score >15 and not benign by both PolyPhen-2 and SIFT) *EIF2AK4* variants in NIHR BRIDGE Study. Transcript: ENST00000263791.5. *EIF2AK4* variants are not shared between PAH patients and controls. Biallelic *EIF2AK4* variants are seen only in PAH cases.

Bold - variants identified in more than one patient in the PAH Cohort. MAF - minor allele frequency

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Project	HGVSc	Consequence	HGVSp	Allele count PAH patients	Allele count non-PAH BRIDGE controls	ExAC MAF	PolyPhen-2	SIFT	CADD Phred Score	*EIF2AK4* genotype
PAH	c.3325G>A	missense variant	p.G1109R	1	0	0.0000082	probably damaging (1)	deleterious (0.02)	35	Additional second (likely trans) variant identified
PAH	c.3884T>G	missense variant	p.L1295R	1	0	Not found in ExAC	probably damaging (1)	deleterious (0)	32	Additional second (likely trans) variant identified
PAH	c.4400dupT	frameshift variant	p.E1468Rfs*14	1	0	Not found in ExAC			36	Additional second (likely trans) variant identified
PAH	c.4418_4421delCAGA	frameshift variant	p.T1473Rfs*17	1	0	0.0000083			36	Additional second (likely trans) variant identified
PAH	c.4769delT	frameshift variant	p.L1590*	1	0	0.0000083			33	Additional second (likely trans) variant identified
PAH	c.281dupA	frameshift variant	p.N94Lfs*8	2	0	Not found in ExAC			35	Homozygous variant
PAH	c.1159_1160delCT	frameshift variant	p.L387Cfs*27	2	0	Not found in ExAC			29.6	Homozygous variant

Supplemental Table 6. Summary of rare (ExAC MAF <0.0001) and predicted deleterious (CADD score >15 and not benign by both PolyPhen-2 and SIFT) *EIF2AK4* variants in NIHR BRIDGE Study. Transcript: ENST00000263791.5. *EIF2AK4* variants are not shared between PAH patients and controls. Biallelic *EIF2AK4* variants are seen only in PAH cases.

Bold - variants identified in more than one patient in the PAH Cohort. MAF - minor allele frequency

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Project	HGVSc	Consequence	HGVSp	Allele count PAH patients	Allele count non-PAH BRIDGE controls	ExAC MAF	PolyPhen-2	SIFT	CADD Phred Score	*EIF2AK4* genotype
PAH	**c.1795G>C**	**missense variant**	**p.G599R**	**4**	**0**	**Not found in ExAC**	**probably damaging (1)**	**deleterious (0)**	**32**	**Homozygous variant**
PAH	**c.3097C>T**	**stop gained**	**p.Q1033***	**3**	**0**	**8.24E-06**			**45**	**Homozygous variant**
PAH	c.3605A>T	missense variant	p.H1202L	2	0	Not found in ExAC	probably damaging (1)	deleterious (0)	31	Homozygous variant
PAH	c.4392dupT	frameshift variant & splice region variant	p.K1465*	2	0	Not found in ExAC			35	Homozygous variant

Supplemental Table 6. Summary of rare (ExAC MAF <0.0001) and predicted deleterious (CADD score >15 and not benign by both PolyPhen-2 and SIFT) *EIF2AK4* variants in NIHR BRIDGE Study. Transcript: ENST00000263791.5. *EIF2AK4* variants are not shared between PAH patients and controls. Biallelic *EIF2AK4* variants are seen only in PAH cases.

Bold - variants identified in more than one patient in the PAH Cohort. MAF - minor allele frequency

Supplemental Table 7. Page 1/4

Age (years)	Gender	Ethnicity	*EIF2AK4* variant HGVSc	Consequence type	*EIF2AK4* genotype	*BMPR2* mutation	Non-protein coding *EIF2AK4* variant	mPAP (mmHg)	Cardiac output (L/min)	FC	FEV_1 (% pred)	FVC (% pred)	KCO (% pred)	Digital clubbing	CT diagnosis	Family history PAH	Pulmonary artery vasodilator therapy	Pulmonary oedema with treatment	Histology assessed
23	M	British	c.3884T>G	missense variant	C Het			52	3.3	3	97	119	33	Yes	Possible PVOD / PCH		PDE5i + ERA + IV Prostanoid	No	
			c.3055_3064delCTGACCAACG	frameshift variant															
48	M	Other	c.4400dupT	frameshift variant	C Het			46	6.4	3	116	120	45	No	CT not available for analysis		ERA + PDE5i + inhaled Prostanoid	No	
			c.1739dupA	frameshift variant															
38	F	Other Asian	c.2827A>G	missense variant	C Het			40	4.5	2				No	CT not available for analysis		ERA + PDE5i	No	
			c.4418_4421delCAGA	frameshift variant															
			c.145-2A>G	splice acceptor variant															

Supplemental Table 7. Phenotypic and genotypic description of patients with a clinical diagnosis of PAH with *EIF2AK4* variants. mPAP – mean pulmonary artery pressure, FC – functional class, FEV_1 – forced expiratory volume in 1s, FVC - forced vital capacity, K_{CO} – transfer coefficient for carbon monoxide, PDE5i – phosphodiesterase type 5 inhibitor, ERA – endothelin receptor antagonist, C Het – compound heterozygous, Hom – homozygous, Het – heterozygous, Unk – unknown

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Age (years)	Gender	Ethnicity	*EIF2AK4* variant HGVSc	Consequence type	*EIF2AK4* genotype	*BMPR2* mutation	Non-protein coding *EIF2AK4* variant	mPAP (mmHg)	Cardiac output (L/min)	FC	FEV^1 (% pred)	FVC (% pred)	KCO (% pred)	Digital clubbing	CT diagnosis	Family history PAH	Pulmonary artery vasodilator therapy	Pulmonary oedema with treatment	Histology assessed
70	F	British	c.1392delT c.257+4A>C	frameshift variant splice region variant & intron variant	C Het			76	6.6	3	101	127	33	Unk	Possible PVOD / PCH		PDE5i + ERA + inhaled Prostanoid	No	
36	F	Indian	c.3605A>T	missense variant	Hom			44	2.7	3	73	83	40	Yes	Possible PVOD / PCH		ERA + PDE5i + inhaled Prostanoid	No	
22	M	Pakistani	c.1795G>C	missense variant	Hom			65	3.0	3	92	93	31	Yes	PAH		ERA + PDE5i + IV Prostanoid	No	Yes
29	M	Pakistani	c.3097C>T	stop gained	Hom			50	4.9	3	99	107	27	Unk	PAH	Sister died from PAH	PDE5i	No	
18	M	Not stated	c.1159_1160delCT	frameshift variant	Hom			92		3	86	82	28	No	Possible PVOD / PCH		ERA + IV Prostanoid	No	
25	F	Pakistani	c.1795G>C	missense variant	Hom			57	5.6	3	82	87	33	No	PAH		PDE5i + ERA	No	

Supplemental Table 7. Phenotypic and genotypic description of patients with a clinical diagnosis of PAH with *EIF2AK4* variants. mPAP – mean pulmonary artery pressure, FC – functional class, FEV_1 – forced expiratory volume in 1s, FVC - forced vital capacity, K_{CO} – transfer coefficient for carbon monoxide, PDE5i – phosphodiesterase type 5 inhibitor, ERA – endothelin receptor antagonist, C Het – compound heterozygous, Hom – homozygous, Het – heterozygous, Unk – unknown

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Age (years)	Gender	Ethnicity	*EIF2AK4* variant HGVSc	Consequence type	*EIF2AK4* genotype	*BMPR2* mutation	Non-protein coding *EIF2AK4* variant	mPAP (mmHg)	Cardiac output (L/min)	FC	FEV_1 (% pred)	FVC (% pred)	KCO (% pred)	Digital clubbing	CT diagnosis	Family history PAH	Pulmonary artery vasodilator therapy	Pulmonary oedema with treatment	Histology assessed
24	F	Not stated	c.2446C>T	stop gained	Het (both on same allele) *			60	5.2	3	96	97	81	Unk	CT not available for analysis	Father and sister died of PAH	Unk	Unk	
			c.3218G>T	missense variant															
39	F	British	c.1072_1073dupGT	frameshift variant	Het			54	3.0	2	87	98	72	No	CT not available for analysis		ERA	No	
40	F	British	c.44C>T	missense variant	Het		c.4303-50delT	43	5.6	2	99	96	109	Unk	Possible PVOD / PCH		ERA	No	
44	M	British	c.2516T>C	missense variant	Het	c.853-2A>G (splice acceptor variant)	c.361-180A>G	53	3.8	3	102	98	54	Unk	PAH		PDE5i + ERA	No	
25	F	British	c.3722A>G	missense variant	Het					3	53	49	41	No	CT not available for analysis		PDE5i + ERA + IV Prostanoid	No	

Supplemental Table 7. Phenotypic and genotypic description of patients with a clinical diagnosis of PAH with *EIF2AK4* variants. mPAP – mean pulmonary artery pressure, FC – functional class, FEV_1 – forced expiratory volume in 1s, FVC - forced vital capacity, K_{CO} – transfer coefficient for carbon monoxide, PDE5i – phosphodiesterase type 5 inhibitor, ERA – endothelin receptor antagonist, C Het – compound heterozygous, Hom – homozygous, Het – heterozygous, Unk – unknown, *maternally inherited

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Age (years)	Gender	Ethnicity	*EIF2AK4* variant HGVSc	Consequence type	*EIF2AK4* genotype	*BMPR2* mutation	Non-protein coding *EIF2AK4* variant	mPAP (mmHg)	Cardiac output (L/min)	FC	FEV_1 (% pred)	FVC (% pred)	KCO (% pred)	Digital clubbing	CT diagnosis	Family history PAH	Pulmonary artery vasodilator therapy	Pulmonary oedema with treatment	Histology assessed
66	F	Not stated	c.4646G>A	missense variant	Het			44	2.1	3	79	100		Unk	PAH		PDE5i + ERA	No	
72	M	British	c.1660G>T	missense variant & splice region variant	Het			30	2.8	3				No	PAH		IV Prostanoid	No	
59	F	Other	c.3711_3713delGAG	inframe deletion	Het			41	3.4	3	68	68	95	Unk	PAH		ERA + PDE5i	No	
48	F	British	c.3604C>T	missense variant	Het	c.2695C>T (stop gained)		57	4.4	4	90	100	61	Unk	PAH		PDE5i + ERA	No	
70	F	Other White	c.220G>A	missense variant	Het			42	5.4	2				Unk	CT not available for analysis		ERA	Unk	

Supplemental Table 7. Phenotypic and genotypic description of patients with a clinical diagnosis of PAH with *EIF2AK4* variants. mPAP – mean pulmonary artery pressure, FC – functional class, FEV_1 – forced expiratory volume in 1s, FVC - forced vital capacity, K_{CO} – transfer coefficient for carbon monoxide, PDE5i – phosphodiesterase type 5 inhibitor, ERA – endothelin receptor antagonist, C Het – compound heterozygous, Hom – homozygous, Het – heterozygous, Unk – unknown

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	PAH patients with *BMPR2* mutations *	**PAH patients with no mutations in PAH associated genes**	**PAH patients with *EIF2AK4* heterozygous variants**	**PAH patients with biallelic *EIF2AK4* mutations**	**PVOD/PCH patients**	**p**
n	64	255	3	7	5	
Age (years)	42 [31 - 52]	53 [39 - 67]	39 [32 - 40]	25 [23 - 38]	63 [27 - 76]	<0.001
Gender (n female [%])	45 [70.3%]	179 [70.2%]	3 [100%]	2 [28.6%]	4 [80%]	0.161
Ethnicity (n white Caucasian [%])	50 [78.1%]	226 [88.6%]	2 [66.7%]	2 [28.6%]	4 [80%]	<0.001
Digital clubbing (n [%])	5 [13.2%]	3 [2.2%]	0 [0%]	2 [40%]	0 [0%]	0.004
BMI	28 [25 - 33]	27 [24 - 31]	24 [24 - 25]	24 [21 - 27]	27 [24 - 32]	0.202

Supplemental Table 8. Phenotype summary of patients with preserved spirometry (FEV_1 > 80 % predicted and FVC > 80 % predicted). PAH patients with biallelic *EIF2AK4* mutations are still younger at diagnosis and have a significantly reduced KCO compared to other groups.
mPAP – mean pulmonary artery pressure, CO – cardiac output, PVR – pulmonary vascular resistance, FEV_1 – forced expiratory volume in 1 second, FVC – forced vital capacity, KCO – transfer coefficient for carbon monoxide, BMI – body mass index. * Also includes the 2 patients with heterozygous *EIF2AK4* variants and a *BMPR2* mutation. Data presented as median [IQR] unless indicated. Percentages were calculated using the number of patients for whom data were available as the denominator.

Supplemental Table 8. Page 2/2						
	PAH patients with *BMPR2* mutations *	**PAH patients with no mutations in PAH associated genes**	**PAH patients with *EIF2AK4* heterozygous variants**	**PAH patients with biallelic *EIF2AK4* mutations**	**PVOD/PCH patients**	**p**
mPAP (mmHg)	56 (15)	51 (18)	54 (8)	57 (20)	57 (7)	0.008
CO (L/min)	3 [3 - 4]	4 [3 - 5]	5 [4 - 5]	5 [4 - 6]	3 [3 - 3]	<0.001
PVR (WU)	14 [10 - 18]	10 [7 - 14]	8 [7 - 9]	9 [8 - 15]	14 [11 - 19]	<0.001
Vasoresponders (n [%])	0 [0%]	18 [21.7%]	0 [0%]	0 [0%]		0.016
FEV_1 (%pred)	97 [88 - 102]	93 [87 - 101]	96 [92 - 97]	97 [89 - 100]	98 [94 - 106]	0.525
FVC (%pred)	102 [96 - 113]	103 [96 - 112]	97 [96 - 98]	107 [90 - 120]	109 [101 - 113]	0.704
KCO (%pred)	80 [71 - 93]	68 [46 - 84]	81 [76 - 95]	33 [30 - 33]	33 [28 - 37]	<0.001
Resting S_AO_2 (%)	96 [94 - 98]	96 [93 - 98]	98 [98 - 99]	91 [90 - 92]	95 [91 - 95]	0.021
S_AO_2 post walk test (%)	95 [90 - 98]	91 [85 - 96]	94 [87 - 96]	80 [75 - 84]	85 [85 - 88]	<0.001

Supplemental Table 8. Phenotype summary of patients with preserved spirometry (FEV_1 > 80 % predicted and FVC > 80 % predicted). PAH patients with biallelic *EIF2AK4* mutations are still younger at diagnosis and have a significantly reduced KCO compared to other groups.
mPAP – mean pulmonary artery pressure, CO – cardiac output, PVR – pulmonary vascular resistance, FEV_1 – forced expiratory volume in 1 second, FVC – forced vital capacity, KCO – transfer coefficient for carbon monoxide, BMI – body mass index. * Also includes the 2 patients with heterozygous *EIF2AK4* variants and a *BMPR2* mutation. Data presented as median [IQR] unless indicated. Percentages were calculated using the number of patients for whom data were available as the denominator.

Supplemental Table 9. Page 1/2				
Group		**All biallelic *EIF2AK4* mutation carriers**	**PVOD with no *EIF2AK4* mutation**	**p**
n		11	10	
Age (years)		26.8 [22.5 - 34.3]	68.3 [63.9 - 72.1]	0.001
Gender (n female [%])		6 [54.5%]	5 [50.0%]	1.000
Ethnicity (n white Caucasian [%])		5 [45.5%]	9 [90.0%]	0.063
mPAP (mmHg)		52 [47 - 63]	48 [42 - 57]	0.342
PCWP (mmHg)		11 [7.5 - 12]	11.5 [9.0 – 12.2]	0.560
FEV_1 (% pred)		93.1 [82.8 - 98.5]	79.0 [72.3 – 91.0]	0.236
FVC (% pred)		95.5 [84.6 - 108.5]	96.0 [73.0 – 101.0]	0.720
KCO (% pred)		32.0 [28.7 – 33.0]	41.4 [36.8 – 54.0]	0.013
Centrilobular ground glass opacification density	None	2 [18.2%]	6 [60.0%]	0.012
	Subtle	2 [18.2%]	3 [30.0%]	
	Present	7 [63.6%]	1 [10.0%]	

Supplemental Table 9. Phenotypic and radiological characteristics of biallelic *EIF2AK4* mutation carriers compared to patients with a clinical diagnosis of PVOD and no *EIF2AK4* mutation.

mPAP – mean pulmonary artery pressure, PCWP – pulmonary capillary wedge pressure, FEV_1 – forced expiratory volume 1 s, FVC – forced vital capacity, KCO – transfer coefficient for carbon monoxide. Data presented as median [IQR] unless stated.

Supplemental Table 9. Page 2/2				
Group		**All biallelic *EIF2AK4* mutation carriers**	**PVOD with no *EIF2AK4* mutation**	**p**
Centrilobular ground glass opacification extent	None	2 [18.2%]	7 [70.0%]	0.007
	<5%	1 [9.1%]	1 [10.0%]	
	5-25%	2 [18.2%]	1 [10.0%]	
	25-50%	1 [9.1%]	1 [10.0%]	
	50-75%	2 [18.2%]	0 [0.0%]	
	75-100%	3 [27.3%]	0 [0.0%]	
Interlobular septal thickening	None	7 [63.6%]	2 [20.0%]	0.068
	Subtle	0 [0.0%]	1 [10.0%]	
	Present	4 [36.4%]	7 [70.0%]	
Mediastinal lymphadenopathy	None	4 [36.4%]	2 [20.0%]	0.635
	Present	7 [63.6%]	8 [80.0%]	
Pleural effusion	None	11 [100.0%]	6 [60.0%]	0.035
	Small	0 [0.0%]	4 [40.0%]	
Neovascularity	None	10 [90.9%]	9 [90.0%]	1.000
	Present	1 [9.1%]	1 [10.0%]	
CT diagnosis	PAH	4 [36.4%]	3 [30.0%]	
	Possible PVOD/PCH	7 [63.6%]	7 [70.0%]	

Supplemental Table 9. Phenotypic and radiological characteristics of biallelic *EIF2AK4* mutation carriers compared to patients with a clinical diagnosis of PVOD and no *EIF2AK4* mutation.
mPAP - mean pulmonary artery pressure, PCWP - pulmonary capillary wedge pressure, FEV_1 - forced expiratory volume 1 s, FVC - forced vital capacity, KCO - transfer coefficient for carbon monoxide. Data presented as median [IQR] unless stated.

Group	Time to assessment 1 (days)	n	Change in 6mwd (m)	Change in FC	Time to assessment 2 (days)	n	Change in 6mwd (m)	Change in FC	Number on prostanoid therapy before the 2nd assessment [%]
PAH *BMPR2*	357 [314 - 386]	21	+69 [20 - 100]	-1 [-1 - -1]	1120 [1055 - 1174]	18	+45 [31 - 115]	-1 [-1 - -0.5]	5 [23%]
PAH biallelic *EIF2AK4*	358 [335 -388]	9	+28 [-13 - 77]	0 [-1 - 0]	1102 [1090 – 1112]	5	+62 [-8 - 132]	0 [0 - 0]	1 [10%]
PAH no mutation	387 [340 - 414]	16	+81 [61 - 151]	-1 [-1 - 0]	1118 [1105 - 1159]	9	+104 [20 - 144]	-1 [-1 - 0]	4 [17%]
p	0.295		0.343	0.039	0.730		0.748	0.044	0.816

Supplemental Table 10. Response to pulmonary artery vasodilator therapies at 1 and 3 years after diagnosis compared to baseline. 6mwd - six-minute walk test distance, FC - functional class. Drop in number of patients between assessment 1 and 2 due to death, transplantation or lack of sufficient follow up time. Data presented as median [IQR] unless stated.

Variable	Hazard Ratio [95% confidence interval]	p
PAH *BMPR2* mutation*	0.148 [0.055 - 0.396]	<0.001
PAH no mutation*	0.179 [0.073 - 0.440]	<0.001
PVOD*	0.393 [0.075 - 2.065]	0.27
Age at diagnosis	1.043 [1.033 - 1.053]	<0.001
Male gender	1.631 [1.222 - 2.179]	<0.001

Supplemental Table 11. Cox proportional hazards model assessing time to death. Patients with a clinical diagnosis of PAH and biallelic *EIF2AK4* mutations had an increased risk of death compared to other PAH patients. Number of patients = 858. Events = 194.
* compared to the PAH biallelic *EIF2AK4* mutation carriers

Variable	Hazard Ratio [95% confidence interval]	p
PAH *BMPR2* mutation*	0.175 [0.066 - 0.462]	<0.001
PAH no mutation*	0.203 [0.083 - 0.501]	<0.001
PVOD*	0.840 [0.222 - 3.193]	0.798
Age at diagnosis	1.036 [1.027 - 1.046]	<0.001
Male gender	1.542 [1.165 - 2.042]	0.002

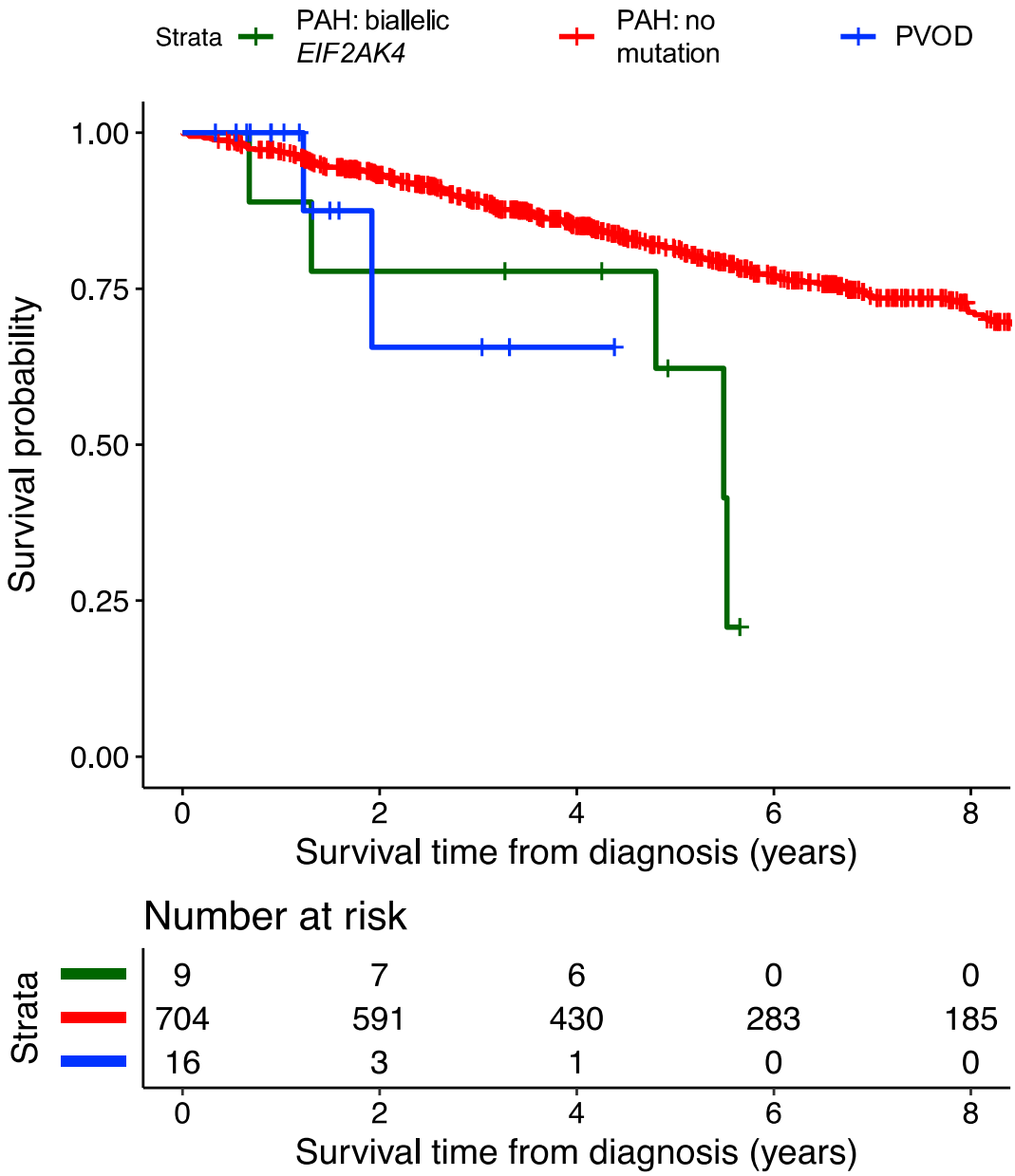
Supplemental Table 12. Cox proportional hazards model assessing time to death or transplantation. Number of patients = 858. Events = 208.
* compared to the PAH biallelic *EIF2AK4* mutation carriers

Variable	Hazard Ratio [95% confidence interval]	p
PAH *BMPR2* mutation*	0.376 [0.080 - 1.763]	0.215
PAH no mutation*	0.456 [0.109 - 1.905]	0.282
PVOD*	1.029 [0.133 - 7.953]	0.978
Age at diagnosis	1.034 [1.020 - 1.046]	<0.001
Male gender	1.515 [1.000 - 2.296]	0.051

Supplemental Table 13. Sensitivity analysis including only prospectively recruited UK patients. Cox proportional hazards model assessing time to death. Number of patients = 608. Events = 95.
* compared to the PAH biallelic *EIF2AK4* mutation carriers

Supplemental Figures

Figure S1



Supplemental Figure Legends:

Figure S1: Kaplan – Meier survival curves showing survival time (time to death) for patients with a clinical diagnosis of PAH or PVOD.

Supplemental References

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