

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

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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 haemangiomas (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:

Pulmonary hypertension

Pulmonary veno-occlusive disease

Genetics, human

EIF2AK4

Prognosis

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 changes associated with PVOD may lead to misclassification of these patients as PAH.

- Genetic testing allows early identification of these patients, facilitating appropriate management.

INTRODUCTION

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 haemangiomas (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 *BMP2* 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). The cohort also 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 mutations, 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 Isaac 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) v84²⁵. 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 database²⁶. 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" respectively²⁷⁻²⁹.

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 patients³⁰. 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. 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 *BMP2* 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 *BMP2* 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 *BMP2* 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 (FEV₁ 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 *BMP2* 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 *BMP2* 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 *BMP2* 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 *BMP2* 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 hemangiomas 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).

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 *EIF2AK4* biallelic 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 *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 haemangiomas. 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 *BMP2* 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*

mutations³². 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, we demonstrated a worse prognosis in patients with a clinical diagnosis of PAH and biallelic *EIF2AK4*. However, 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 *BMP2R* 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 *BMP2R* 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 *BMP2R* 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 PAH³⁷. 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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The NIHR BioResource – Rare Diseases Consortium

UK National Cohort Study of Idiopathic and Heritable PAH

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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 FEV₁ < 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).

Tables

	PAH patients with <i>BMPR2</i> mutations *	PAH patients with no mutations in PAH associated genes	PAH patients with <i>EIF2AK4</i> heterozygous variants	PAH patients with biallelic <i>EIF2AK4</i> 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 _A O ₂ (%)	96 [94 - 97]	96 [93 - 97]	98 [98 - 98]	91 [90 - 94]	94 [91 - 95]	0.010
S _A O ₂ post walk test (%)	94 [90 - 97]	92 [85 - 96]	94 [84 - 96]	78 [75 - 82]	88 [85 - 89]	<0.001

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.

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.

	Group	PAH patients with <i>BMPR2</i> mutations	PAH patients with no mutations in the previously reported PAH genes	PAH patients with heterozygous <i>EIF2AK4</i> variants	PAH patients with biallelic <i>EIF2AK4</i> 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%]	
	None	17 [81.0%]	18 [85.7%]	4 [100.0%]	5 [71.4%]	4 [28.6%]	0.001

Interlobular septal thickening	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%]	
Table 2. Radiological features and consensus radiological diagnosis of PAH patients in the CT substudy. Data presented as n [%].							