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Phenotype and outcome of PAH patients carrying a *TBX4* mutation

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ABSTRACT

Introduction

TBX4 mutation cause small patella syndrome (SPS) and/or pulmonary arterial hypertension (PAH). The characteristics and outcomes of PAH associated with *TBX4* mutations are largely unknown.

Methods

We report the clinical, functional, radiologic, histologic and haemodynamic characteristics and outcomes of heritable PAH patients carrying a *TBX4* mutation from the French PH Network.

Results

Twenty patients were identified in 17 families. They were characterized by a median age at diagnosis of 29 (0-76) year-old and a female to male ratio of 3. Most of the patients were in NYHA functional class III or IV (70%) with a severe hemodynamic impairment (median pulmonary vascular resistance of 13.6 [6.2-41.8] Wood Units). Skeletal signs of SPS were present in 80% of cases. Half of the patients had mild restrictive or obstructive limitation and diffusing capacity for carbon monoxide was decreased in all patients. High-resolution computed tomography showed bronchial abnormalities, peri-bronchial cysts, mosaic distribution and mediastinal lymphadenopathies. PAH therapy was associated with significant clinical improvement. At follow-up (median 76 months), two patients died and two underwent lung transplantation. One-, three- and five-year event-free survival rates were 100%, 94% and 83%, respectively. Histologic examination of explanted lungs revealed alveolar growth abnormalities, major pulmonary vascular remodelling similar to that observed in idiopathic PAH, and accumulation of cholesterol crystals within the lung parenchyma.

Conclusion

PAH due to *TBX4* mutations may occur with or without skeletal abnormalities across a broad age range from birth to late adulthood. PAH is usually severe and associated with bronchial and parenchymal abnormalities.

Key Words:

Genetics, Genetic counselling, Pulmonary arterial hypertension, Small patella syndrome, *TBX4*.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a severe, progressive and uncommon disease affecting small pulmonary arteries. Pulmonary vascular remodelling is mainly characterized by abnormal proliferation of endothelial cells, smooth muscle cells, myofibroblasts and pericytes, leading to increased pulmonary arterial pressure and right ventricular failure [1, 2]. Several medical conditions are related to the development of PAH, such as connective tissue diseases, congenital heart diseases, portal hypertension, and HIV infection [3]. In the absence of associated condition, exposure to drugs and toxins causing PAH or of a familial history of PAH, the disease is classified as idiopathic [3]. The main cause of heritable PAH is mutations in the *bone morphogenetic protein receptor type 2* gene (*BMPR2*), which was first described in 2000. Since then, novel mutations have been identified in different genes of interest (*TBX4*, *ATP13A3*, *GDF2*, *SOX17*, *AQP1*, *ACVRL1*, *SMAD9*, *ENG*, *KCNK3*, *CAV1*, *GDF2*, *BMP10*) [4].

In 2013, array comparative hybridization and sequencing of a population of children presenting with PAH associated with mental retardation and/or dysmorphic features led to the identification of a new gene of interest: *T-box transcription factor 4* (*TBX4*) [5]. The *TBX4* gene is a member of the T-box gene family encoding transcription factors that play varied and important roles throughout development, especially in the development and branching of the lungs and in lower limb formation [6–9]. Mutations in this gene cause small patella syndrome (SPS, also called coxo-podo-patellar syndrome), an autosomal dominant skeletal dysplasia affecting the lower limbs, which is characterized by hypoplasia or aplasia of the patella, ossification defects of the ischia and inferior pubic rami, and anomalies of the feet such as a large gap between the first and the second toes [10].

To date, *TBX4* mutation is reported to be one of the main genetic causes of PAH in children and is mainly reported in childhood-onset PAH [11–14]. Because of its pivotal role in embryogenesis, especially in the lungs and lower limbs, heritable PAH patients carrying *TBX4* mutations may present with a distinct phenotype. To test this hypothesis, we have studied the clinical, functional, radiologic, histologic and haemodynamic characteristics as well as long-term outcomes of PAH patients carrying a *TBX4* mutation from the French Pulmonary Hypertension (PH) Network.

METHODS

Patients selection

We conducted a retrospective population-based study of PAH patients carrying a *TBX4* mutation from the registry of the French PH Network (French PH Referral Center, Hôpital Bicêtre, Université Paris Sud, Le Kremlin-Bicêtre and its 25 associated centres across France). This registry was set up in agreement with French bioethics laws (Commission Nationale de l'Informatique et des Libertés n°842063) [6]. Genetic counselling was offered to all patients with idiopathic, familial, or drug-induced PAH [16]. All patients provided written informed consent prior to genetic analysis.

Genetic analysis

PAH predisposing genes, including the *TBX4* gene, were screened by NGS-based gene panel analysis as previously described [17, 18]. Briefly, a custom gene panel including established PAH and PVOD genes (*BMPR2*, *TBX4*, *EIF2AK4*, *CAV1*, *KCNK3*, *SMAD9*, *ACVRL1*, *ENG*, *BMP9*) was used for NGS-targeted capture. Libraries were prepared according to the KAPA Library preparation protocol for Illumina platforms, captured using the SeqCap EZ Choice Library (Roche/NimbleGen) following the manufacturer's protocol and sequenced using the MiSeq system (Illumina). Data are analyzed by a bioinformatic pipeline developed by GenoDiag Inc, allowing identification of variants and CNVs. Each base must be covered by at least 40 reads to be validated. All variants of interest are validated by Sanger sequencing. CNV are validated by MLPA when available or by Q-PCR. *TBX4* genetic testing is performed since 2014.

Clinical, functional, haemodynamic and radiologic characteristics

We reviewed clinical data (age, medical history and physical examination), dyspnoea assessed by modified New York Heart Association (NYHA) functional class, non-encouraged 6-minute walk distance (6MWD), and pulmonary functional tests (PFTs) including diffusing capacity of the lung for carbon monoxide corrected for haemoglobin (DLCO).

Hemodynamic measurements at RHC included mean pulmonary artery pressure (mPAP), pulmonary artery wedge pressure (PAWP), right atrial pressure (RAP), and mixed venous oxygen saturation (SvO2). Cardiac output (CO) was measured by the standard thermodilution technique. Cardiac index (CI) was calculated as cardiac output divided by body surface area. PVR was calculated as [(mPAP-PAWP)/CO]. Precapillary PH was defined as mPAP ≥ 25 mmHg and PAWP ≤ 15 mmHg and pulmonary vascular resistance (PVR) > 3 WU [1].

High-resolution computed tomography (HRCT) of the chest was independently analysed by an expert radiologist (MRJ) who was blinded to clinical and haemodynamic data.

Follow-up and clinical outcomes

Medical therapies approved for PAH (prostacyclin derivatives, endothelin receptor antagonists and phosphodiesterase type-5 inhibitors) were given to the patients according to the clinical judgement and discretion of the individual treating physicians. Clinical, functional, and haemodynamic follow-up data were collected in the French PH registry. Time to death or lung transplantation was recorded.

Statistical analysis

The analyses were carried out using the SPSS Base 20.0 statistical software package (SPSS Inc., Chicago, Ill). The variables were expressed as medians (min-max) for quantitative variables and as numbers (percentages) for categorical variables. We used the Wilcoxon signed rank test to compare the NYHA functional class before and after treatment and the Wilcoxon matched-pair sign rank test to compare quantitative data before and after treatment. Overall survival was calculated from the time of the diagnosis of PAH and was estimated by the Kaplan-Meier method. Correlations between data were studied with Spearman’s rank correlation coefficient.

RESULTS

Patient population

Since 2014, 448 index patients (excluding related cases) were screened for PAH predisposing genes including *TBX4*. We identified 20 PAH (4.5%) patients in 17 unrelated families carrying heterozygous mutations in the *TBX4* gene bringing the frequency of *TBX4* mutations in France at 6% and 3% in childhood-onset PAH and adult-onset PAH respectively. Fourteen mutations were point mutations, two were copy number variations and one was a variant located in the promoter region classified as a variant of unknown significance (VUS) (**Table 1**). However, the patient carrying this VUS presented with typical SPS foot abnormalities leading to the genetic diagnosis of *TBX4-linked PAH.* The mutations of eleven patients have been previously reported [12, 17–19].

In this cohort, nine (45%) cases presented with childhood-onset PAH (< 18 years old). Sixteen patients were sporadic cases, and four were from the same family (**Figure 1**). Median age at PAH diagnosis was 29 (0-76) year-old: six (0-17) year-old in the childhood-onset group and 56 (24-76) year-old in the adult-onset group. Repartition of age at diagnosis was presented in **Figure 2**. A female predominance was observed (female-male ratio of 3), especially in the childhood-onset group, in which all patients were females.

Two patients (10%) presented with atrial septal defects, and one had patent ductus arteriosus. Among them, one child developed PAH nine months after percutaneous closure of the atrial septal defect. Four patients had a history of mild asthma.

Clinical and functional characteristics

At diagnosis, two (10%) patients were in NYHA functional class II, 11 (55%) were in class III and three (15%) were in class IV. NYHA functional class was not evaluable in four (20%), children because of their age. At diagnosis, the 6MWD was 388 meters (224-613) in the whole cohort, 392 (235-585) in adults and 290 (224-613) in children. Forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, total lung capacity (TLC) were presented in **Table 2**. Fourteen patients had complete PFTs showing normal values in 7 (50%) patients, mild obstructive limitation in 4 (28.6%) patients and mild restrictive limitation in 3 patients (21.4%). Moreover, DLCO was decreased in all patients

with a median of 57.5% (43-69) (**Table 2**). This decrease was not associated with tobacco exposure since only two patients were smokers (14 and 10 pack-years, respectively).

Haemodynamic assessment at diagnosis

All patients underwent RHC at PAH diagnosis showing severe precapillary PH with an elevated mPAP of 59.5 (29-87) mmHg, a normal PAWP of 8 mmHg (4-14), a decreased CI of 2.7 (1.48-7.60) $L.min^{-1}.m^{-2}$ and an increased PVR of 13.6 (6.2-41.8) Wood Units (**Table 2**). No patient had acute vasodilator responses according to current international guidelines [1]. No significant differences in haemodynamic characteristics were observed between children and adults (data not shown). We found no significant correlations between PVR and DLCO (r=-0.24; p=0.47), FEV_1 (r=0.12; p=0.71), FVC (r=-0.14; p=- 0.63) or PaO_2 (r=0.24; p=0.58).

Analysis of high-resolution CT of the chest

Fifteen (75%) patients had analysable high-resolution CT of the chest at diagnosis (**Table 3, Figure 3**). Fourteen CTs (93.3%) presented pulmonary abnormalities. Nine (60%) showed central and peripheral bronchial abnormalities, including thickened and irregular walls (n=7; 46.7%), bronchial and/or tracheal diverticula (n=5; 33.3%), mucoid impactions (n=3; 20%) and moniliform and cylindrical bronchiectasis (n=2; 13.3%). Four (26.7%) CT images showed the presence of peri-bronchial cysts communicating, in most cases, with bronchi, and hilar or mediastinal lymphadenopathies were observed in six (40%) patients. Parenchymal abnormalities were mainly mosaic distribution (n=6; 40%), subpleural septal lines (n=4; 26.6%), micronodules (n=2; 13.3%) and ground glass opacities (n=2; 13.3%). Two CTs (13.3%) showed micronodules and sub-pleural neovascularity-like lesions. We found no significant differences in lung abnormalities, highlighted by HRCT of the chests, between childhood-onset PAH and adult-onset PAH (data not shown).

Lung histopathology

Lung samples were available in three patients (**Supplemental table 1**). One sample was a surgical lung biopsy, and two were lung explants (**Figure 4**). All three showed major pulmonary arterial remodelling similar to that observed in idiopathic PAH, with plexiform lesions in two (66%) (**Supplemental table 1**). Parenchymal abnormalities were found in all

patients and accounted for patchy peribronchiolar and peripheral fibrosis, lymphoid nodules, peri-bronchial inflammation with cholesterol clefts and hypertrophy of smooth muscle. Alveolar septa were also interrupted and appeared rarefied, resulting in an “emphysematous dystrophia” appearance. These changes are potentially related to small airway remodelling and altered lung development, as previously described in such patients [20].

Skeletal anomalies

Clinical data on skeletal abnormalities were available in 15 (75%) out of the 20 patients, and 12 of them (80%) showed clinical abnormalities. Nine (75%) patients showed a unique skeletal abnormality, two (17%) presented with two abnormalities and one (8%) with three abnormalities. No skeletal abnormalities were identified in three patients (20%) of 14, 62 and 56 year-old respectively; one of these three patients had normal lower limbs and pelvic radiographs. Ten patients, including five children and five adults, presented with foot abnormalities characterized by a large gap between the first and the second toes and flat feet. Disorders of the knees, such as congenital dislocation of the patella or knee dysplasia, were found in five patients (33%, including two children and three adults). One patient showed a lack of ossification of the ischia on pelvic radiography (**Table 1**).

All the mutated members of the familial case and two parents (**Figure 1**, III2 and III3), obligate carriers of the familial *TBX4* mutation, presented with features of SPS.

Response to PAH therapy

Nineteen patients received PAH therapy. Thirteen patients were reevaluated with RHC in the first year after initiation of treatment with a median delay of six (3-11) months (**Table**). At reassessment, four patients (31%) were treated with endothelin receptor antagonist (ERA) monotherapy; two patients (15.2%) were treated with phosphodiesterase type 5 inhibitor (PDE5i) monotherapy; five patients (38.6%) were treated with oral dual combination of ERA and PDE5i; one patient (7.6%) was treated with dual combination therapy of ERA and prostacyclin derivative; and one patient (7.6%) received triple combination therapy including an ERA, PDE5i and prostacyclin derivative. Among the patients who received PAH therapy, three (22.8%) were treated with calcium channel blockers (CCB), despite the absence of acute vasodilator response (**Table 4**).

At reassessment, a clinical improvement was observed with improved NYHA functional class ($p<0.001$) and increased 6MWD ($p=0.01$). However, there was no significant hemodynamic improvement in mPAP, CI or PVR (**Table 4**).

Outcomes

At the last follow-up, 12 patients (60%) were treated with triple combination therapy (ERA, PDE5i and prostacyclin derivative), and six (30%) received oral dual oral combination therapy (ERA and PDE5i). One patient was treated with PDE5i and CCB. One patient received only CCB during 5 years. After a median follow-up of 56 (6-239) months, two patients (10%) had died (one 67 year-old patient died from right heart failure after 53 months of follow-up, and the other patient died at 74 year-old from an unknown cause after 23 months of follow-up). Two patients underwent double lung transplantation after 7 and 22 years of follow-up, respectively. The 1-, 3- and 5-year event-free survival rates were 100%, 94% and 83%, respectively (**Figure 5**).

DISCUSSION

We describe a cohort of 20 PAH patients carrying a *TBX4* mutation across a broad age range from birth to late adulthood from a nationwide PH registry. Our report describes the phenotype and outcome of a French mixed cohort of childhood- and adult-onset PAH associated with *TBX4* mutation. PAH due to *TBX4* gene mutations seem to occur with a bimodal distribution mode in our cohort, comprising two main peaks of occurrence: an early onset in childhood as previously described [5, 12, 14] and a later presentation in adulthood **(Figure 2)**. This observation is supported by the fact that *TBX4* mutations are identified in 6% of pediatric index cases and in 3% of adult index cases. This consideration cannot be stated formally given the limited size of our population. However, a recent study of exome sequencing in a large cohort of 2572 adult and pediatric PAH find the same type of presentation distribution in PAH patients carrying a *TBX4* mutation [21]. A female predominance was observed, as described in other forms of heritable PAH [22, 23]. Clinical features of SPS were found in most patients but were frequently non-obvious. At diagnosis, patients presented with severe precapillary PH, usually associated with bronchial, parenchymal and vascular abnormalities, as demonstrated by HRCT of the chest and lung histology. After initiation of PAH therapies, a statistically significant clinical improvement was observed with no significant haemodynamic changes. Even if prognosis seems to be better than in PAH associated with *BMPR2* mutation, prognosis remained severe with two deaths and two lung transplantations at follow-up.

A high penetrance of *TBX4* mutation for skeletal abnormalities has been described [5]. In our cohort, 80% of patients presented with signs of SPS. However, signs were absent or non-obvious in some patients. Indeed, three out of 19 patients had no skeletal abnormalities, illustrating that PAH may be the only clinical manifestation of *TBX4* mutation mimicking idiopathic PAH. Therefore, personal and familial signs of SPS must be systematically searched in every patient newly diagnosed with idiopathic or familial PAH, and *TBX4* mutations should be screened even in the absence of skeletal abnormalities. In the literature, this ascertainment was previously made by Zhu et al., who identified 13 cases of PAH associated with a *TBX4* mutation including a single case of SPS; and by Navas et al., who identified four PAH patients carrying a *TBX4* mutation with no skeletal abnormalities

identified [13, 24]. By contrast, the penetrance of *TBX4* mutation for PAH is incomplete, as previously reported with other PAH predisposing genes. In the present series, we have identified a small proportion of familial cases (4 patients from the one family and 16 sporadic cases) in comparison with what is usually observed in PAH associated with *BMPR2* mutation [16]. In the reported family all *TBX4* mutation carriers presented with SPS, but only four female members developed PAH, at 1, 3, 17 and 76 years-old, demonstrating that the manifestation of the disease is independent of the mutation type (**Figure 1**).

The hemodynamic characteristics in our population are broadly similar to previous reports showing severe precapillary PH at diagnosis without acute vasodilator response according to current guidelines. However, some patients presented with partial vasoreactivity in accordance with what has been reported in paediatric series [14]. Among the four patients who received CCB therapy, only one presented stable disease while the three others have become worse requiring the use of PAH treatments. This haemodynamic profile was similar to the results observed in other heritable PAH populations, such as *BMPR2* or *ACVRL1* mutation carriers, for whom acute vasoreactivity is classically absent [22, 23].

We observed congenital heart diseases in 15% of our cases (two atrial septal defects and one patent ductus arteriosus): 2 in the 9 childhood-onset PAH and 1 in the 11 adult-onset PAH. The pediatric report by Galambos et al. described 36.8% atrial septal defects, 21% patent ductus arteriosus, 5.3% ventricular septal defects and 5.3% patent foramen ovale [14]. These differences may be explained at least in part by a higher incidence of copy number variation in the paediatric population. Indeed, the copy number variations generally encompassed the whole *TBX4* gene plus some other genes, including the *TBX2* gene, which plays an important role in the embryologic development of the heart and is associated with cardiac septal defects [25, 26].

One may hypothesize that precapillary PH in patients carrying *TBX4* mutation could be a consequence of developmental disorders of the lungs and should be classified as PH due to chronic respiratory diseases (group 3 of the PH classification) [27]. Analysis of HRCT of the chest demonstrated that 60% of patients presented with bronchial abnormalities, with central and peripheral thickening, irregularity of the bronchial walls and presence of tracheal or bronchial diverticula in one-third of patients. PFTs revealed an obstructive respiratory disease in 28.6% of patients and that 28.6% of patients showed a decreased FEV_1<80%.

Furthermore, four patients had mild asthma history. Histological assessment of lung samples in three patients confirmed an abnormal distal lung development with emphysematous-like dystrophia appearing as disruption and rarefaction of the bronchiolar septation, abnormalities of bronchi with the presence of tracheal and bronchial diverticula and interstitial fibrosis. Furthermore, histologic examinations showed the presence of cholesterol cleft inclusions located in the perivascular connective tissue. This aspect is not a common feature of PAH but has been previously described in heterogeneous PH populations in which it is impossible to formally eliminate the presence of underlying *TBX4* mutation or any developmental disorder [28–30]. These findings are reminiscent of those reported in the paediatric cohort of Galambos et al. [14]. Moreover, macroscopically, lung explants from our patients had a small size, compatible to a certain degree with pulmonary hypoplasia, a lung development disorder classically defined by a ratio of lung weight to body weight less than 0.012 after 28 weeks of gestation [20]. Finally, this hypothesis may still be supported by the low DLCO, which is not usually as pronounced in idiopathic and heritable PAH [31]. These abnormalities may be related to pulmonary development disorders due to *TBX4* mutations. However, some patients did not have CT abnormalities but developed PAH, arguing for the presence of isolated pulmonary vascular disease. Indeed, histologic assessment revealed pulmonary arterial remodelling as classically observed in PAH, including plexiform lesions (**Figures 3B, 3C**) similar to those observed in idiopathic or heritable PAH [2]. Moreover, we did not show significant correlations between PVR, reflecting the vascular anomalies, and, FEV_1, FVC or PaO_2, reflecting lung parenchymal involvement or DLCO reflecting the involvement of alveolo-capillar barrier. This finding argues for a specific involvement of pulmonary arteries in this heritable form of PAH and against a mechanism merely due to parenchymal involvement.

Even if they might have started early during lung development, the pulmonary vascular involvement could progress slowly in *TBX4* mutation carriers. This could explain the relative long-term stability with PAH treatments. Despite initially severe PAH requiring a combination of PAH therapies and, in turn, the use of triple combination therapy in 60% of patients, the clinical status improved despite the absence of significant hemodynamic improvement. The overall survival rates of our cohort seems to be better than those observed in other forms of idiopathic and heritable PAH, but they must be interpreted with caution because of the

retrospective nature of the study, the small size of the studied population and the variable duration in follow-up [32].

Most of the radiologic or histologic abnormalities observed in our patients could be related to the embryologic roles of the *TBX4* gene. *In utero*, TBX4 is abundantly expressed in the pulmonary mesenchyme at critical stages of development, where the transcription factor activates genes that are essential for branching morphogenesis, including *FGF10* and *Nkx2.1*. The association of *TBX4* mutations with PAH further raises the possibility that TBX4 regulates genes that influence pulmonary vascular development and function [33]. Using a transgenic mouse line (*Tbx4* lung enhancer), it was recently shown that angiogenic vessels originating from the extra-pulmonary site initially serve as a stem and trigger lung mesenchymal stem cell differentiation and commitment to endothelial progenitor cells, which then coalesce into pre-existing vessels and become the major source for lung vasculature [34]. In alveolar capillary dysplasia, a disease mainly caused by *FOXF1* mutations and characterized by pulmonary vein misalignment, capillary paucity and alveolar misdevelopment, decreased FOXF-1 and/or its downstream transcription factor TBX4 disrupt lung microvessel formation and homing to alveolar epithelium [35]. The involvement of FGF signalling in early lung development has been thoroughly investigated. FGF9 positively regulates the expression of *Fgf10* via the upregulation of TBX4 in the lung mesenchyme. Epithelium- and mesothelium-derived FGF9 induce proliferation in both the epithelial and mesenchymal compartments of the developing lung and are also involved in pulmonary vascular development [36]. It has been shown that TBX4 is highly specific in lung fibroblasts and that TBX4 is required for cell proliferation and collagen gel contraction capacity [37]. Transcriptome analysis revealed that TBX4 could broadly regulate fibroblast-related pathways and partly contribute to super-enhancer-mediated transcriptional programmes. *TBX4* knockdown and subsequent RNA-seq were performed in adult fibroblasts. Among the significantly regulated genes, *KCNK6*, *CYP1B1*, *HMOX1* and *ESM1* have been previously shown to be important in PAH pathogenesis [38]. Thus, TBX4 deficiency may compromise lung development and pulmonary vasculature in particular, through insufficient angiogenesis related in part to low FGF expression. In adults, TBX4 is mainly expressed in pulmonary fibroblasts, where it regulates genes and pathways involved in PAH.

The main limitations of this study are first of all the retrospective data collection with a potential underestimation of pediatric cases due to a lack of exhaustivity in genetic screening in early death. Moreover, the population is of limited size, even if it remains the largest adult PAH population of *TBX4* mutation carriers to date. Of note, it is unlikely to gather a much larger cohort of patients, since the prevalence of *TBX4* mutation currently ranges from 2.8% and 7.7% in childhood-onset PAH and from 1.5% and 2.4% in adult-onset PAH [12, 13, 17, 18]. Mutations in the *TBX4* gene have probably been also underdiagnosed because, until recently, physicians were not systematically looking for skeletal abnormalities in PAH patients. Moreover, mutations in the *TBX4* gene have not been tested in all PAH cases, due to its recent association with pulmonary vascular disease. It has been difficult to interpret the hemodynamic response to PAH therapy in this study because of incomplete hemodynamic follow-up in children PAH. Last, the pathologic assessments brought major information on pulmonary vascular and parenchymal involvements but this assessment was limited to a small number of patients, which makes it difficult to generalize the results to the whole population.

In conclusion, PAH due to *TBX4* mutation is an autosomal dominant disease with incomplete penetrance for PAH and/or SPS and generally occurs in sporadic cases. PAH in this population is severe and occurs across a broad age range from birth to late adulthood. Heritable PAH associated with *TBX4* mutations often presents with bronchial and parenchymal radiological abnormalities and low DLCO.

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FIGURES LEGEND

FIGURE 1. Family tree of the familial case, whose four members presented with pulmonary arterial hypertension (PAH) and skeletal anomalies corresponding with incomplete small patella syndrome (SPS) and carried a mutation of the TBX4 gene.

FIGURE 2. Age at PAH diagnosis in the reported PAH cohort

FIGURE 3. High Resolution CT of the chest of patients carrying TBX4 mutation

A: 5-mm thick transverse CT section of the upper lobes, viewed as a maximum intensity projection, in a 34 year-old female patient showing bilateral micronodules suggestive of bronchiolitis.
B: 1-mm thick transverse CT section of the upper lobes, viewed as a minimum intensity projection (same patient as A). Presence of a large, multiloculated diverticula (arrow), directly connected to the posterior wall of the trachea (arrowhead). Note the additional presence of emphysema in both lungs.
C: 2mm-thick coronal lung image obtained in the posterior part of the chest in a 24 year-old male, showing dilated subpleural pulmonary vessels (arrowheads).
D: 1-mm thick transverse CT section (lung image) obtained at the level of the tracheal bifurcation in a 17 year-old female patient. Note the presence of a thin-walled cyst (arrow), adjacent to a peripheral bronchial division in the posterior segment of the right upper lobe.
E: 3-mm thick transverse CT section (mediastinal image) obtained at the level of the right bronchus intermedius (same patient as D). Marked dilatation of right and left bronchial arteries (arrows).
F: Coronal oblique reformation of the chest, viewed as a minimum intensity projection (magnified view of the left hilum) in a 56 year-old male patient. Note the presence of bronchial wall irregularities and numerous adenolectases (arrows) in segmental and subsegmental bronchi of the left upper lobe.

FIGURE 4. Lung histopathology of lung explant from a PAH patient with a *TBX4* mutation

The histological analysis of lung explants showed pulmonary vascular remodelling with plexiform lesions and organized thrombi of some elastic pulmonary arteries. Small pulmonary arteries appeared thickened with marked intimal fibrosis (A). Plexiform lesions were also found close to or within the lumen of small pulmonary arteries (B, C). Eventually, organized thrombi were also identified in some elastic-type pulmonary arteries (D).
Parenchymal alterations were also observed with patchy peribronchiolar, and subpleural fibrosis were identified (E, F). The fibrosis incorporated lymphoid nodules, remodelled vessels and cholesterol cleft deposits (F, star). Eventually, alveolar alterations with rarefaction of alveolar septa resulting in an emphysematous pattern were also noticed (G). Dilation and remodelling of bronchial vessels were additionally found (H).

FIGURE 5. Time to death or lung transplantation of PAH patients carrying TBX4 mutation

The event-free survival was estimated by the Kaplan-Meier method.

Table 1: *TBX4* mutations and signs of SPS identified in PAH patients.

Case	Family	Nucleotide change	Amino acid change	SPS signs
1	1	c.143dup	p.Pro50Thrfs*24	NA
2	2	c.781C>T	p.Arg261*	feet anomalies knee dysplasia
3	3	c.231G>A	p.Trp77*	No abnormities
4	4	c.1119C>G	p.Tyr373*	feet anomalies knee dysplasia
5	4	c.1119C>G	p.Tyr373*	feet anomalies
6	4	c.1119C>G	p.Tyr373*	feet anomalies
7	4	c.1119C>G (obligate carrier)	p.Tyr373* (obligate carrier)	feet anomalies
8	5	c.1021G>C	p.Asp341His	feet anomalies
9	6	c.677C>A	p.Ser226Tyr	NA
10	7	c.153_181del	p.Val54Hisfs*10	knee dysplasia
11	8	c.1458dup	p.Pro487Alafs*16	No abnormities
12	9	c.(549+1_550-1)_(702+1_703-1)del	-	feet anomalies left knee hemarthrosis lack of ossification of ischia
13	10	c.121G>T	p.Gly41*	NA
14	11	Del 17q23.1q23.2(58.1-60.3 Mb)	-	feet anomalies
15	12	c.-584G>A	-	feet anomalies
16	13	c.1160_1167del	p.Thr387Argfs*29	feet anomalies
17	14	c.1119C>A	p.Tyr373*	knee dysplasia
18	15	c.1112dup	p.Pro372Serfs*14	No abnormities
19	16	c.1112dup	p.Pro372Serfs*14	NA
20	17	c.143dup	p.Pro50Thrfs*24	NA

NA: non-available

TABLE 2: Clinical, functional, hemodynamic and biological data of PAH at diagnosis in patients carrying a *TBX4* mutation

		PAH with *TBX4* mutation (n=20)
Age, *years*		29 (0-76)
Gender, *female/male*		15/5
Familial / sporadic PAH		4/16
NYHA functional class, I-II		2 (12.5%)
III-IV		14 (87.5%)
6MWD, *m*	n=14	388 (224-613)
Haemodynamic parameters		
mPAP, *mmHg*	n=20	59.5 (29-87)
PAWP, *mmHg*	n=19	8 (4-14)
CI, $L.min^{-1}.m^{-2}$	n=18	2.71 (1.48-7.60)
PVR, *wood units*	n=18	13.6 (6.2-41.8)
$\mathbf{PVR_i}$, *wood units*.m^{-2}	n=18	19.6 (4.7-46.2)
TPR, *wood units*	n=18	16.3 (7.5-50.3)
Acute vasodilator response	n=17	0
Pulmonary function tests		
$\mathbf{FEV_1}$, *% pred*	n=14	85.5 (50-109)
FVC, *% pred*	n=14	89.5 (62-124)
$\mathbf{FEV_1/FVC}$, *%*	n=14	75 (58-91)
TLC, *% pred*	n=14	93.5 (71-112)
DLCO, *% pred*	n=12	57.5 (43-69)
Arterial blood gases at ambient room air		
$\mathbf{PaO_2}$, *mmHg* (n=8)	n=8	73.3 (61-108)
$\mathbf{PaCO_2}$, *mmHg* (n=8)	n=8	35.5 (29.5-41)
Increased BNP, *>50 pg.mL*$^{-1}$ or **NT-proBNP**, *>300 pg.mL*$^{-1}$	n=12	9 (75%)

Data are presented as the median (min-max).
% pred: percentage of predicted value, 6MWD: 6-Minute Walk Distance, BNP: brain natriuretic peptide, CI: cardiac index, DLCO: diffusing capacity for carbon monoxide

corrected for haemoglobin concentration, FEV_1: Forced expiratory volume in one second, FVC: Forced vital capacity, NYHA: New York Heart Association, mPAP: mean pulmonary artery pressure, NT-pro-BNP: N-terminal pro-brain natriuretic peptide, PaCO2: partial pressure of carbon dioxide in arterial blood, PaO2: partial pressure of oxygen in arterial blood, PAWP: pulmonary artery wedge pressure, PVR: pulmonary vascular resistance, PVR_i: pulmonary vascular resistance indexed on body surface, TLC: total lung capacity, TPR: total pulmonary resistance

TABLE 3: Characteristics of high-resolution CT of the chest in 15 PAH patients carrying a *TBX4* mutation

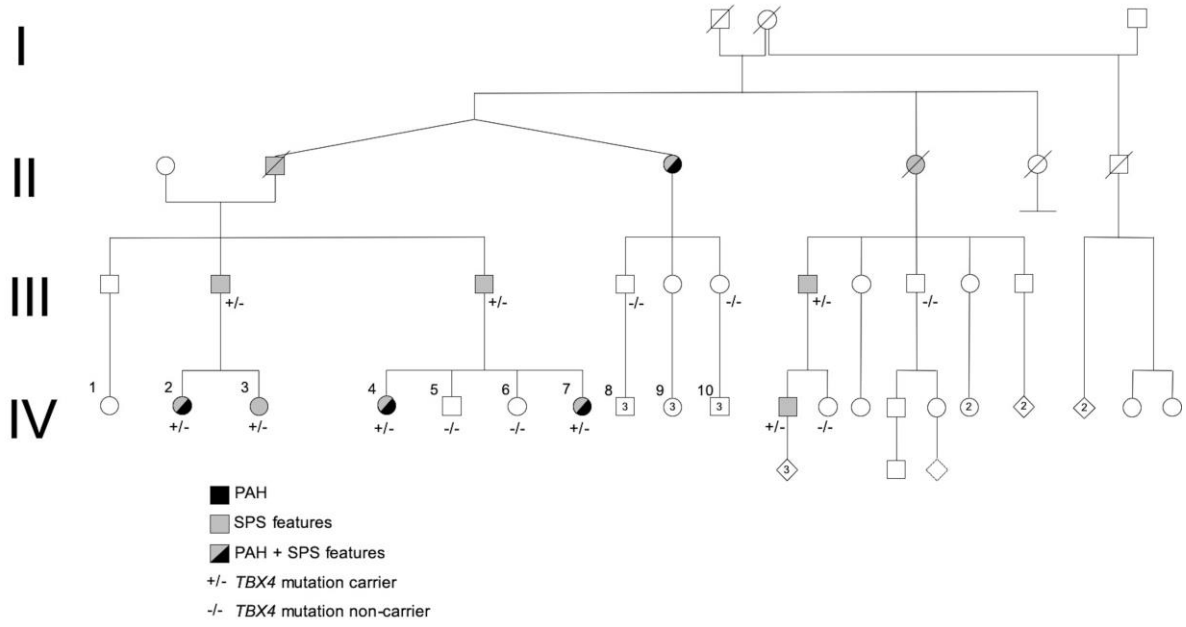
	HRCT of the chest
Bronchial lesions	**9 (60%)**
Thickened and irregular bronchial walls	7 (46.7%)
Tracheal and/or bronchial diverticula	5 (33.3%)
Cysts	4 (26.7%)
Mucoid impactions	3 (20%)
Parenchymal lesions	**13 (86.7%)**
Mosaic distribution	6 (40%)
Subpleural septal lines	4 (26.6%)
Centrilobular micronodules	2 (13.3%)
Ground glass opacities	2 (13.3%)
Condensation	1 (6.7%)
Lymphadenopathies	**6 (40%)**
Mediastinal	6 (40%)
Hilar	3 (20%)
Systemic hypervascularisation	**3 (20%)**
Bronchiolar	3 (20%)
Non-bronchiolar	0
Not analysable	4 (26.7%)

HRCT: high resolution computed tomography

TABLE 4: Clinical, functional, and hemodynamic data at diagnosis and reassessment after initiation of PAH therapy

	Diagnosis (n=13)	**Second assessment** (n=13)	p value
Time between diagnosis and second assessment, *months*	6 (3-11)		
Childhood-onset PAH/Adult-onset PAH	4 / 9		
NYHA functional class, I-II	1 (7.5%)	9 (69%)	< 0.01
III-IV	12 (92.5%)	4 (31%)	
6MWD, *m* (n=12)	388 (224-585)	478 (270-538)	0.01
mPAP, *mmHg*	59 (34-83)	47 (25-64)	0.07
PAWP, *mmHg*	8 (4-14)	8 (4-14)	0.72
CI, $L.min^{-1}.m^{-2}$	2.8 (1.48-7.6)	2.85 (1.82-5.3)	0.62
PVR, *wood units*	11.7 (6.2-41.8)	8.4 (2.2-15.8)	0.17
PVR_i, *wood units*.m^{-2}	18.3 (4.7-29.4)	14.4 (3.3-21.4)	0.15
TPR, *wood units*	14.4 (7.5-50.3)	10.7 (3.7-19.2)	0.15
PAH-specific therapy			
ERA monotherapy		4 (31%)	
PDE5i monotherapy		2 (15.2%)	
ERA + PDE5i		5 (38.6%)	
ERA + Prostacyclin derivative		1 (7.6%)	
ERA + PDE5i + Prostacyclin derivative		1 (7.6%)	

Data are presented as the median (min-max). Comparisons between groups were analysed by the Wilcoxon matched-pair sign rank test and the Wilcoxon signed rank test.
6MWD: 6-Minute Walk Distance, CCB: calcium channel blocker, CI: cardiac index, ERA: endothelin receptor antagonist, NYHA: New York Heart Association, mPAP: mean pulmonary artery pressure, PAWP: pulmonary artery wedge pressure, PDE5i: phosphodiesterase type 5 inhibitors, PVR: pulmonary vascular resistance, PVR_i: pulmonary vascular resistance indexed on body surface, TPR: total pulmonary resistance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PAH
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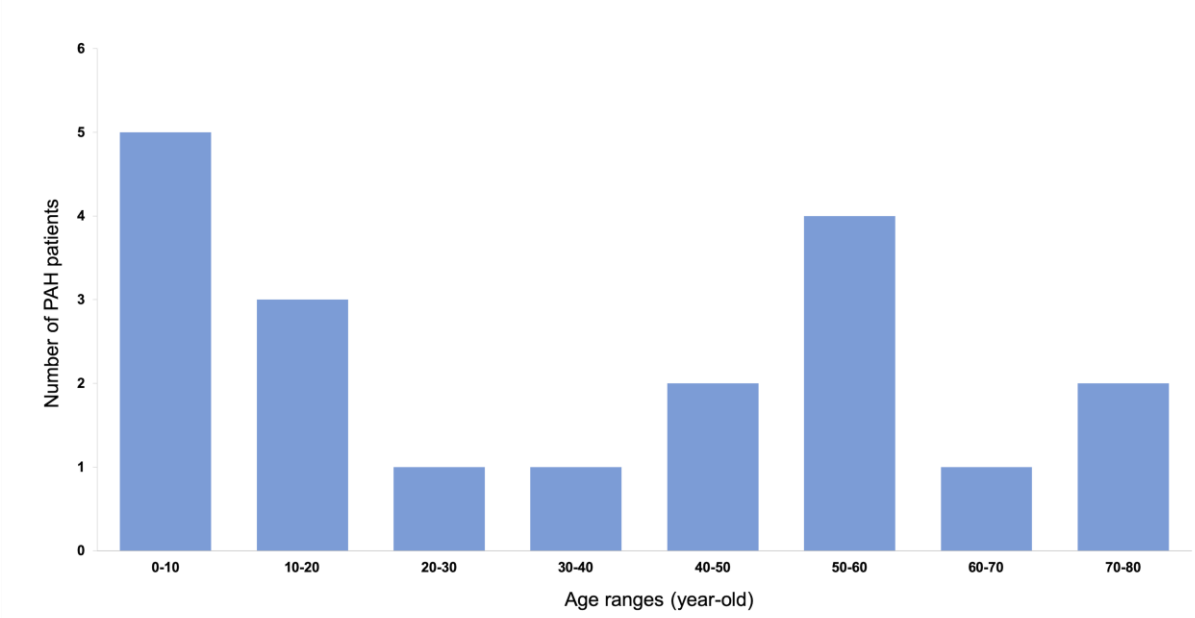
+/- TBX4 mutation carrier
-/- TBX4 mutation non-carrier


Number of PAH patients
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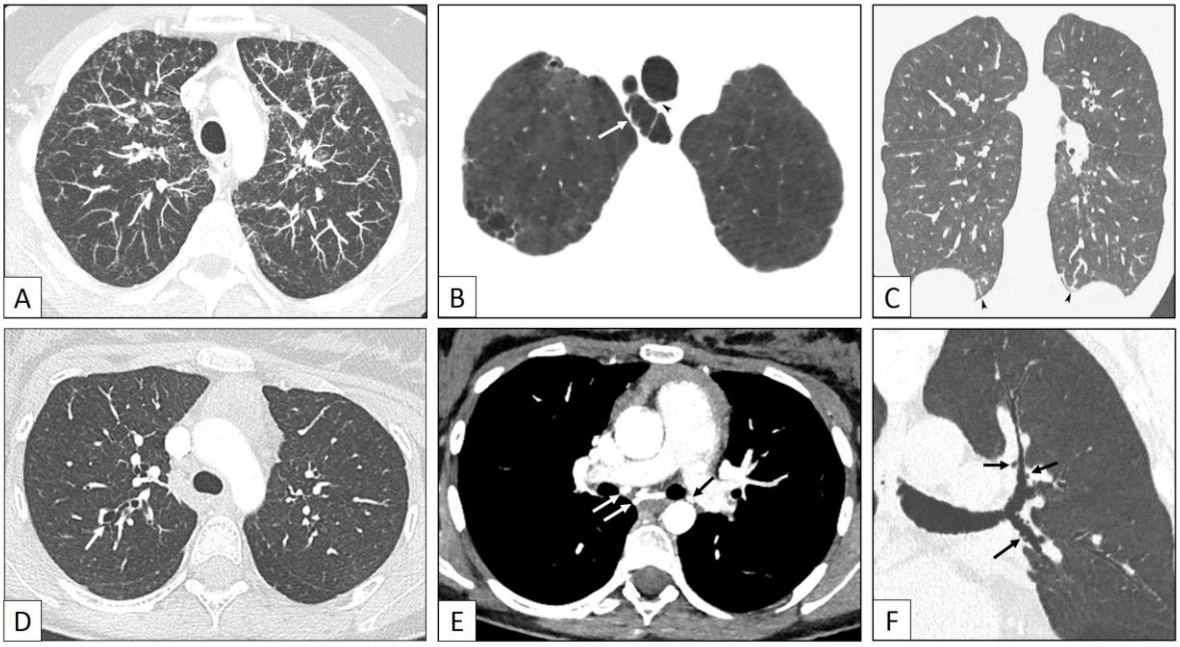
Age ranges (year-old)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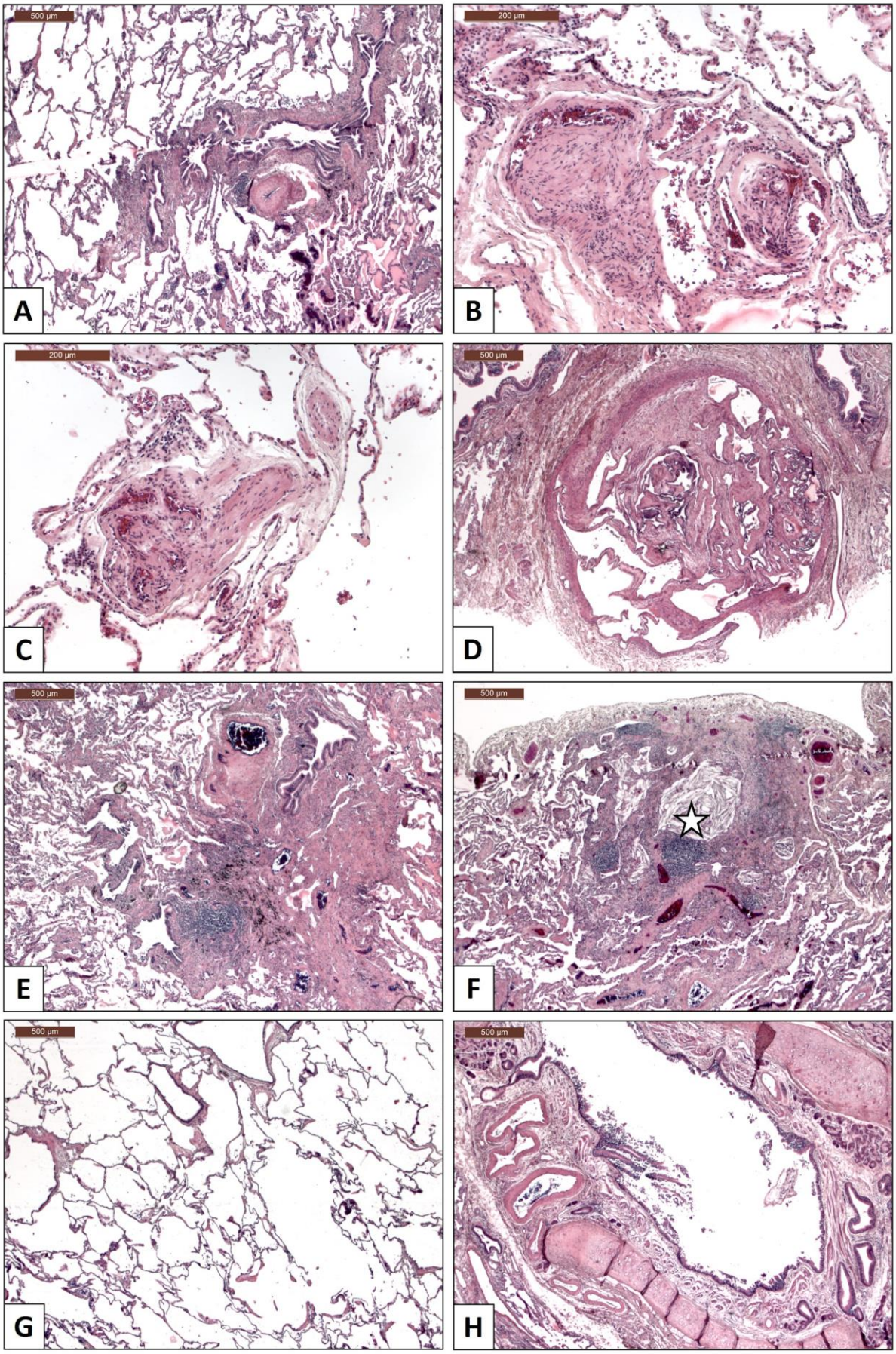
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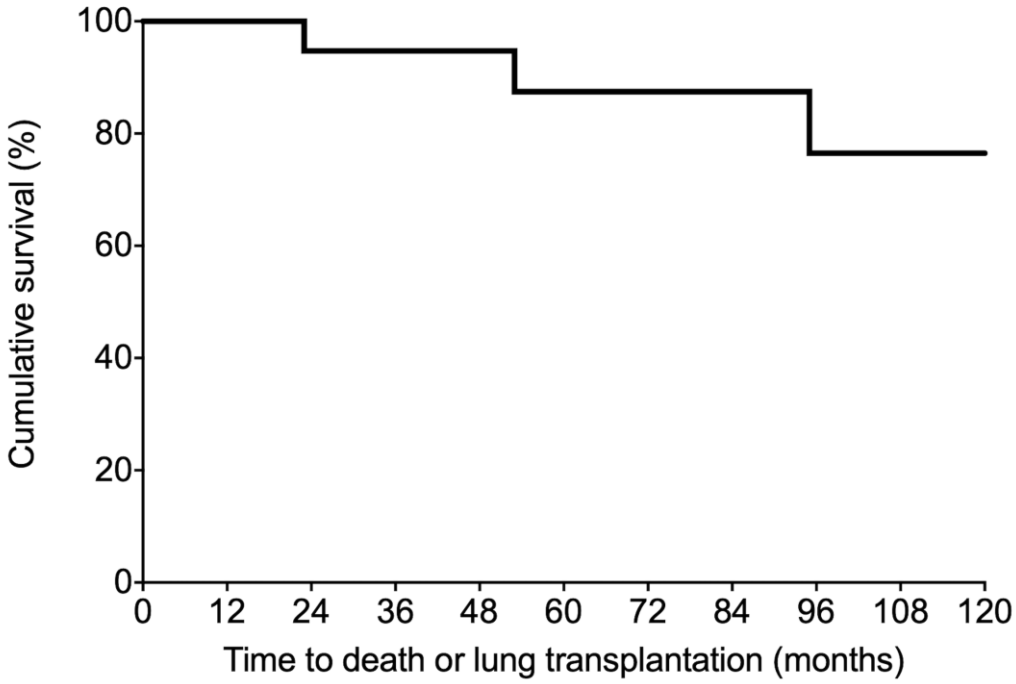

n	20	20	19	18	14	12	12	12	8	8	7

Supplemental table 1: Histopathological features of lung biopsy and explants.

	Patient 12 (surgical biopsy)	**Patient 3** (lung explant)	**Patient 20** (lung explant)
***TBX4* mutation (nucleotide change)**	c.(549+1_550-1)_(702+1_703-1)del	c.231G>A	c.143dup
Lung macroscopy	NA	Small size	Small size
Alveolar structure:			
Alveolar septa	Thin alveolar septa		
Emphysematous changes	Yes		
Interstitial fibrosis and inflammation	Patchy, peribronchiolar and perivascular		
Bronchial anomalies	Peri-bronchial inflammation with cholesterol clefts and hypertrophy of smooth muscle		
Vascular remodeling:			
- Small pulmonary arteries (<500μm)			
Intimal and medial thickening	Yes	Yes	Yes
Plexiform lesions	No	Numerous	Numerous
- Large (elastic-type pulmonary arteries)			
Intimal thickening	NA	Mild	Mild
Organized thrombi		No	Yes

NA: not available