An emerging phenotype of pulmonary arterial hypertension patients carrying *SOX17* variants

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PAH due to *SOX17* variants is a severe phenotype associated with CHD, haemoptysis and radiological anomalies. Histopathology shows severe pulmonary arterial remodelling and malformations affecting pulmonary vessels and systemic arteries. https://bit.ly/3yWSYUP

Cite this article as: Montani D, Lechartier B, Girerd B, *et al*. An emerging phenotype of pulmonary arterial hypertension patients carrying *SOX17* variants. *Eur Respir J* 2022; 0: 2200656 [DOI: 10.1183/13993003.00656-2022].

Received: 28 March 2022
Accepted: 17 May 2022

Abstract

Background The phenotype of pulmonary arterial hypertension (PAH) patients carrying *SOX17* pathogenic variants remains mostly unknown.

Methods We report the genetic analysis findings, characteristics and outcomes of patients with heritable PAH carrying *SOX17* variants from the French Pulmonary Hypertension Network.

Results 20 patients and eight unaffected relatives were identified. The median (range) age at diagnosis was 17 (2–53 years), with a female:male ratio of 1.5. At diagnosis, most of the patients (74%) were in New York Heart Association Functional Class III or IV with severe haemodynamic compromise, including a median pulmonary vascular resistance of 14.0 (4.2–31.5) WU. An associated congenital heart disease (CHD) was found in seven PAH patients (35%). Patients with CHD-associated PAH were significantly younger at diagnosis than PAH patients without CHD. Four patients (20%) suffered from recurrent haemoptysis requiring repeated arterial embolisations. 13 out of 16 patients (81%) for whom imaging was available displayed chest computed tomography abnormalities, including dilated, tortuous pulmonary vessels, ground-glass opacities as well as anomalies of the bronchial and nonbronchial arteries. After a median (range) follow-up of 47 (1–591) months, 10 patients underwent lung transplantation and one patient benefited from a heart–lung transplantation due to associated CHD. Histopathological analysis of lung explants showed a congested lung architecture with severe pulmonary arterial remodelling, subpleural vessel dilation and numerous haemorrhagic foci.

Conclusions PAH due to *SOX17* pathogenic variants is a severe phenotype, frequently associated with CHD, haemoptysis and radiological abnormalities. Pathological assessment reveals severe pulmonary arterial remodelling and malformations affecting pulmonary vessels and thoracic systemic arteries.

Introduction

Pulmonary arterial hypertension (PAH) is an uncommon and severe disease affecting small pulmonary arteries. Although the exact pathophysiology of PAH remains obscure, recent advances have provided a better understanding of cellular and molecular drivers of pulmonary vascular remodelling, including endothelial cell dysfunction, smooth muscle cell abnormalities, inflammation and immune system dysregulation, and imbalance in receptors and ligands of the transforming growth factor-β superfamily [1–3]. Pulmonary vascular remodelling leads to pre-capillary pulmonary hypertension (PH) and progressive right ventricular failure [4]. The main causes of familial/heritable PAH are pathogenic variants in the *BMPR2* gene, which were first described in 2000 [5, 6]. Since then, a number of novel pathogenic variants have been identified in several genes (*TBX4*, *ATP13A3*, *GDF2*, *SOX17*, *AQP1*, *ACVRL1*, *SMAD9*, *ENG*, *KCNK3*, *CAV1*, *GDF2* and *BMP10*) [5].

Gräf *et al*. [7] recently reported that heterozygous variants in the *SOX17* gene were over-represented in a large PAH cohort, with nine patients harbouring *SOX17* pathogenic variants among 1038 PAH index patients. By performing whole-exome sequencing in a PAH cohort with congenital heart disease (CHD), Chung and coworkers [8, 9] identified *SOX17* as a candidate risk gene in ∼3% of patients, suggesting that rare variants in genes regulated by SOX17 also contribute to PAH-CHD. However, the phenotype of PAH patients carrying *SOX17* pathogenic variants remains mostly unknown.

In the present study, we report the clinical, functional, radiological, histological and haemodynamic characteristics as well as the long-term outcomes of PAH patients and relatives from the French PH Network carrying *SOX17* variants.

Methods

Patient selection

We conducted a retrospective population-based study of PAH patients carrying *SOX17* variants from the French PH Network and its National Registry (adult coordinating PH reference centre, Hôpital Bicêtre, AP-HP; paediatric PH competence centre, Hôpital Necker, AP-HP; and 25 PH competence centres across France). This Registry was set up in agreement with French bioethics laws (Commission Nationale de l'Informatique et des Libertés agreement 842063). As previously described [10], genetic counselling was offered to all patients with idiopathic, familial or drug-induced PAH and to first-degree relatives of PAH patients with identified pathogenic variants. All patients provided written informed consent prior to genetic analysis.

Genetic analysis

PAH-predisposing genes, including the *SOX17* gene, were screened by next-generation sequencing (NGS)-based gene panel analysis as previously described [11], Briefly, a custom gene panel including established PAH and pulmonary veno-occlusive disease (PVOD) genes (*BMPR2*, *TBX4*, *EIF2AK4*, *CAV1*, *KCNK3*, *SMAD9*, *ACVRL1*, *ENG*, *GDF2*, *KDR*, *SOX17*, *KDR*, *BMP10*, *AQP1*, *ATP13A3* and *ABCC8*) was used for NGS targeted capture using the HyperCap workflow (Roche/NimbleGen, Pleasanton, CA, USA) following the manufacturer's protocol and sequenced on Illumina platforms (Illumina, San Diego, CA, USA). Data were analysed by a bioinformatic pipeline developed by GenoDiag (Paris, France), which allowed the identification of variants and copy number variants. Each base must be covered by at least 30 reads to be validated. All variants of interest were validated by Sanger sequencing. The *SOX17* DNA sequence was compared with the reference sequence (NM_0022454.3).

Clinical, functional, haemodynamic and radiological characteristics

We reviewed clinical data (age, medical history and physical examination), dyspnoea assessed by modified New York Heart Association (NYHA) Functional Class, 6-min walk distance (6MWD) and pulmonary function tests results, including diffusing capacity of the lung for carbon monoxide corrected for haemoglobin (D_{LCO}).

Haemodynamic measurements obtained during right heart catheterisation (RHC) included mean pulmonary arterial pressure (mPAP), pulmonary arterial wedge pressure (PAWP) and right atrial pressure. Cardiac output (CO) was measured by the standard thermodilution technique or by both the Fick principle and thermodilution when an associated CHD was present. The cardiac index (CI) was calculated as CO/body surface area. Pulmonary vascular resistance (PVR) was calculated as (mPAP−PAWP)/CO. Pre-capillary PH was defined as mPAP >20 mmHg, PAWP ⩽15 mmHg and PVR ⩾3 WU [6]. We *a priori* hypothesised that CHD-associated PAH patients had a different phenotype compared with non-CHD-associated PAH patients and consequently compared the two subgroups.

High-resolution computed tomography (HRCT) of the chest and CT pulmonary angiography (CTPA) in 16 PAH patients and eight relatives carrying a *SOX17* pathogenic variant were analysed by an expert radiologist (M.R-J.) blinded to the clinical diagnosis (PAH patient or healthy *SOX17* variant carrier).

Outcomes, lung transplantation and pathological assessment

Medical therapies approved for PAH (prostacyclin derivatives, endothelin receptor antagonists (ERAs) and phosphodiesterase-5 inhibitors (PDE5is)) were administered according to the clinical judgement and discretion of treating physicians. Time to death or lung transplantation was recorded. Eight lung samples, comprising seven lung explants and one surgical biopsy, from seven patients were analysed.

Statistical analysis

Quantitative variables were expressed as median (minimum–maximum range) and categorical variables were expressed as number (percentage). Comparisons of continuous values were performed using the Mann–Whitney test. Time to death or transplantation was calculated from the time of the diagnosis of PAH and was estimated by the Kaplan–Meier method. The analyses were performed using Prism 8 (GraphPad, San Diego, CA, USA).

Results

Genetic testing

We analysed the *SOX17* gene by targeted panel sequencing in a series of 452 PAH index patients referred to our clinical molecular laboratory for genetic diagnosis of PAH. A *SOX17* pathogenic or likely pathogenic variant (American College of Medical Genetics and Genomics (ACMG) class 4 and 5) was identified in 14 (3.1%) index patients, corresponding to 12 sporadic idiopathic PAH and two familial PAH with cases identified over two or three generations (figure 1a). Parental DNA samples were available for four index cases and *SOX17* variants were identified *de novo* in two patients (table 1). Genetic counselling and testing was offered to 18 first-degree relatives and eight of them (44.4%) carried *SOX17* pathogenic variants (table 1).

13 distinct *SOX17* pathogenic or likely pathogenic variants were identified. Among these, nine had never been described (table 1). Two were truncating variants, c.788dup, p.(Glu264Glyfs*101) and c.499_520del, p.(Leu167Trpfs*213), leading to a loss of the SOX C-terminal domain of the SOX17 protein, which includes the β-catenin-binding domain. The c.499_520del, p.(Leu167Trpfs*213) variant was identified in two unrelated index patients and was previously described in two distinct patients [7]. The c.788dup, p.(Glu264Glyfs*101) variant had never been described, but a deletion of this same amino acid leading to a truncated protein, p.(Pro263Argfs*124), was previously described [12]. The remaining 11 pathogenic or likely pathogenic variants were missense variants. 10 were classified as likely pathogenic variants according to ACMG guidelines (ACMG class 4) [13, 14] since they are located in the high mobility group (HMG) box, a conserved region known as a mutational hotspot of the *SOX17* gene (figure 1b). *In silico* prediction favours a deleterious effect and they are not found or are found at a very low frequency in the Genome Aggregation Database (gnomAD) database (table 1). One variant, c.416C>T, p.(Pro139Leu), was classified as a pathogenic variant (ACMG class 5) since parental DNA analysis established that it was a *de novo* variant. Q3

Three additional *SOX17* variants were identified in three patients with sporadic idiopathic PAH. These variants were classified as ACMG class 3 since they were not located in the HMG box (figure 1b).

Patient population

20 PAH patients carrying a *SOX17* variant were identified in the French PH Registry: 15 sporadic PAH and five familial PAH from two families (four *SOX17* variants confirmed by genetic testing and one obligate carrier) (table 2). The median age at diagnosis was 17 (2–53) years, with a female predominance (female:male ratio 1.5). Half of the patients carrying a *SOX17* variant (n=10) presented with childhood-onset PAH (age <18 years). The distribution of age at diagnosis is presented in figure 2a. Associated CHD was found in 35% of *SOX17* variant carriers (n=7), including four atrial septal defects (ASDs) of the ostium secundum type and three ventricular septal defects (VSDs). *SOX17* variant carriers with CHD-associated PAH were significantly younger at PAH diagnosis compared with patients without CHD (median age at diagnosis 10 (2–21) *versus* 26 (8–53) years; p=0.004) (figure 2b). A patent foramen ovale (PFO) was found in three additional patients. Recurrent haemoptysis was reported in four (20%) patients, requiring multiple bronchial artery embolisations in three of them (table 2).

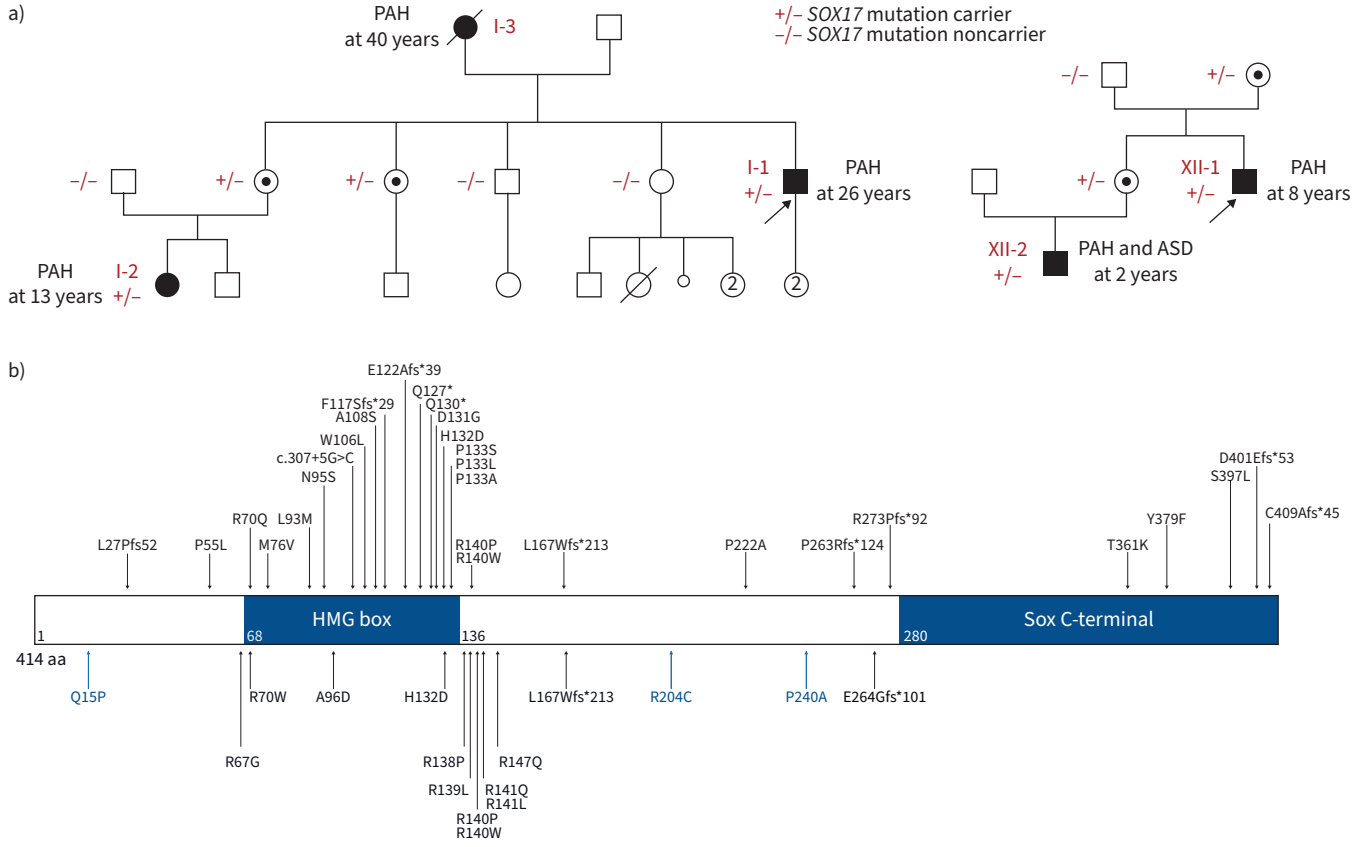

FIGURE 1 Family trees of the familial cases and identified *SOX17* variants. a) Family trees of the familial cases: Family I (left) and Family I XII (right) (table 1). Family I included three pulmonary arterial hypertension (PAH) patients (filled shapes), two of whom had identified *SOX17* mutations. The third PAH patient died at age 40 years before mutation identification. Two asymptomatic carriers were identified. Family XII included two PAH patients diagnosed at age 8 and 2 years. Atrial septal defect (ASD) was identified in the younger PAH patient. Two asymptomatic carriers were identified. b) *SOX17* gene representation and identified variants. Upper lane: variants previously published [11, 12, 16, 31]; lower lane: variants identified in this study; blue arrows: variant of uncertain significance. HMG: high mobility group; aa: amino acids. Q11 Q12 Q13 Q14 Q15

Eight first-degree relatives carrying a *SOX17* pathogenic variant were identified. All of them were asymptomatic and reported no medical history of cardiorespiratory disease. In those healthy subjects, echocardiography did not reveal any sign suggesting PH or CHD.

Clinical, functional and haemodynamic assessment findings at PAH diagnosis

At diagnosis, 74% of patients carrying a *SOX17* variant were in NYHA Functional Class III or IV. 6MWD at diagnosis was available in nine patients aged ⩾13 years, showing a median distance of 460 (285–600) m. Pulmonary function tests were available at diagnosis in 13 patients, showing a reduced D_{LCO} (63% (44–97%) of predicted values) and arterial oxygen tension (P_{aO_2}) of 73 (49–97) mmHg. Q4

RHC at diagnosis was available for all patients and revealed severe pre-capillary PH, with a markedly elevated mPAP of 64.4 (33–105) mmHg, a normal PAWP of 7 (1–14) mmHg, a CI of 2.9 (1.7–4.4) $L \cdot min^{-1} \cdot m^{-2}$ and an increased PVR of 15.6 (4.2–31.5) WU (table 3). Acute vasodilator testing performed in 15 patients showed a mean PVR decrease of 24% (0–55%), but only two fulfilled the criteria for acute vasodilator response according to guidelines [4, 6]. CHD-associated PAH patients had a median PVR of 15.1 WU compared with 12.1 WU in PAH patients without CHD (p=0.135). Q5 Q6 Q7

TABLE 1 Identified *SOX17* variants

Patient	Relatives	HGVS c.	HGVS p.	gnomAD MAF (%)	CADD Phred score	Inheritance	ACMG class	Type of PAH
I-1/I-2	2	c.422G>A	p.(Arg141Gln)	NF	34	ND	4	Familial
II		c.287C>A	p.(Ala96Asp)	NF	32	ND	4	Sporadic
III	2	c.208C>T	p.(Arg70Trp)	NF	35	Maternal	4	Sporadic
IV		c.499_520del[#,¶]	p.(Leu167Trpfs*213)	NF	ND	*De novo*	5	Sporadic
V		c.610C>T	p.(Arg204Cys)	NF	33	ND	3	Sporadic
VI		c.499_520del[#,¶]	p.(Leu167Trpfs*213)	NF	ND	ND	5	Sporadic
VII		c.440G>A	p.(Arg147Gln)	NF	29.8	ND	4	Sporadic
VIII	2	c.199C>G	p.(Arg67Gly)	0.0032	26.8	Maternal	4	Sporadic
IX		c.416C>T	p.(Pro139Leu)	NF	29.9	*De novo*	5	Sporadic
X		c.422G>T	p.(Arg141Leu)	NF	33	ND	4	Sporadic
XI		c.788dup	p.(Glu264Glyfs*101)	NF	ND	ND	5	Sporadic
XII-1/XII-2	2	c.413G>C	p.(Arg138Pro)	NF	32	ND	4	Familial
XIII		c.419G>C[¶]	p.(Arg140Pro)	NF	32	ND	4	Sporadic
XIV		c.718C>G	p.(Pro240Ala)	NF	15.9	ND	3	Sporadic
XV		c.44A>C	p.(Gln15Pro)	0.0005	25.8	ND	3	Sporadic
XVI		c.418C>T[¶]	p.(Arg140Trp)	NF	35	ND	4	Sporadic
XVII		c.394C>G[¶]	p.(His132Asp)	NF	26.2	ND	4	Sporadic

A CADD Phred score ⩾20 indicates the 1% most deleterious variants in the genome sequence. HGVS: Human Genome Variation Society (coding DNA reference sequence (c.) and protein reference sequence (p.)); gnomAD: Genome Aggregation Database; MAF: minor allele frequency; CADD: Combined Annotation Dependent Depletion; ACMG: American College of Medical Genetics and Genomics; NF: not found, ND: not determined; PAH: pulmonary arterial hypertension. [#]: this variant was found in two distinct unrelated patients; [¶]: variants previously described in the literature.

Radiological findings

16 patients carrying a *SOX17* variant had analysable HRCT and CTPA at PAH diagnosis (table 4 and figure 3). Eight unaffected relatives carrying a *SOX17* variant had analysable HRCT and CTPA. 13 patients (81%) and two healthy relatives (25%) had abnormal radiological findings. In PAH patients, HRCT showed dilated and tortuous pulmonary vessels in 13 PAH patients (81%), most commonly associated with adjacent ground-glass opacities (75%) and micronodular scissural abnormalities (75%). Bronchial, nonbronchial and mediastinal systemic arteries were dilated in 11 (69%), six (38%) and five (31%) PAH patients, respectively. Systemic pulmonary shunts were noticed in six (38%) PAH patients. Two healthy relatives (25%) had mild radiological abnormalities (dilated and tortuous pulmonary vessels) (table 4).

Pathological assessment of the lungs

Eight lung samples were available in seven patients carrying a *SOX17* variant: one surgical lung biopsy and seven lung explants (figures 4 and 5, and supplementary table S1). The main histopathological features were severe pulmonary arterial remodelling with plexiform lesions (eight out of eight (100%)), a congestive parenchyma (eight out of eight (100%)), and pleural and subpleural vessel dilation (eight out of eight (100%)) in addition to dilated bronchial arteries (five out of eight (63%)). The overall architecture of the lungs was preserved, although congested, with numerous foci of alveolar haemorrhages. Singular millimetric fibrovascular lesions were frequently identified adjacent to the plexiform lesions, as previously described in other forms of heritable PAH due to *BMPR2* mutations [15]. Septal veins were often dilated, occasionally with thickened vessel walls. Pulmonary venous remodelling was, however, heterogeneous, without signs suggestive of PVOD or pulmonary capillary haemangiomatosis. Significant dilation of bronchial systemic arteries was noticed, with enlarged peribronchial vasa vasora. In two lung explants, bronchial artery embolisation material was detected (figure 4h) and the embolising agent was identified within a subpleural vein in one patient (figure 5c). In three patients, there was a thickened pleura associated with haemorrhagic pleural and subpleural lesions, suggesting a haemothorax. Pulmonary infarction sequelae were also noticed in these three patients.

Follow-up and outcomes

After a median follow-up of 46 (1–591) months, more than half of the patients carrying a *SOX17* variant (n=11) received a lung transplantation (10 double lung and one heart–lung transplantation due to uncorrectable CHD). Transplantation was performed with a median delay from diagnosis of 114 (7–167) months. Eight patients (40%) were alive and treated with PAH drugs after a median follow-up of 29 (1–591) months.

TABLE 2 Clinical characteristics of signs of pulmonary arterial hypertension (PAH) patients carrying a *SOX17* mutation (n=20)

Patient	Sex	Age at PAH diagnosis (years)	CHD/ PFO	Haemoptysis	Other medical conditions
I-1	Male	26	No	Yes	
I-2	Female	13	No	No	
I-3	Female	40	PFO	No	
II	Female	21	ASD	No	Stroke
III	Female	35	No	No	
IV	Female	6	ASD	No	
V	Female	24	No	No	
VI	Male	32	PFO	No	
VII	Female	13	VSD	No	
VIII	Female	12	VSD	Yes (2 BAEs)	Epistaxis
IX	Female	36	No	Yes	Recurrent miscarriages, optic neuromyelitis
X	Female	10	ASD	Yes (3 BAEs)	
XI	Female	3	VSD	No	
XII-1	Male	8	PFO	No	
XII-2	Male	2	ASD	No	
XIII	Female	18	No	No	
XIV	Male	27	No	No	
XV	Male	53	No	No	
XVI	Male	10	No	No	
XVII	Male	16	No	Yes (4 BAEs)	

CHD: congenital heart disease; PFO: patent foramen ovale; ASD: atrial septal defect; VSD: ventricular septal defect; BAE: bronchial artery embolisation.

At last follow-up or before death or lung transplantation, 45% of the patients carrying a *SOX17* variant (n=9) were treated with triple PAH therapy associating an ERA, a PDE5i and a prostacyclin analogue, 40% (n=8) were receiving dual combination therapy (ERA in combination with either PDE5i or prostacyclin analogue) and one remained on monotherapy (ERA). Two patients with an acute vasodilator response were initiated on calcium channel blocker monotherapy: the first patient died suddenly within 1 month of diagnosis, while the second patient had a satisfactory long-term response to calcium channel blocker monotherapy.

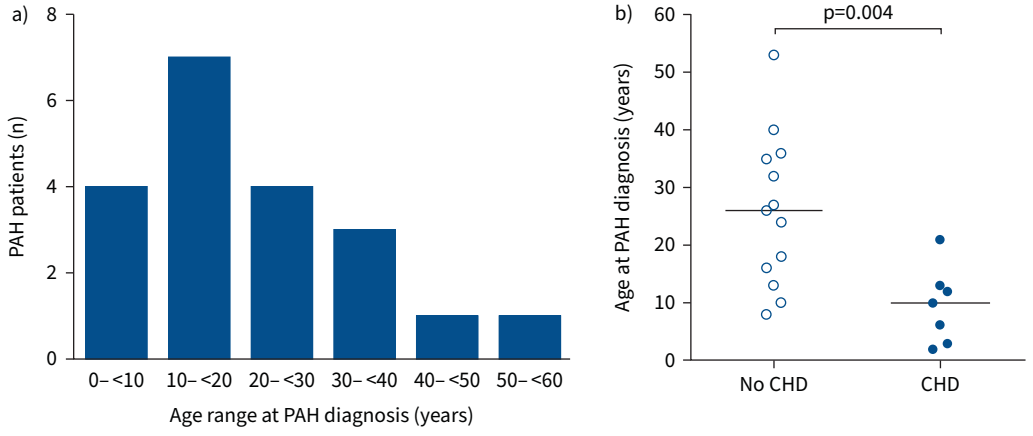

FIGURE 2 Patient age at pulmonary arterial hypertension (PAH) diagnosis. a) Age at diagnosis of the whole cohort (n=20). Median age at diagnosis was 17 (2–53) years. b) Age at diagnosis according to the presence of congenital heart disease (CHD). Patients with CHD-associated PAH were significantly younger. Median is indicated.

TABLE 3 Clinical, functional and haemodynamic assessment parameters at the diagnosis of pulmonary arterial hypertension (PAH) in patients carrying a *SOX17* mutation Q24

Patient	Age at PAH diagnosis (years)	NYHA FC	mPAP (mmHg)	CI ($L \cdot min^{-1} \cdot m^{-2}$)	PVR (WU)	Acute NO response	ΔPVR (%)	6MWD (m)	P_{aO2} (mmHg)	D_{LCO} (% pred)
I-1	26	II	79	3.00	16.4	Yes	−40	580	67	44
I-2	13	III	68	2.65	17.3	No		391	93	69
I-3	40	IV	70	1.80	23.5				70	
II	21	III	92	1.70	31.5				54	50
III	35	IV	66	1.70	17.7	No	−5	373	57	72
IV	6	III	81	2.90	29.6	No	−28	322		
V	24	III	54	2.96	9.0	Yes	−55	491	97	74
VI	32	III	58	2.10	12.0	No	−28	460	61	62
VII	13	II	76	3.00	15.1	No	−27	285	49	60
VIII	12	III	105	4.40	12.8	No	−28			
IX	36	III	41	3.80	4.2				79	60
X	10	III	87	1.90	30.0		−31			
XI	3	III	51	3.90	12.8	No	−29		78	
XII-1	8	II	49	3.71	8.2	No	−20	370	87	97
XII-2	2		38	5.38	9.6	No	−9			
XIII	18	III	80	2.30	20.3	No		460	84	63
XIV	27	II	37	3.00	7.5	No	−10	525	92	66
XV	53	III	43	2.20	10.3		0		54	56
XVI	10	III	80	3.25	17.3	No	−20			
XVII	16	II	33	2.50	6.2	No	−24	600	92	81

NYHA: New York Heart Association; FC: Functional Class; mPAP: mean pulmonary arterial pressure; CI: cardiac index; PVR: pulmonary vascular resistance; NO: nitric oxide; ΔPVR: percentage of PVR decrease after NO challenge; 6MWD: 6-min walk distance; P_{aO_2}: arterial oxygen tension; D_{LCO}: diffusing capacity of the lung for carbon monoxide corrected for haemoglobin concentration.

Lung transplantation procedures were associated with a relatively high frequency of complications. Grade 3 primary graft dysfunction occurred in five of the 11 transplanted patients (45%). Moreover, lung transplantation early outcomes were remarkable with an haemothorax complicating three out of 11 procedures (27%).

Discussion

We report the phenotype and outcomes of 20 childhood-onset and adult-onset PAH patients carrying a *SOX17* variant. These patients are remarkable because they present with a variety of cardiovascular characteristics including severe pulmonary arterial remodelling with plexiform lesions, dilated systemic bronchial and nonbronchial arteries, and CHD. Indeed, ASD or VSD was observed in one-third of our patients, mainly in childhood-onset disease, underscoring that PAH patients presenting with an associated

TABLE 4 Characteristics of high-resolution computed tomography of the chest in 16 pulmonary arterial hypertension (PAH) patients and eight asymptomatic relatives carrying a *SOX17* mutation Q25

	PAH patients (n=16)	Relatives (n=8)
Dilated and tortuous pulmonary vessels	13 (81)	2 (25)
Ground-glass halos adjacent to pulmonary vessels	12 (75)	1 (13)
Micronodular scissural abnormalities	12 (75)	0
Diffuse ground-glass opacities	11 (69)	0
Dilated bronchial arteries	11 (69)	1 (13)
Dilated nonbronchial systemic arteries	6 (38)	0
Systemic pulmonary shunts	6 (38)	0
Mediastinal systemic hypervascularisation	5 (31)	0

Data are presented as n (%).

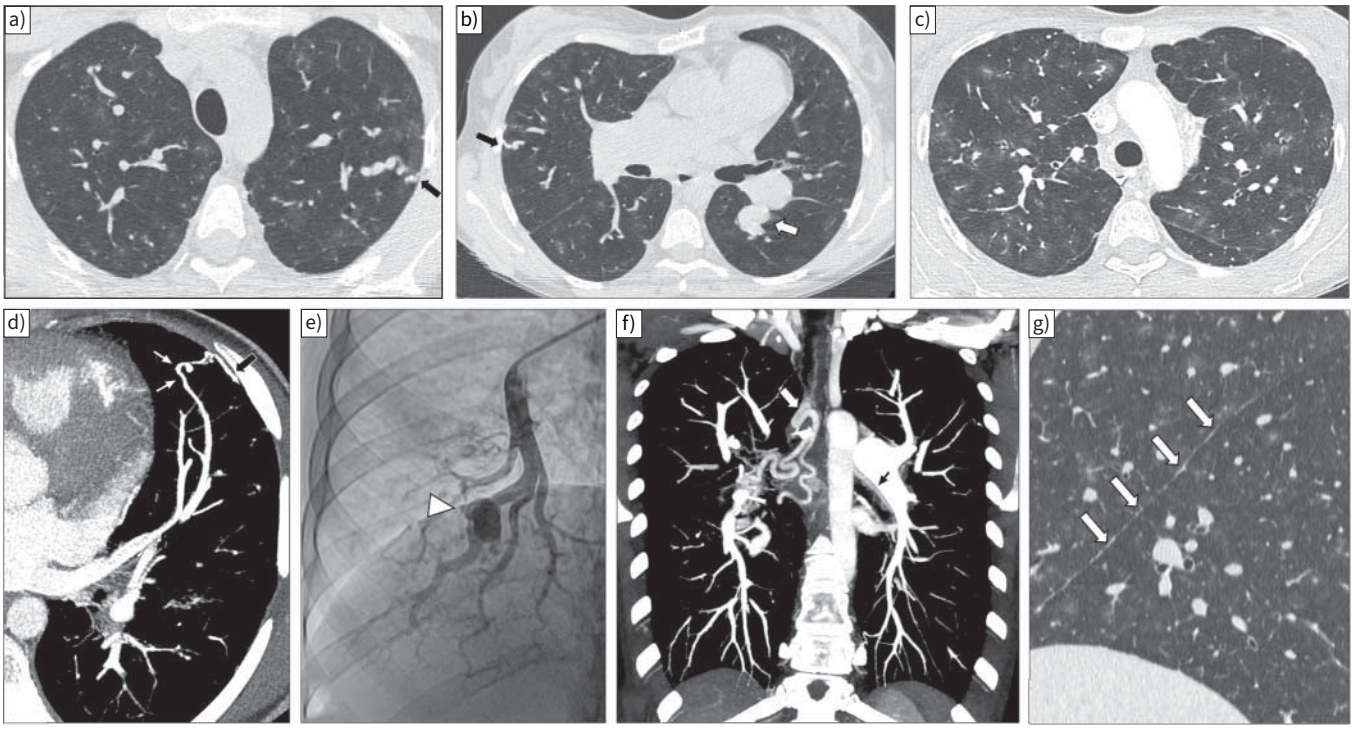

FIGURE 3 Representative high-resolution computed tomography (HRCT) of the chest, CT pulmonary angiography (CTPA) and pulmonary angiogram of pulmonary arterial hypertension patients carrying a *SOX17* pathogenic variant. a–c) Thin-collimated HRCT of the chest showing a) subpleural dilated and b) tortuous pulmonary vessels (black arrows) and c) ground-glass opacities. d) The black arrow points to direct communication between a dilated distal pulmonary artery and a dilated intercostal artery. b, e) Aneurysmal dilatation identified on b) CTPA (white arrow) and e) pulmonary angiography (arrowhead). f) Marked dilatation of proximal bronchial arteries is frequently observed (white and black arrows). g) The presence of numerous fissural irregularities (white arrows) suggests the additional presence of dilated systemic vessels at the pleural surface. Q17 Q18

CHD may have a heritable disease caused by a *SOX17* pathogenic variant. Lung HRCT of the chest and CTPA detected ground-glass opacities and abnormal pulmonary arteries, systemic bronchial and nonbronchial arteries. More than half of our patients presented with severe clinical and haemodynamic impairment refractory to PAH therapy justifying lung transplantation, which was associated with frequent complications (notably primary graft dysfunction and haemothorax). Lung histopathology showed severe pulmonary arterial remodelling, subpleural vessel dilation and numerous haemosiderin-laden macrophages. Q8

The radiological and histopathological findings depicted in our cohort are likely to be related to the embryological role of SOX17. Members of the SRY-related HMG box (SOX) family are shown to be essential for the regulation of numerous developmental processes, where SOX17 acts as a crucial regulator in pulmonary vascular morphogenesis [16, 17] and cardiovascular development [18]. SOX17 is a transcription factor implicated in the regulation of various cell developmental processes, notably endoderm formation and tumour angiogenesis [16, 19], but is also required for the normal development of the pulmonary vasculature [17, 20]. Although SOX17 has a well-demonstrated role in embryogenesis, PAH occurrence at adult age in some patients suggests that SOX17 is also implicated in pulmonary vascular remodelling in adults. Approximately 20 SOX genes have been characterised and three SOX group F genes (*SOX7*, *SOX17* and *SOX18*) are coexpressed in vascular endothelial cells [21]. In animal models, SOX7, SOX17 and SOX18 have overlapping roles in postnatal neovascularisation, and vascular endothelial-specific deletion of all three leads to massive oedema [22]. However, SOX17 plays a key role in developing and maintaining arterial endothelial cell specificity and integrity [16]. SOX17 is selectively expressed in arteries (but not in veins) and *SOX17* knockout reduces the expression of arterial-specific genes and induces the expression of venous-specific genes [16]. In a mouse postnatal retina model, the inhibition of *Sox17* expression in endothelial cells led to strong vascular hypersprouting, a loss of arterial integrity and large arteriovenous malformations [16, 23]. These studies support the critical role of SOX17 in angiogenesis, maintenance of vascular homeostasis and arterial specificity [20]. The SOX family is characterised by a highly conserved HMG box, a sequence-specific DNA-binding domain, where most of

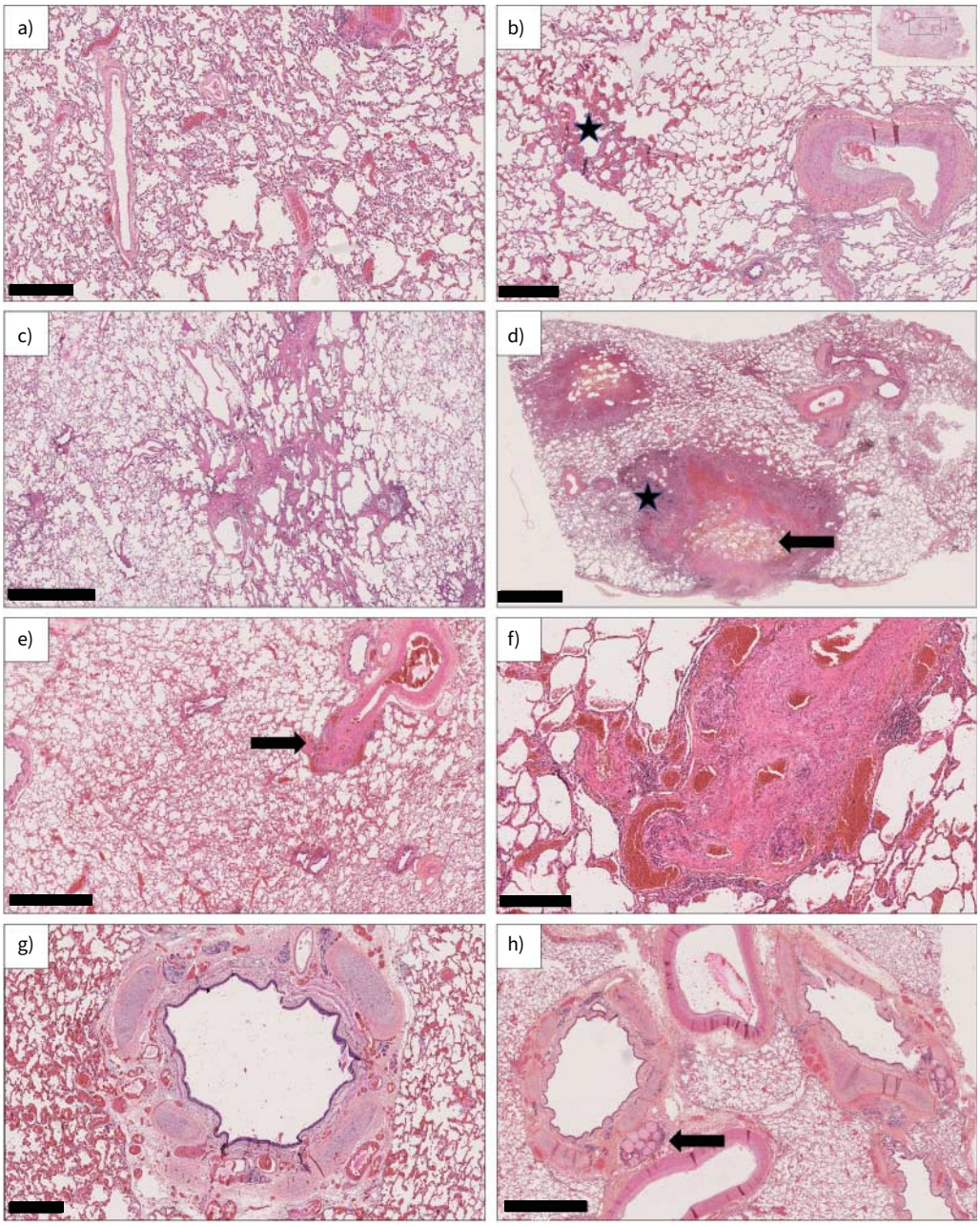

FIGURE 4 Histopathology of lung explants from pulmonary arterial hypertension (PAH) patients carrying a *SOX17* variant. a) Lung parenchyma overview from a *SOX17* mutated PAH explant. Gross architecture is not altered. Alveolar septa are enlarged by congestion and cell density appears augmented by alveolitis. No capillary haemangiomatosis foci are visible. b) Foci of capillary congestion and dilation (star) are easily found within the lung parenchyma. c) Parenchymal scarring (with fibrosis and alveolar collapse), likely subsequent to a haemorrhagic infarction. d) Subpleural haemorrhagic infarction displaying a necrotic core (arrow) with peripheral resorptive histiocytic infiltrate (star). e) Plexiform lesions adjacent to the pulmonary artery are easily found (arrow). f) Enlarged image focused on the plexiform lesion showing fibrosis and vessel dilation. g) Bronchial vessels and capillaries are dilated. h) Bronchial vessels are markedly dilated. Embolizing material is seen in the bronchial vessels (arrow). Scale bars: a) 500 µm; b, g) 600 µm; c, e, h) 2 mm; d) 3 mm; f) 400 µm. Q19

the missense variants are located. Site-directed mutagenesis studies have shown that missense mutations within this region can impair both direct DNA binding [24] and SOX17/β-catenin protein complex interactions [25], demonstrating that sequence alteration within this domain has a strong functional impact on the SOX17 protein.

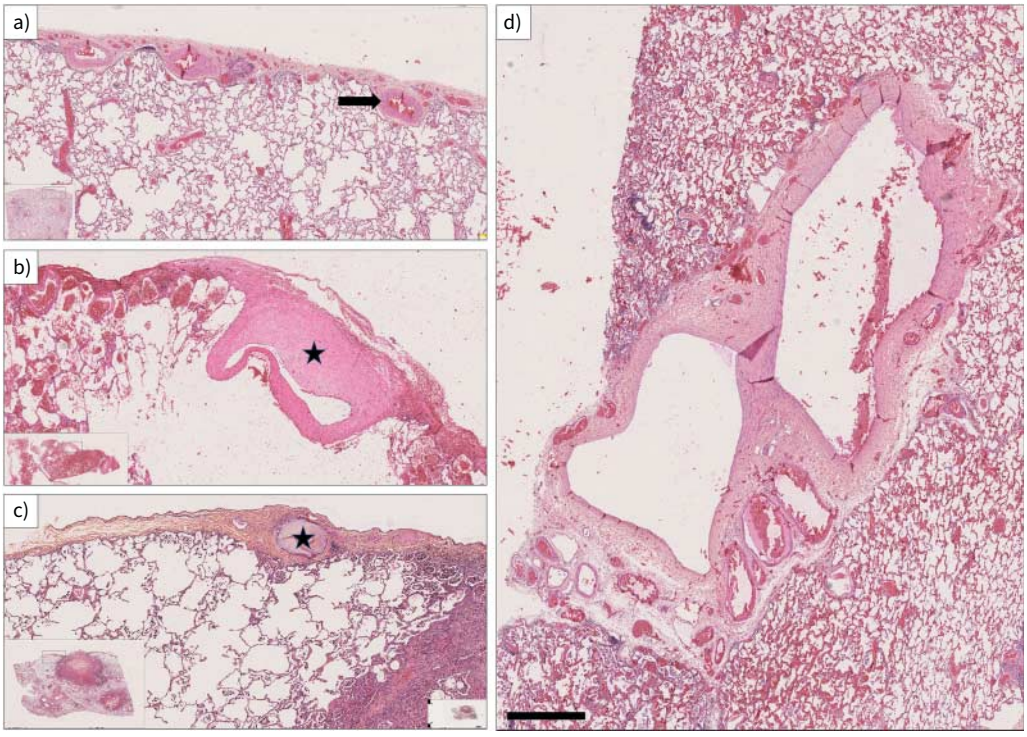

FIGURE 5 Histopathology of lung explants from pulmonary arterial hypertension patients carrying a *SOX17* variant. a, b, d) Subpleural veins are markedly thickened and dilated (arrow in a; star in b). c) Bronchial artery embolisation material is found in the subpleural vein in this explant (star). Scale bar: a) 1 mm.

We identified a *SOX17* pathogenic variant in two unrelated patients with familial PAH. Segregation analysis was then performed in seven additional family members of these two patients, and the *SOX17* variant was found in the two PAH patients and in four unaffected relatives (figure 1a). Considering these family trees and the higher frequency of the sporadic PAH cases, we conclude that PAH due to *SOX17* mutations has an autosomal transmission with incomplete penetrance. This genetic inheritance, as well as the female predominance, are consistent with what has been described previously for other PAH predisposition genes such as *BMPR2*, the leading cause of heritable PAH [5, 26, 27]. PAH due to *SOX17* mutations was relatively rare in previously reported idiopathic or familial PAH cohorts, with *SOX17* variants found in ∼0.9% and ∼0.7% in reports of 1038 [7] and 413 PAH patients [8], respectively. In our present study, ACMG class 4 and 5 variants were identified in 3.1% of PAH index patients. We intentionally included ACMG class 3 variants in our study in order to have a more exhaustive cohort, although their pathogenicity is not demonstrated. It appears, however, that one of the two patients harbouring a class 3 variant (Patient XV) had a severe haemodynamic impairment (PVR 10.3 WU) and underwent double lung transplantation 7 months after diagnosis (with pathological abnormalities similar to what was noticed in other patients). The role of *SOX17* in PAH was only recently reported and its mutation frequency was undoubtedly underestimated until now. Genetic screening in patients with CHD-PAH was not routinely done before evidence of the role of certain development genes such as *TBX4*, *SOX17* and *KDR* was demonstrated. In the French PH Referral Centre, children and adults with CHD-PAH are systematically screened with a genetic panel comprising those genes, which explains an increased identification of these mutations in our cohort.

Rare deleterious *SOX17* variants have also been identified in ∼3.2% of patients with CHD-associated PAH in another cohort of 256 patients, suggesting an over-representation of *SOX17* variants in this form of PAH [8]. These results are in accordance with those of our report, in which one-third of *SOX17* variant carriers with PAH presented with an associated CHD, a finding reminiscent of that observed with other developmental genes associated with heritable PAH, such as *TBX4* [26, 28]. Saba *et al.* [29] demonstrated that Sox17 mesoderm-specific loss of function in mouse embryos is associated with cardiac defects, which could explain this frequent association. Interestingly, *SOX17* variant carriers with PAH and CHD were significantly younger at PAH diagnosis (childhood onset) compared with *SOX17* variant carriers with isolated PAH (adulthood

onset) (figure 2). We hypothesise that two non-mutually exclusive mechanisms could explain this difference. First, coexisting PAH and CHD may be the consequence of a major development impairment caused by dysfunction of SOX17, a key player of cardiac and pulmonary vascular development [18, 20]. Second, CHD could act as a second hit triggering accelerated pulmonary vascular remodelling in a predisposed dysfunctional pulmonary circulation related to *SOX17* mutation.

At diagnosis, SOX*17* variant carriers with PAH presented with severely compromised haemodynamic parameters, with a median mPAP of 64.4 mmHg and a median PVR of 14.0 WU. These parameters are in line with the haemodynamic severity reported in large series of PAH patients with a *BMPR2* mutation [27, 30]. HRCT of the chest and CTPA identified a *SOX17* variant carrier radiological phenotype with numerous vascular abnormalities (dilated and tortuous vessels associated with micronodular and ground-glass opacities), which are not usual findings of idiopathic PAH [31]. Pathological examination can explain these radiological findings, with evidence of a congested architecture, dilated vessels (notably in the subpleural zones) and severe pulmonary arterial remodelling with plexiform lesions. Moreover, ground-glass opacities seen at HRCT are most likely explained by the micro-haemorrhagic foci depicted in lung histopathology. Q9

SOX17-associated vascular malformations are associated with recurrent haemoptysis, frequently requiring bronchial artery embolisation and eventually listing for lung transplantation. Increased haemoptysis risk was already described in *BMPR2* mutation carriers [15]. Considering the risk of severe life-threatening haemoptysis in *SOX17* mutation carriers, further research is needed to evaluate the value of preventive systemic artery embolisations once such vascular malformations are identified. However, such preventive procedures would not be exempt from potential risks, at least in part due to the presence of arteriovenous shunts. These shunts are seen on CTPA and further supported by identification of embolisation material in subpleural veins in a lung explant following embolisation, supporting the presence of an anatomical communication between bronchial arteries and pulmonary veins (figure 5).

The overall prognosis of PAH patients carrying a *SOX17* mutation is poor, with more than half of our patients receiving transplantation at follow-up and one early death in a patient treated only with calcium channel blockers. Transplant-free survival is, however, difficult to evaluate since diagnosis was made in several prevalent PAH cases. Our data suggest a high risk of complications following lung transplantation, notably haemothorax and primary graft dysfunction, that should be firmly confirmed in larger multicentre studies. Periprocedural haemorrhagic complications may be explained at least in part by lung histopathology showing dilated and fragile vessels, notably within the subpleural space (supplementary table S1). Patients carrying a *SOX17* pathogenic variant should thus be managed by a trained lung transplantation team with extreme caution before and immediately after lung transplantation, highlighting the relevance of genetic screening and imaging of the chest of such PAH patients.

Healthy carriers of pathogenic *SOX17* variants had no or mild radiological abnormalities and they had no evidence of pre-symptomatic cardiac malformation. This wide phenotypic variability within the same family harbouring the same mutation has already been observed in PAH associated with mutations in developmental genes, especially in small patella syndrome associated with *TBX4* mutations [26]. Genetic counselling and screening of first-degree relatives is recommended in PH guidelines [4, 6]. This is particularly true in healthy carriers of *SOX17* variants when the risks of PAH and CHD are considered. Longitudinal follow-up of healthy *SOX17* variant carriers will reveal whether pulmonary vascular anomalies and PH may occur with time. Screening procedures remain to be defined in these individuals since the penetrance of PAH and CHD in *SOX17* variant carriers is unknown. We previously demonstrated that echocardiography and N-terminal pro-brain natriuretic peptide blood level analysis may be insufficient tools to effectively screen for PH in asymptomatic relatives harbouring *BMPR2* mutations [32]. More exhaustive strategies, including cardiopulmonary exercise testing, D_{LCO} measurement and ECG, might be useful screening tools in first-degree relatives of patients with heritable PAH [32]. The vascular phenotype associated with *SOX17* pathogenic variants suggests that CTPA should be encouraged in first-degree relatives. Echocardiography screening of CHD of first-degree relatives of patients carrying a *SOX17* pathogenic variant should also be considered, allowing detection of asymptomatic CHD and timely management.

In conclusion, *SOX17* pathogenic variants are associated with a severe form of heritable PAH that can be associated with a variety of cardiac and thoracic vessel anomalies. Although our cohort was of relatively limited size, our data clearly demonstrate a unique phenotype of heritable PAH related to *SOX17* pathogenic variants. International prospective registries are warranted to further reveal and understand the phenotype of rare variants associated with PAH, such as *SOX17* pathogenic variants. Identification of signs highly suggestive of *SOX17* pathogenic variants is critical since this phenotype is associated with an

augmented risk of various complications, such as recurrent haemoptysis. Furthermore, the role of SOX17 in pulmonary vascular development and remodelling is of growing interest, as restoring both its gene expression and signalling might constitute a future therapeutic solution for this severe disease.

Acknowledgements: We thank all physicians from the French Pulmonary Hypertension Network (PulmoTension) and the French PAH Patient Association (HTaPFrance).

Conflict of interest: D. Montani reports grants from Acceleron, Janssen and Merck; consulting fees from Acceleron; lecture honoraria from Bayer, Janssen and Merck; outside the submitted work. A. Boucly reports grants from Acceleron, Janssen and MSD; lecture honoraria from Janssen and Merck; travel support from Janssen; outside the submitted work. D. Bonnet reports grants and consulting fees from Janssen and Novartis; participation on an advisory board for Lupin; outside the submitted work. M. Humbert reports grants from Acceleron, Janssen and Merck; consulting fees from Acceleron, Janssen, Merck, Altavant, MorphogenIX and Bayer; lecture honoraria from Janssen and Merck; participation on advisory boards for Acceleron, Janssen, Merck and United Therapeutics; outside the submitted work. All other authors have nothing to disclose.

Support statement: B. Lechartier is a recipient of a grant from the Fondation Juchum and the Ligue Pulmonaire Vaudoise.

References

1 Humbert M, Guignabert C, Bonnet S, *et al.* Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J* 2019; 53: 1801887.
2 Huertas A, Tu L, Humbert M, *et al.* Chronic inflammation within the vascular wall in pulmonary arterial hypertension: more than a spectator. *Cardiovasc Res* 2020; 116: 885–893.
3 Lechartier B, Berrebeh N, Huertas A, *et al.* Phenotypic diversity of vascular smooth muscle cells in pulmonary arterial hypertension: implications for therapy. *Chest* 2022; 161: 219–231.
4 Galiè N, Humbert M, Vachiery J-L, *et al.* 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). *Eur Respir J* 2015; 46: 903–975.
5 Morrell NW, Aldred MA, Chung WK, *et al.* Genetics and genomics of pulmonary arterial hypertension. *Eur Respir J* 2019; 53: 1801899.
6 Simonneau G, Montani D, Celermajer DS, *et al.* Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur Respir J* 2019; 53: 1801913.
7 Gräf S, Haimel M, Bleda M, *et al.* Identification of rare sequence variation underlying heritable pulmonary arterial hypertension. *Nat Commun* 2018; 9: 1416.
8 Zhu N, Welch CL, Wang J, *et al.* Rare variants in *SOX17* are associated with pulmonary arterial hypertension with congenital heart disease. *Genome Med* 2018; 10: 56.
9 Welch CL, Austin ED, Chung WK. Genes that drive the pathobiology of pediatric pulmonary arterial hypertension. *Pediatr Pulmonol* 2021; 56: 614–620.
10 Girerd B, Montani D, Jaïs X, *et al.* Genetic counselling in a national referral centre for pulmonary hypertension. *Eur Respir J* 2016; 47: 541.
11 Eyries M, Montani D, Nadaud S, *et al.* Widening the landscape of heritable pulmonary hypertension mutations in paediatric and adult cases. *Eur Respir J* 2019; 53: 1801371.
12 Zhu N, Pauciulo MW, Welch CL, *et al.* Novel risk genes and mechanisms implicated by exome sequencing of 2572 individuals with pulmonary arterial hypertension. *Genome Med* 2019; 11: 69.
13 Richards S, Aziz N, Bale S, *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015; 17: 405–424.
14 Amendola LM, Jarvik GP, Leo MC, *et al.* Performance of ACMG-AMP variant-interpretation guidelines among nine laboratories in the Clinical Sequencing Exploratory Research Consortium. *Am J Hum Genet* 2016; 98: 1067–1076.
15 Ghigna M-R, Guignabert C, Montani D, *et al. BMPR2* mutation status influences bronchial vascular changes in pulmonary arterial hypertension. *Eur Respir J* 2016; 48: 1668–1681.
16 Corada M, Orsenigo F, Morini MF, *et al.* Sox17 is indispensable for acquisition and maintenance of arterial identity. *Nat Commun* 2013; 4: 2609.
17 Lange AW, Haitchi HM, LeCras TD, *et al.* Sox17 is required for normal pulmonary vascular morphogenesis. *Dev Biol* 2014; 387: 109–120.

18 Francois M, Koopman P, Beltrame M. *SoxF* genes: key players in the development of the cardio-vascular system. *Int J Biochem Cell Biol* 2010; 42: 445–448.
19 Yang H, Lee S, Lee S, *et al.* Sox17 promotes tumor angiogenesis and destabilizes tumor vessels in mice. *J Clin Invest* 2013; 123: 418–431.
20 Wu Y, Wharton J, Walters R, *et al.* The pathophysiological role of novel pulmonary arterial hypertension gene *SOX17*. *Eur Respir J* 2021; 58: 2004172.
21 Sakamoto Y, Hara K, Kanai-Azuma M, *et al.* Redundant roles of *Sox17* and *Sox18* in early cardiovascular development of mouse embryos. *Biochem Biophys Res Commun* 2007; 360: 539–544.
22 Zhou Y, Williams J, Smallwood PM, *et al.* Sox7, Sox17, and Sox18 cooperatively regulate vascular development in the mouse retina. *PLoS One* 2015; 10: e0143650.
23 Corada M, Orsenigo F, Morini MF, *et al.* Sox17 is indispensable for acquisition and maintenance of arterial identity. *Nat Commun* 2013; 4: 2609.
24 Sinner D, Kordich JJ, Spence JR, *et al.* Sox17 and Sox4 differentially regulate beta-catenin/T-cell factor activity and proliferation of colon carcinoma cells. *Mol Cell Biol* 2007; 27: 7802–7815.
25 Banerjee A, Ray S. Structural insight, mutation and interactions in human beta-catenin and SOX17 protein: a molecular-level outlook for organogenesis. *Gene* 2017; 610: 118–126.
26 Thoré P, Girerd B, Jaïs X, *et al.* Phenotype and outcome of pulmonary arterial hypertension patients carrying a *TBX4* mutation. *Eur Respir J* 2020; 55: 1902340.
27 Sztrymf B, Coulet F, Girerd B, *et al.* Clinical outcomes of pulmonary arterial hypertension in carriers of *BMPR2* mutation. *Am J Respir Crit Care Med* 2008; 177: 1377–1383.
28 Galambos C, Mullen MP, Shieh JT, *et al.* Phenotype characterisation of *TBX4* mutation and deletion carriers with neonatal and paediatric pulmonary hypertension. *Eur Respir J* 2019; 54: 1801965.
29 Saba R, Kitajima K, Rainbow L, *et al.* Endocardium differentiation through Sox17 expression in endocardium precursor cells regulates heart development in mice. *Sci Rep* 2019; 9: 11953.
30 Evans JDW, Girerd B, Montani D, *et al. BMPR2* mutations and survival in pulmonary arterial hypertension: an individual participant data meta-analysis. *Lancet Respir Med* 2016; 4: 129–137.
31 Remy-Jardin M, Ryerson CJ, Schiebler ML, *et al.* Imaging of pulmonary hypertension in adults: a position paper from the Fleischner Society. *Eur Respir J* 2021; 57: 2004455.
32 Montani D, Girerd B, Jaïs X, *et al.* Screening for pulmonary arterial hypertension in adults carrying a *BMPR2* mutation. *Eur Respir J* 2021; 58: 2004229.

Q10

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Q9 Check "median mPAP of 64.4 mmHg".

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