Phenotype characterisation of *TBX4* mutation and deletion carriers with neonatal and paediatric pulmonary hypertension

Csaba Galambos[1,19], Mary P. Mullen[2,19], Joseph T. Shieh[3], Nicolaus Schwerk[4], Matthew J. Kielt[5], Nicola Ullmann[6], Renata Boldrini[7], Irena Stucin-Gantar[8], Cristina Haass[9], Manish Bansal[10], Pankaj B. Agrawal[11], Joyce Johnson[12], Donatella Peca[7], Cecilia Surace[7], Renato Cutrera[6], Michael W. Pauciulo[13], William C. Nichols[13], Matthias Griese[14], Dunbar Ivy[15], Steven H. Abman[16], Eric D. Austin[5] and Olivier Danhaive[17,18]

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***TBX4* mutations and deletions are associated with abnormal distal lung development, persistent pulmonary hypertension of the newborn, paediatric pulmonary hypertension, multiple congenital anomalies and developmental disabilities** http://bit.ly/2UXDrl3

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ABSTRACT Rare variants in the T-box transcription factor 4 gene (*TBX4*) have recently been recognised as an emerging cause of paediatric pulmonary hypertension (PH). Their pathophysiology and contribution to persistent pulmonary hypertension in neonates (PPHN) are unknown. We sought to define the spectrum of clinical manifestations and histopathology associated with *TBX4* variants in neonates and children with PH.

We assessed clinical data and lung tissue in 19 children with PH, including PPHN, carrying *TBX4* rare variants identified by next-generation sequencing and copy number variation arrays.

Variants included six 17q23 deletions encompassing the entire *TBX4* locus and neighbouring genes, and 12 likely damaging mutations. 10 infants presented with neonatal hypoxic respiratory failure and PPHN, and were subsequently discharged home. PH was diagnosed later in infancy or childhood. Three children died and two required lung transplantation. Associated anomalies included patent ductus arteriosus, septal defects, foot anomalies and developmental disability, the latter with a higher prevalence in deletion carriers. Histology in seven infants showed abnormal distal lung development and pulmonary hypertensive remodelling.

TBX4 mutations and 17q23 deletions underlie a new form of developmental lung disease manifesting with severe, often biphasic PH at birth and/or later in infancy and childhood, often associated with skeletal anomalies, cardiac defects, neurodevelopmental disability and other anomalies.

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Introduction

Pulmonary arterial hypertension (PAH) is a rare condition in infants and children, with a prevalence ranging between 4.8 and 8.1 cases per million [1–3], that leads to progressive right heart failure and high mortality despite recent progress in diagnosis and treatment [4]. PAH is a precapillary condition, and a subtype of pulmonary hypertension (PH). Although PAH is heterogeneous, genetic defects relevant to pulmonary circulation underlie the majority of familial PAH cases and a significant subset of idiopathic PAH cases. Mutations in the bone morphogenetic protein (BMP) receptor type 2 gene (*BMPR2*) and other BMP-associated genes are found in ∼70% of familial PAH cases, and 20% of idiopathic PAH cases in adults and children [5–8]. Recent studies have revealed a high prevalence of variants in T-box transcription factor 4 (*TBX4*), the gene associated with small patella syndrome (SPS) [9], in paediatric PAH [8, 10–12].

In the perinatal period, neonates may present with a form of PH known as persistent pulmonary hypertension of the newborn (PPHN), a condition with different underlying aetiologies causing persistent elevation of pulmonary vascular resistance and failure to transition from a fetal to postnatal circulatory pattern. PPHN is more common than paediatric PAH, with an incidence of 0.18%, 20% of which is seemingly idiopathic [13]. Although PPHN is mostly reversible, with a mortality <10%, a small subset of cases typically unresponsive to therapy have developmental lung diseases [14]. Recently, *TBX4* rare variants were described in three neonates with hypoxic respiratory failure caused by developmental lung disease [15, 16], expanding the spectrum of manifestations associated with these gene defects.

Given the potential importance of *TBX4* expression during pulmonary development and the association between *TBX4* and paediatric PH, we collected data from 19 paediatric patients with identified *TBX4* variants and sought to more precisely determine the spectrum of manifestations in infants and children.

Methods

This series consists of cases selected from January 2014 to December 2017 from various clinical centres (supplementary table S1) on the basis of PH initially diagnosed by right heart catheterisation (RHC) in seven cases or echocardiography in 12 cases (table 1) during infancy or childhood and the presence of a *TBX4* rare variant identified *via* clinical or research testing. Small nucleotide variants (SNVs) were identified by next-generation sequencing either from certified clinical laboratories or custom research panels, or by Sanger sequencing (supplementary table S1). For missense variants, the functional impact on protein structure was assessed by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and Combined Annotation Dependent Depletion v1.3 (CADD) [17]. Minor allele frequency (<0.05) was checked searching the Exome Aggregation Consortium database [18]. Variants were compared to the ClinVar [19] and ClinGen [20] databases. Copy number variants (CNVs) were determined by chromosomal arrays. Variant significance was determined following the American College of Medical Genetics guidelines for CNVs [21] and SNVs [22]. De-identified patient data, including biometrics, family and neonatal history, initial and subsequent diagnostic studies and functional data, follow-up, and outcome, were extracted from registries or medical records (supplementary table S1). Clinically obtained lung tissue, when available, was re-analysed by a single pathologist (CG). This study was conducted in compliance with local institutional review boards.

Affiliations: [1]Dept of Pathology and Laboratory Services, University of Colorado School of Medicine and Children's Hospital Colorado, Aurora, CO, USA. [2]Dept of Cardiology, Boston Children's Hospital and Pediatrics Harvard Medical School, Boston, MA, USA. [3]Institute for Human Genetics, Medical Genetics, University of California San Francisco, UCSF Benioff Children's Hospital, San Francisco, CA, USA. [4]Clinic for Pediatric Pulmonology, Allergology and Neonatology, Hannover Medical School, Hannover, Germany. [5]Division of Pediatric Pulmonology, Vanderbilt University Medical Center, Nashville, TN, USA. [6]Division of Pediatric Pulmonology, Bambino Gesù Children's Hospital, Rome, Italy. [7]Dept of Laboratory Medicine, Bambino Gesù Children's Hospital, Rome, Italy. [8]Division of Perinatology, University Medical Centre, Ljubljana, Slovenia. [9]Division of Neonatology, San Pietro-Fatebenefratelli Hospital, Rome, Italy. [10]Division of Pediatric Cardiology, University of Iowa Children's Hospital, Iowa City, IA, USA. [11]Division of Newborn Medicine and Genetics and Genomics, Harvard School of Medicine, Boston Children's Hospital, Boston, MA, USA. [12]Dept of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA. [13]Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Dept of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA. [14]Division of Pediatric Pulmonology, Ludwig-Maximilians-University, Munich, Germany. [15]Division of Pediatric Cardiology, Dept of Pediatrics, University of Colorado School of Medicine and Children's Hospital Colorado, Aurora, CO, USA. [16]Division of Pulmonary Medicine, Dept of Pediatrics, University of Colorado School of Medicine and Children's Hospital Colorado, Aurora, CO, USA. [17]Division of Neonatology, University of California San Francisco Benioff Children's Hospital, San Francisco, CA, USA. [18]Division of Neonatology, Catholic University of Louvain, Brussels, Belgium. [19]Contributed equally to this work as joint first authors.

Correspondence: Olivier Danhaive, Division of Neonatology, Saint-Luc University Hospital, Catholic University of Louvain, 10 Avenue Hippocrate, Brussels B-1200, Belgium. E-mail: olivier.danhaive@ucsf.edu

TABLE 1 Clinical characteristics

Case	Sex	Ethnicity	Neonatal course							Postnatal PH# course				
			GA weeks BW g Z-score	Clinical presentation	ECMO days	iNO days	MV days	Home days	Medication		Initial diagnosis	Tissue sample	Cardiovascular medication	Age at follow-up/ death: outcome
1	Male	Caucasian	36 2000 −1.70	PPHN¶ RDS	Yes 12	Yes 35	Yes 45	Yes 60	O_2 PDE5i	E	3 months: hypoxaemia 4 m: RSV infection, HRF, ECMO	4 month biopsy	O_2, PDE5i	5 months: death from hypoxic respiratory failure
2	Male	Caucasian	38 3090 −0.15	Omphalocele Transient tachypnoea	No	No	Yes	Yes 17	No	E	5 months: hypoxaemia	-	O_2	4 years: stable mild PH, ILD
3	Female	Caucasian	36 2790 0.42	PPHN	No	Yes 6	Yes 6	Yes 41	O_2	E	2 months: hypoxaemia	-	PDE5i, ERA	10 years: stable mild PH
4	Female		40	Normal	No	No	No	Yes		C	4 years: intermittent hypoxaemia 5 years: PDA closure Lack of follow-up until age 18	-	O_2, PDE5i, treprostinil	29 years: death from refractory PH
5	Female		40	PPHN Pneumothorax	Yes 10	Yes	Yes	Yes 45	No	E	2.5 months: hypoxaemia	-	O_2, PDE5i, treprostinil, epoprostenol, CCB	15 years: stable PH
6	Female	Caucasian	39 1/7 3.136 −0.27	RDS	No	No	No	Yes 12	O_2	C	6 months: hypoxaemia, dyspnoea	-	ERA, PDE5i, O_2	11 years: severe PH
7	Female	Caucasian	37 4/7 1890 −2.34	PPHN	No	Yes 1	Yes 13	Yes 180	O_2	E	1.5 months: hypoxaemia, dyspnoea	3 month biopsy	O_2, iNO, PDE5i, digoxin	8 months: death, PH crisis during surgery (Meckel's diverticulectomy)
8	Female	Caucasian	40	Normal	No	No	No	Yes	No	C	3 years: dyspnoea, fatigability, syncope	-	O_2, *s.c.* treprostinil, PGE5i	12 years: severe PH
9	Female	Caucasian	40+	PPHN MAS	Yes 5	Yes	Yes 15	Yes 30	No	C	2.5 years: hypoxaemia, syncope	-	O_2, PDE5i, ERA, *s.c.* treprostinil	7 years: severe PH
10	Male	Caucasian	40 3490 −0.17	PPHN	Yes 2	Yes 2	Yes 5	Yes 30	O_2	C	18 months: hypoxaemia	2 years: biopsy 18 years: explant	O_2, ERA, PDE5i, CCB	18 years: end-stage PH, heart–lung transplantation
11	Female		40	Normal	No	No	No	Yes	No	C	8 years: PDA right to left	-	ERA	

Continued

TABLE 1 Continued

Case	Sex	Ethnicity	Neonatal course							Postnatal PH[#] course				
			GA weeks BW g Z-score	Clinical presentation	ECMO days	iNO days	MV days	Home days	Medication		Initial diagnosis	Tissue sample	Cardiovascular medication	Age at follow-up/ death: outcome
12	Female	Caucasian	39 1/7 3450 0.13	PPHN OI: 40 RDS Pneumothorax	No	Yes	Yes 11	Yes 64	O_2	E	2.5 years: hypoxaemia, chILD[§]	2 months: biopsy	O_2, PDE5i, ERA	11 years: end-stage chILD, heart–lung transplantation
13	Female	Caucasian	40 3/7 3510 0.04	PPHN OI: 78 RDS	No	Yes 7	Yes 10	Yes 27	O_2	E	1 month: chILD	1.5 months: biopsy	O_2	4 years: severe chILD, mild PH
14	Male	Caucasian	40 3/7 3820 0.33	PPHN OI: 57 RDS	No	Yes 5	Yes 5	Yes 14	No	E	1 month: persistent tachypnoea	-	PDE5i	3 months: chILD, no PH, short follow-up
15	Female	Caucasian	39 2950 –0.62	RDS Pneumothorax	No	No	No	Yes 15		E	5 months: persistent chILD following RSV infection	-	No	7 months: bronchiolitis 10 years: chILD, no PH
16	Female	Caucasian	40	RDS Pneumothorax	No	No	No	Yes 7	No	E	1 month: persistent tachypnoea	-	PDE5i	10 years: no residual PH
17	Male	Caucasian	38 2040 –2.65	Normal	No	No	No	Yes	No	C	12 years: hypoxaemia during knee surgery	-	O_2, PDE5i, ERA, *i.v.* treprostinil	21 years: moderate PH
18	Male	Caucasian	36 5/7 2450 –1.00	PPHN OI: 58 RDS	No	Yes 6	Yes 9	Yes 92	O_2	E	7 years: chILD	7 years: biopsy	O_2, PDE5i, ERA	9 years: moderate PH
19	Female	Caucasian	40 3075 –0.71	Normal	No	No	No	Yes	No	E	5 months: chILD	7 years: biopsy	O_2	10 years: ch ILD, moderate PH

PH: pulmonary hypertension; GA: gestational age; BW: birth weight; ECMO: extracorporeal membrane oxygenation; iNO: inhaled nitric oxide; MV: mechanical ventilation; PPHN: persistent pulmonary hypertension of the newborn; RDS: respiratory distress syndrome; O_2: oxygen therapy; PDE5i: phosphodiesterase 5 inhibitor; E: echocardiography; RSV; respiratory syncytial virus infection; HRF: hypoxic respiratory failure; ILD: interstitial lung disease; ERA: endothelin receptor antagonist; C: cardiac catheterisation; PDA: patent ductus arteriosus; MAS: meconium aspiration syndrome; CCB: calcium channel blocker; *s.c.*: subcutaneous; OI: oxygenation index; chILD: childhood interstitial lung disease. [#]: PH was defined by a mean pulmonary artery pressure >25 mmHg >3 months of age; each of these children had a pulmonary artery wedge pressure <15 mmHg and pulmonary vascular resistance index >3 WU·m^2 consistent with pulmonary arterial hypertension, requiring right heart catheterisation for accurate, quantitative diagnosis, although Doppler echocardiography provides reliable qualitative data with the benefit of less invasiveness [25]; [¶]: the echocardiography criteria for PPHN were estimated right ventricle systolic pressure ⩾ 2/3 systemic pressure by direction and velocity of ductus arteriosus flow and/or two-dimensional interventricular septum position and/or peak tricuspid regurgitant jet velocity [52]; [+]: maternal methamphetamine use; [§]: chILD was diagnosed per American Thoracic Society criteria (at least three of the following four criteria are present: 1) respiratory symptoms (cough, rapid and/or difficult breathing, or exercise intolerance), 2) respiratory signs (tachypnoea, adventitious sounds, retractions, digital clubbing, failure to thrive or respiratory failure), 3) hypoxaemia and 4) diffuse abnormalities on a chest radiograph or computed tomography scan, after exclusion of the common diseases that can cause developmental lung disorders as the primary diagnosis [53]).

Results

Genotype characterisation

18 different heterozygous variants were identified in the 19 patients (including two siblings) which consisted of six CNVs involving the 17q23.2 locus and 12 *TBX4* SNVs (table 2). The CNVs comprised two sizes of ~2.2 Mb and ~3.6–3.7 Mb encompassing the whole *TBX4* coding sequence plus several other genes (figure 1a). The 2.2 Mb CNV (cases 1, 3, 4 and 5) is a recurrent 17q23.1q23.2 deletion due to segmental duplications that has been previously described [23] and reported in ClinGen. The larger CNV (cases 2 and 6) has only been reported once in ClinVar. The SNVs are novel. Among these, 10 are likely-gene-disrupting variants, including frameshift indel (cases 7–11), premature stop-gain (cases 12–14) and canonical splice site (cases 15 and 16) variants, and three are missense variants affecting the T-box DNA binding domain consensus (cases 17–19) (figure 1b). The two nonsense and two of the frameshift variants located downstream of the T-box domain, in the absence of experimental data demonstrating their gene disrupting effect, were classified as likely pathogenic. The three missense mutations were considered likely pathogenic on the basis of the PolyPhen-2 and/or CADD scores of 0.85–1 and $\geqslant$10–20 respectively, conservation of the amino acid position across all vertebrate species, and complete (for p.Gly106 and p.Leu186) or moderate (for p.Val218) conservation in the T-box domain of the 13 human TBX proteins. Of the eight variants in which inheritance was determinable, three (37%) were *de novo* and five (62%) were familial, with carrier siblings affected with SPS (case 18) or determined to have previously had PAH (cases 13 and 14), and carrier mothers affected with SPS (cases 12 and 15), PAH (case 15) or asymptomatic (case 16). Although the number and nature of tested genes varied from centre to centre (supplementary table S1), no *BMPR2*, forkhead box F1 (*FOXF1*) or other PH-related pathogenic gene variants could be found in any tested patient.

Clinical phenotype and outcomes

All patients were born term or late preterm (median 40.0 weeks, interquartile range (IQR) 38.0 weeks), with a female to male ratio of 2.16:1, similar for CNVs and SNVs (tables 1 and 3). Median birthweight was normal for gestational age (median 3075 g, IQR 2450 g), although three neonates were small for gestational age (birthweight z-score<−1.28) [24]. 11 neonates required invasive respiratory support. The most frequent presentation was PPHN in 10 out of 19 neonates (53%), which was severe in eight cases (oxygenation index>25 or need for extracorporeal membrane oxygenation (ECMO)). Four neonates (10%) presented with transient respiratory distress without PH; the remaining five had an uneventful neonatal course. All patients survived their newborn intensive care unit course and were discharged home at a median age of 37 days (range 7–180 days), six with home oxygen and two with sildenafil. In two patients with PPHN history, right ventricular systolic pressures (RVSP) remained elevated after the neonatal period despite therapy and they both died in infancy, at 5 and 8 months. In the remaining eight with PPHN, PH appeared to improve or resolve within the first months of life. Later in childhood, the 17 surviving patients underwent a cardiology evaluation, often for new-onset hypoxaemia or for cardiorespiratory symptoms (table 1). These patients were diagnosed with PH (median age 1.5 years, IQR 0.17 years). The duration of follow-up at the time of this report varied between patients, this study being retrospective (median 10.0 years, IQR 4.7 years). Three patients evolved to end-stage lung disease despite multiple vasodilator therapies: one died at 29 years old and two underwent heart–lung transplantation, one at 11 years and one at 18 years old. 11 patients continue to have chronic PH at their last evaluation, despite the use of multiple PH-targeted therapies in seven patients, a single PH-targeted therapy in one patient, and treatment with supplemental oxygen alone in three patients. PH had resolved at the last follow-up in the remaining three patients (cases 14, 15 and 16: 3 months–10 years), one of whom was medication-free whereas two remained on single vasodilator therapy. 10 out of the 13 patients whose information was retrievable (77%) had skeletal anomalies, including SPS with its typical foot anomaly [9]. Other neurological and developmental disorders included autism, microcephaly, neurosensory deficits and muscular tone anomalies.

Cardiac imaging and haemodynamic analysis

Seven of the patients were assessed by echocardiography alone, while 12 underwent at least one RHC during their treatment course (table 4). 10 patients (53%) had systemic or suprasystemic RVSP in the neonatal period. Eight had patent ductus arteriosus (PDA), which persisted beyond the neonatal period in four patients. Two of these had left-to-right shunting at initial PH diagnosis: one was surgically closed at age 4 years (case 4), and one was haemodynamically insignificant (case 5). Two patients (cases 5 and 11) had right-to-left shunting at the diagnostic RHC persisting at follow-up. An atrial septal defect was present in eight patients (42%). In the 12 patients in whom cardiac catheterisation was performed (median age 1.5 years, IQR 0.17 years), high mean pulmonary artery pressures (mPAP) (60.0 mmHg, IQR 57.5mm Hg) and pulmonary vascular resistance indices (median 16.6 Wood units, IQR 10.7 Wood units) were

TABLE 2 Genetic rare variants and phenotype

Case	TBX4 nucleotide and protein variant	Genomic position (Hg19; Chr17)#	Putative mechanism and consequence	Genotype and inheritance	ExAC allele frequency, CADD score	ACMG classification	Skeletal anomalies	Other anomalies	Neurological and psychomotor deficits
1	CNV	17q23.1q23.2 (58 078 171–60 316 749)x1	Gene deletion haploinsufficiency	Heterozygous *De novo*	n/a	Pathogenic (ClinGen)	Not known	Hypothyroidism, cortisol deficiency	Hypertonia
2	CNV	17q22q23.2(56 623 275–60 285 107)x1	Gene deletion haploinsufficiency	Heterozygous Not known	n/a	Likely pathogenic (ClinVar)	Facial dysmorphism, foot anomaly, not known	ASD, omphalocele	Developmental delay, seizures, nystagmus
3	CNV	17q23.1q23.2 (57 972 342–60 472 864)x1	Gene deletion haploinsufficiency	Heterozygous Not known	n/a	Likely pathogenic (ClinGen)	Joint contractures, foot anomaly	ASD	Mild developmental delay, hearing loss, two-vessel cord, vesicoureteral reflux
4	CNV	17q23.1(58 313 766–60 315 220)x1	Gene deletion haploinsufficiency	Heterozygous Not known	n/a	Likely pathogenic (ClinGen)	Foot anomaly	PDA (ligation at 4 years)	Mild developmental delay
5	CNV	17q23.1q23.2 (57 992 012–60 330 998)x1	Gene deletion haploinsufficiency	Heterozygous Not known	n/a	Likely pathogenic (ClinGen)	SPS, foot anomaly	PDA, ASD, VSD	Developmental delay, nystagmus
6	CNV	17q22q23.2(56 429 075–60 181 763)x1	Gene deletion haploinsufficiency	Heterozygous Not known	n/a	Likely pathogenic	Club foot	ASD, gastrostomy	Microcephaly, hearing loss, esotropia, nystagmus, severe developmental delay
7	c.251_delG p.(Gly84Alafs*4)	59 534 962	Indel Frameshift Loss of function	Heterozygous *De novo*	ExAC AF: 0	Pathogenic	Not known	Transient PDA, failure to thrive, Meckel diverticulum	Developmental delay
8	c.847dupC p.(Gln283Profs*103)	59 557 506	Indel Frameshift Loss of function	Heterozygous Not known	ExAC AF: 0	Likely pathogenic	Not known		No
9	c.146delG p.(Gly49Aspfs*39)	59 533 997	Indel Frameshift Loss of function	Heterozygous Not known	ExAC AF: 0	Likely pathogenic	Not known	ASD, obstructive apnoea	No
10	c.538_547delCCCTTTGGCC p.(Pro180Ilefs*45)	59 545 007–59 545 016	Indel Frameshift Loss of function	Heterozygous Not known	ExAC AF: 0	Likely pathogenic (ClinVar)	SPS, foot anomaly		No
11	c.1112–1113insC p.(Pro371Profs*15)	59 560 348–59 560 349	Indel Frameshift Loss of function	Heterozygous Not known	ExAC AF: 0	Likely pathogenic (ClinVar)	Foot anomaly	PDA	No

Continued

TABLE 2 Continued

Case	TBX4 nucleotide and protein variant	Genomic position (Hg19; Chr17)[#]	Putative mechanism and consequence	Genotype and inheritance	ExAC allele frequency, CADD score	ACMG classification	Skeletal anomalies	Other anomalies	Neurological and psychomotor deficits
12	c.1054C>T p.(Arg352*)	59 560 290	Nonsense AA substitution Loss of function	Heterozygous Heritable: (M) c.1054C>T with SPS	ExAC AF: 0	Likely pathogenic	SPS, pelvis and foot anomaly	ASD, ILD	No
13[¶]	c.1018C>T p.(Arg340*)	59 557 677	Nonsense AA substitution Loss of function	Heterozygous Heritable: both siblings carrying p.(Arg340*); parents not tested	ExAC AF: 0	Likely pathogenic	No	Transient PDA and PFO, ILD	No
14[¶]	c.1018C>T p.(Arg340*)	59 557 677	Nonsense AA substitution Loss of function	Heterozygous Heritable: both siblings carrying p.(Arg340*); parents not tested	ExAC AF: 0	Likely pathogenic	No	Transient PFO and PDA, ILD	Unknown
15	c.792-1G>C	59 557 450	Splice site AA substitution Loss of function	Heterozygous Heritable: (M) c.792-1G>C with SPS, PH, ILD	ExAC AF: 0	Likely pathogenic	Foot anomaly	Mandibular angioma	No
16	c.702+1G>A	59 556 141	Splice site AA substitution Loss of function	Heterozygous Heritable: (M) c.702+1G>A	ExAC AF: 0	Likely pathogenic (ClinVar)	Not known	No	No
17	c.316G>A p.(Gly106Ser)	59 534 214	Missense AA substitution	Heterozygous *De novo*	ExAC AF: 0.000008236 CADD: 11.0 P2: 1.000	Likely pathogenic	SPS, pelvis and foot scoliosis	PDA, short stature, angiofibromas, keratoconus	ADHD, autism
18	c.557T>G p.(Leu186Arg)	59 555 995	Missense AA substitution	Heterozygous Heritable: two siblings carrying p.(Leu186Arg), with SPS	ExAC AF: 0 CADD: 28.8 P2: 1.000	Likely pathogenic (ClinVar)	Pelvis, vertebral and foot anomaly	ASD, PDA, short stature, facial dysmorphism (long philtrum, hypertelorism)	Moderate developmental delay, hypotonia
19	c.652G>A p.(Val218Met)	59 556 090	Missense AA substitution	Heterozygous Not tested	ExAC AF: 0.0001153 CADD: 24.2 P2: 0.635	Likely pathogenic	No	PFO, short stature, hearing loss	Microcephaly

TBX4: T-box transcription factor 4; Hg19: *Homo sapiens* genome assembly GRCh37; Chr17: chromosome 17; ExAC: Exome Aggregation Consortium; CADD: Combined Annotation Dependent Depletion; ACMG: American College of Medical Genetics; CNV: copy number variant; n/a: not available; ASD: atrial septal defect; PDA: patent ductus arteriosus; SPS: small patella syndrome; VSD: ventricular septal defect; AF: allele frequency; AA: amino acid; M: mother; ILD: interstitial lung disease; PFO: patent foramen ovale; PH: pulmonary hypertension; P2: PolyPhen2; ADHD: attention deficit hyperactivity disorder. [#]: genomic annotations were based on NC_000017.11: *homo sapiens* chromosome 17, GRCh38.p7 primary assembly; transcript sequence NM_001321120.1; protein sequence NP_001308049.1; [¶]: siblings.

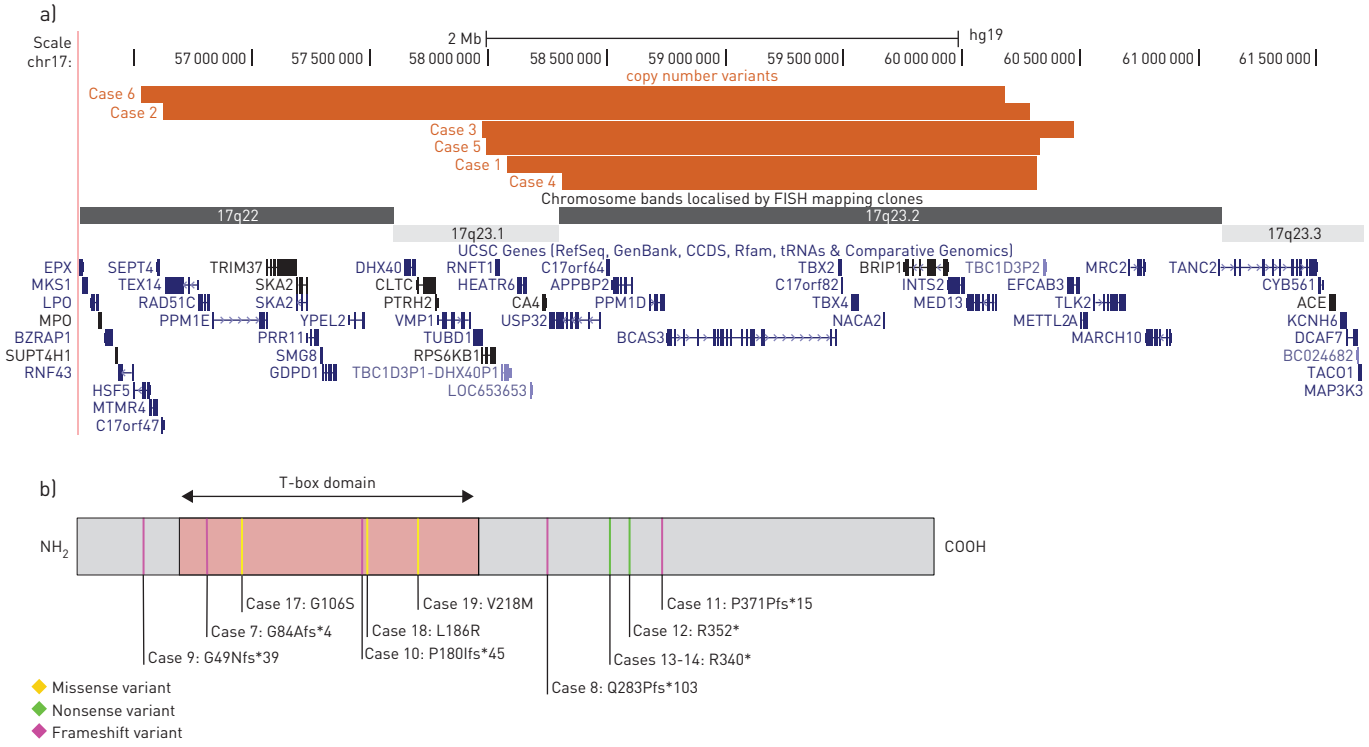

FIGURE 1 Position of 17q23 deletions and *TBX4* mutations. a) Genomic deletions identified in the 17q23.2 region. The smallest deletion overlap region encompasses the following coding genes: *MED13* (subunit of the large Mediator complex, ubiquitous), *INTS2* (subunit of the Integrator complex, low lung expression), *BRIP1* (a member of the *DEAH* helicase family contributing to *BRCA1* activity, ubiquitous), *BCAS3* (associated with angiogenesis and related processes such as cell adhesion, extracellular matrix organisation, peptidase activity and transforming growth factor-β signalling, ubiquitous with high expression in lung [49]), *TBX4* (transcription factor involved in several developmental processes including hindlimb, lung and allantois), *TBX2* (transcription factor involved in patterning of several organs, including heart, brain and limbs [42]), *PPM1D* (protein phosphatase that regulates the DNA damage response pathway, mostly expressed in brain and associated with developmental disability in human [50], *APPBP2* (ubiquitous, interacts with microtubules and is associated with transport and/or processing) and *USP32* (an ubiquitin-specific protease). Among these genes, only *TBX4*, *TBX2* and *PPM1D* have rare variants described in human disease. University of California Santa Cruz (UCSC) Genome Browser (GRCH37/Hg19) [51]. b) Mutation positions in TBX4 protein. The TBX4 protein is represented in grey, with the T-box domain in pink. The three missense variants (yellow) are located in the T-box domain. Three of the frameshift variants (purple) are located either upstream or inside the T-box domain, while two are located downstream. The two nonsense mutations (green) are located downstream. FISH: fluorescent *in situ* hybridisation; CCDS: Consensus Coding Sequence project; tRNA: transfer RNA.

demonstrated. However, only six out of 12 patients met all criteria for a strict diagnosis of PAH based on American Thoracic Society guidelines [25]. In the six patients with serial RHCs, mPAP and pulmonary vascular resistance indices values were equally elevated or increased at follow-up (data not shown). Among the 10 patients who underwent acute vasoreactivity testing, eight (80%) failed to show a decrease in mPAP of at least 10 mmHg to <40 mmHg [25]. Six had a reduced pulmonary-to-systemic blood flow ratio, indicating significant right-to-left shunts. When performed, pulmonary angiography revealed diffuse anatomical and vascular anomalies, including tortuous pulmonary arterioles, abnormal capillary blush, small pulmonary veins and venules, and pulmonary venous obstruction in one patient (not shown).

Phenotypic characteristics and variant type

Table 3 compares the clinical, functional features between the six CNV and the 13 SNV carriers. Although we observed a greater prevalence of associated cardiac and foot anomalies in CNV carriers, only developmental disability reached statistical significance (100% *versus* 33%, p=0.029), in line with others' findings [10]. We also observed a trend for greater RHC functional severity in SNV carriers, although this may reflect an older age at first catheterisation in that group. Supplementary table S3, which compares published 17q23 deletions inclusive and exclusive of the *TBX2/TBX4* loci including our series, shows a greater prevalence of congenital heart defects (57% *versus* 0%, p=0.02) and a similar trend for the presence of PH (57% *versus* 17%, p=0.16) for *TBX2/TBX4*-inclusive deletions.

Imaging studies

Thoracic images could be only collected in a subset of cases (figure 2, supplementary table S2). Neonatal chest radiography (n=5) showed lung hypoplasia (figure 2a), air leaks and/or ground-glass opacities; chest

TABLE 3 Clinical and functional differences in patients with chromosomal deletions *versus* SNVs

Clinical and functional features		All	Subjects with CNV	Subjects with SNV	p-value[#]
Subjects n		19	6	13	
Biometrics	Female	13 (68%)	4 (67%)	9 (69%)	1
	Birth weight g[¶]	3075 (2450)	2754 (523)	2896 (706)	0.36
	Gestational age weeks	40.0 (38.0)	38.5 (36.5)	40.0 (39.0)	0.12
	Birth weight z-score[¶]	−0.27 (−1.00)	0.42 (0.90)	0.77 (1.06)	0.57
Presentation	PPHN	10 (53%)	3 (50%)	7 (54%)	1
	Age at PH diagnosis years	2.69 (3.56)	0.92 (1.38)	3.45 (3.92)	0.16
	Death or transplant	5 (26%)	2 (33%)	3 (23%)	1
Associated anomalies	CHD[+]	7 (39%)	5 (83%)	5 (42%)	0.15
	Toe anomaly	13 (68%)	4 (100%)	6 (67%)	0.49
	Developmental disability	17 (89%)	5 (100%)	4 (33%)	0.029
Right heart catheterisation	Performed n	12	4	8	
	MPAP mmHg	67.8±27.1	56.0±33.5	76.3±21.9	0.23
	Cardiac index $L \cdot min^{-1} \cdot m^{-2}$	3.3±0.9	3.5±1.0	3.1±0.8	0.49
	PVRI WU	23.2±18.7	18.4±19.4	26.7±18.9)	0.47
	Vasodilator responsiveness[§]	2 (16%)	1 (25%)	1 (14%)	1

Data are presented as n (%), median (interquartile range) or mean±SD, unless otherwise stated. CNV: copy number variant; SNV: small nucleotide variant; PPHN: persistent pulmonary hypertension of the newborn; PH: pulmonary hypertension; CHD: congenital heart defect; MPAP: mean pulmonary arterial pressure; PVRI: pulmonary vascular resistance index; WU: Wood units. [#]: p-value for comparison of the group with CNVs *versus* SNVs; t-test for numerical values, Fisher test for categorical values; [¶]: birth weight only available for 13 patients; [+]: CHD was not determined in one patient due the presence of patent ductus arteriosus and patent foramen ovale at <1 month of age, not re-tested subsequently; [§]: vasodilator responsiveness categorised according to Revised Barst Criteria [31].

radiography in infancy and early childhood (n=4) showed a pattern of septal thickening with multifocal areas of dysventilation, bronchial thickening and ground-glass opacities (figure 2b). Computed tomography (CT) scans obtained between 1 and 18 years of age (n=5) showed a spectrum of findings, including multifocal ground-glass opacities, honeycombing and alternating focal cystic changes, and condensed areas and nodules suggesting lobular and lobar fibrosis (figure 2c–f).

Lung histopathology

Pathologic material was available for seven patients, and histological features of lung development and vessel remodelling were analysed semi-quantitatively (table 5). All samples showed diffuse alveolar growth abnormality and variable degrees of pulmonary artery wall remodelling with or without fibrointimal proliferation. No plexiform lesions or vessel necrosis were noted. In patients who had severe symptoms at an early age and underwent biopsy in the neonatal period (cases 1, 7, 12 and 13; figure 3a–i), the histology showed severe disruption of distal lung development characterised by delayed lobular growth with dilated distal airspaces and immature-appearing alveoli without secondary septa, often lined by reactive cuboidal epithelial cells. The distal airspaces appeared enlarged with simplified alveoli. In all cases, there were signs of thickened interstitium; three showed the presence of pale and immature mesenchymal cells as observed in pulmonary interstitial glycogenosis [26], and three had patchy interstitial fibrosis. All had evidence of pulmonary arterial hypertensive remodelling. Back-to-back bronchiolar profiles were seen in two cases and one showed the presence of bronchial vessel recruitment, including intrapulmonary bronchopulmonary anastomoses (IBAs). Overall, these structural changes point to severe disruption of all compartments of distal lung development, reflecting growth arrest during the canalicular or early saccular stage. The lung histology of patients who underwent biopsy in childhood (cases 10a, 18 and 19, and 10b-explant; figure 3j–r) showed evidence of recruited bronchial vascular system, including IBAs and expanded bronchial veins and capillaries, in addition to alveolar simplification and pulmonary artery remodelling. Features of airway remodelling and functional compromise were variably present, characterised by airway wall thickening, increased number of intra-alveolar macrophages and multinucleated giant cells with cholesterol crystals (not shown).

A longitudinal histological analysis was possible in case 10 (biopsy at 2 years and transplant at 18 years). The most striking histological findings included the progression of compromised airway/alveolar growth, characterised by multifocal, markedly underdeveloped and tortuous back-to-back bronchiolar structures, similar to those seen in congenital pulmonary airway malformations [27] or acinar dysplasia (AD) [15]. In addition, pulmonary arteries, lymphatic vessels, airways and pleural vessels showed marked medial wall thickening, and areas of bone formation were noted in the subsequent explant suggesting mesenchymal

TABLE 4 Echocardiography and catheterisation data at initial diagnosis

Case	Original echocardiography		Heart defects	Original right heart catheterisation data														
				Haemodynamics at baseline								Haemodynamics on maximum O_2/iNO vasodilation[#]						
	Age years	Estimated RVSP mmHg		Age years	mPAP mmHg	MSP mmHg	PCWP mmHg	PVRi	RA mmHg	CI L·min^{-1}·m^{-2}	Qp/Qs	mPAP mmHg	PCWP mmHg	PVRi	RA mmHg	CI L·min^{-1}·m^{-2}	Qp/Qs	Response
1	0.3	Suprasystemic	None	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2	0.5	>2/3 systemic	ASD	1.0	22	56	7.5	4.14	6	2.9	0.91	18	6	5.59	6	2.4	0.89	N
3	0.15	Systemic	ASD	0.2	38	48	7	6.7	5	4.3	0.9	30	7	2.8	5	6.1	1.4	Y
4	4	<Systemic[¶]	PDA[¶]	18	102	92	24	50	15	2.4	0.6	75	n/a	18.6	n/a	2	1.8	N
5	0.2	Suprasystemic	PDA, ASD, VSD	0.3	80	73	n/a	24	6	4.5	0.6	70	10	23	n/a	2.8	0.9	N
6	0.5	Systemic	ASD	0.5	38	n/a	n/a	7.0	4	n/a	1.1	n/a	n/a	4.5	n/a	n/a	n/a	n/a
7	0.1	Suprasystemic	None	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
8	4.4	Suprasystemic	None	4.4	83	60	11	27.4	6	2.6	1	78	9	23.4	7	2.9	1	N
9	2.5	Suprasystemic	ASD	2.4	99	58	16	65.1	9	1.8	0.8	n/a	n/a	27.9	n/a	4.6	0.5	N
10	1.5	>2/3 systemic	None	1.5	66	70	10	16.5	6	4.0	1.0	23	n/a	3.2	n/a	n/a	1.0	Y
11	7.7	Suprasystemic	PDA	7.7	63	60	8	18.3	5	3.8	0.8	63	8	19.6	6	3.1	0.9	N
12	2.5	Suprasystemic	None	7.0	111	92	n/a	36.1	2	2.4	1.12	110	102	35.5	n/a	2.6	0.9	N
13	0.1	>2/3 systemic	ASD	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
14	0.1	>2/3 systemic	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
15	5	>2/3 systemic (30)	None	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
16	0.1	>2/3 systemic	None	0.2	57	63	7	12.0	4	3.4	1.2	19	6	4.4	5	2.9	1.0	N
17	12	Suprasystemic	PDA	13.0	55	57	5	13.1	2	3.8	1.0	48	7	9.7	2	4.2	1.0	N
18	7	Suprasystemic (120)	ASD	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
19	10	>2/3 systemic (35)	PFO	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Median	1.5			1.5	63.0	60.0	7.8	16.5	5.0	3.1	1.0	48.0	7	9.7	5	2.9	1	
IQR	0.2			0.4	50.7	57.5	7.0	10.7	4.0	2.5	0.8	24.7	6.7	4.5	5	2.6	0.9	

O_2: oxygen; iNO: inhaled nitric oxide; RVSP: right ventricle systolic pressure; mPAP: mean pulmonary artery pressure; MSP: main systemic pressure; PCWP: post-capillary wedge pressure; PVRI: pulmonary vascular resistance index; RA: right atrial pressure; CI: cardiac index; Qp/Qs: pulmonary-to-systemic blood flow ratio; n/a: not available; IQR: interquartile range; ASD: atrial septal defect; PDA: patent ductus arteriosus beyond neonatal period; VSD: ventricular septal defect; PFO: patent foramen ovale. [#]: maximum vasodilation testing was performed by exposure to fraction of insipired O_2 100% and iNO 40 ppm; responsiveness was defined as a decrease in mPAP of at least 10 mmHg to achieve values <40 mmHg with a normal or increase in CO and a decrease or no change in pulmonary vascular resistance/systemic vascular resistance ratio [25]; [¶]: echocardiography report could not be retrieved, but mild pulmonary hypertension was reported in the medical record, prior to PDA closure at age 5. The patient was subsequently lost to follow-up and untreated until age 18, when right heart catheterisation revealed very elevated mPAP and pulmonary vascular resistance with intracardiac right-to-left shunting.

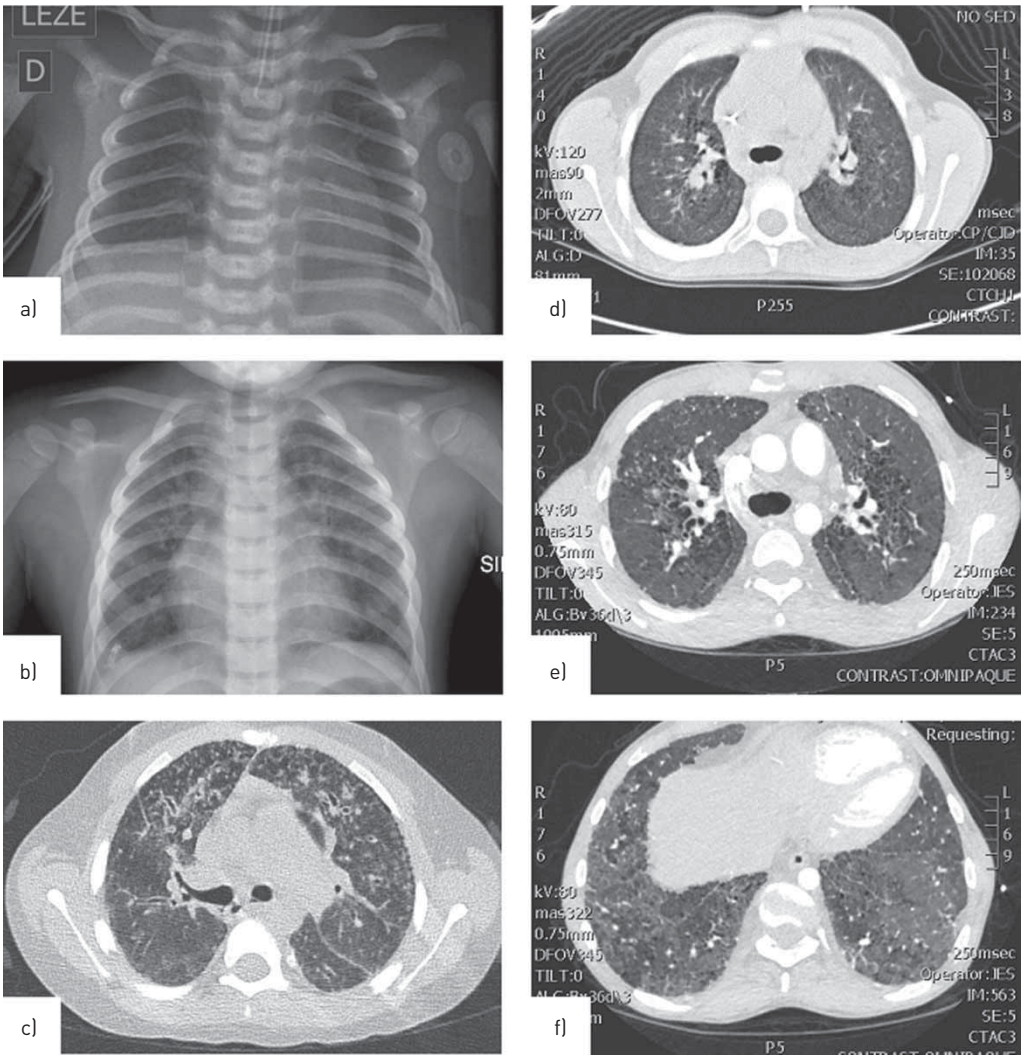

FIGURE 2 Lung imaging. a) Case 1: chest radiography on day 1 with hyperlucent, hypoplastic lungs with poor vascular markings. b, c) Case 19: chest radiography at age 3 years showing ground-glass opacities and bronchial thickening (b); chest computed tomography (CT) at age 10 years showing septal and bronchial wall thickening, honeycombing and diffuse nodules (c). d–f) Case 10: chest CTs at d) age 8 years showing diffuse ground-glass opacity and septal thickening, enlarged pulmonary arteries and scattered emphysema; and e, f) age 18 years showing markedly increased interstitial fibrosis with areas of central mosaic density in upper and lower lobes, small cystic areas with some bronchiectasis, some areas of lungs appearing less involved and a parenchymal bulla present in the lingula.

maldevelopment. Evolving interstitial thickening with fibrosis, IBA recruitment and development of interstitial capillary proliferation were also noted.

Discussion

TBX4 variant carriers are at risk for abnormal distal lung development, PPHN, paediatric-onset PH, multiple congenital anomalies including congenital heart defects and a typical foot malformation, and developmental disabilities. The majority of our patients (63%) presented with a biphasic clinical course consisting of PPHN and neonatal respiratory failure with apparent resolution around 1 month of age, followed by chronic PH later in infancy or early childhood. It is notable that this form of PH fits a precapillary phenotype; however, given the degree of concurrent lung irregularities, these individuals would not technically meet traditional criteria for World Health Organization Classification Group 1 PH (PAH), and might well be classified as Group 3 (PH due to chronic lung disease and/or hypoxia) [6]. Our description of developmental lung disease in patients with *TBX4* variants suggests that associated PH may have several causal associations including chronic respiratory disease and hypoxia in addition to idiopathic PH. Given the difficulty of defining these aetiologies in our retrospective series, we are using the term PH (*versus* PAH) for *TBX4*-associated vascular disease.

TABLE 5 Semi-quantitative analysis of histological features of seven cases contrasted to age at biopsy and outcome

Case	Age at biopsy	Outcome	Alveolar simplification	Wide interstitium	PIG-like cells	Interstitial fibrosis[#]	Back-to-back bronchioli[¶]	Thickened PA muscular wall[+]	PA fibrointimal proliferation	Intrapulmonary bronchopulmonary anastomosis and bronchial vessel recruitment
1[§]	4 months	Death at 5 months	Diffuse	Diffuse	Diffuse	None	Focal	Moderate	Absent	Absent
7[§]	3 months	Death at 8 months	Diffuse	Diffuse	Patchy	Patchy	Absent	Moderate	Moderate	Absent
12[§]	2 months	Lung transplant at 11 years	Diffuse	Patchy	Absent	Patchy	Multifocal	Moderate	Absent	Present
13[§]	1.5 months	Severe ILD at 4 years	Diffuse	Patchy	Diffuse	Patchy	Absent	Moderate	Absent	Absent
10a[f##]	2 years	Lung transplant at 18 years	Diffuse	Absent	Absent	Absent	Absent	Moderate	Absent	Present
10b[f##]	2 years	Lung transplant at 18 years	Diffuse	Diffuse	Absent	Diffuse	Multifocal	Moderate	Absent	Present
18[f]	7 years	Moderate PH at 9 years	Diffuse	Diffuse	Absent	Patchy	Absent	Moderate	Absent	Present
19[f]	7 years	Moderate PH at 10 years	Diffuse	Absent	Absent	Absent	Absent	Moderate	Moderate	Present

PIG: pulmonary interstitial glycogenosis; PA: pulmonary artery; ILD: interstitial lung disease; PH: pulmonary hypertension. [#]: patchy, <50% of parenchyma; diffuse, >50% of parenchyma; [¶]: focal, three foci; multifocal, >3 foci; [+]: moderate, PA muscle wall thickness estimated 40–70% of diameter; [§]: biopsies obtained during neonatal period and showed grimmer prognosis; [f]: biopsies obtained during childhood and showed better outcomes; [##]: biopsy at age 2 and transplant at age 18, allowing longitudinal assessment of evolving histologic features (see details in text).

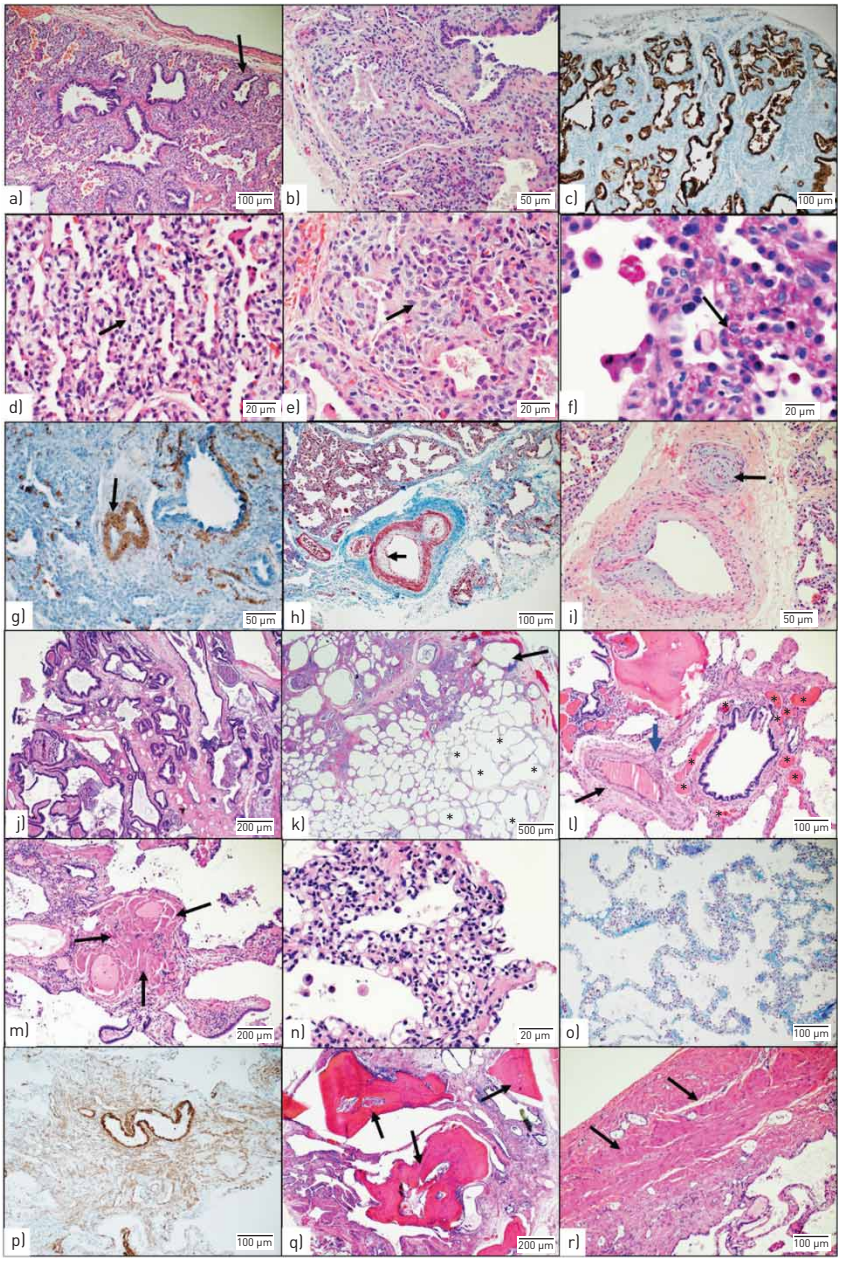

FIGURE 3 Representative histopathology images. Early biopsies obtained in the neonatal period show markedly underdeveloped respiratory lobules (a, b; hematoxylin and eosin (H+E) stain), with small rounded immature alveoli, some with canalicular and others with saccular shape (c; cytokeratin stain). A terminal bronchiole reaches the septum (arrow on a), indicating markedly delayed alveolar development. The interstitium is widened by the presence of immature, pale mesenchymal cells (arrows on d, e; H+E stain); these cells are PAS positive (arrow on f; PAS stain), resembling the features of those of pulmonary interstitial glycogenosis cells. Pulmonary hypertensive remodelling of pulmonary arteries with muscular hypertrophy (arrow on g; smooth muscle actin stain) along with fibrointimal proliferation (arrow on h; trichrome stain) (i; H+E stain) evolving to complete pulmonary arterial obstruction (arrow on i) is identified. Samples from childhood (j–r) showed lack of alveolar development evidenced by multiple foci of back-to-back bronchiolar profile (j; H+E stain), and a terminal bronchiole extending to the pleura surface (arrow on k; H+E stain). There were diffuse alveolar growth abnormalities characterised by large, dilated and simplified alveoli (examples are labelled with asterisks on k). Marked vascular changes included pulmonary arterial hypertensive remodelling (black arrow on l; H+E stain); recruitment of bronchial vasculature including intrapulmonary bronchopulmonary anastomosis (blue arrow on l), bronchial veins and microvessels (asterisks on l); and marked muscular hypertrophy of lymphatic vessels (arrows on m; H+E stain). Pathologic remodelling of the interstitium is seen with the presence of proliferating capillaries (n; H+E stain), fibrosis (o; trichrome stain), focal smooth muscle proliferation (p; smooth muscle actin stain) and multiple areas of bone formation (arrows on q; H+E stain). In one case (10b) marked pleural smooth muscle muscularisation is noted (arrows on r; H+E stain).

Diffuse developmental lung disorders are rare diseases related to aberrations in primary mechanisms of lung airway and vascular development, and include such diagnoses as AD, congenital alveolar dysplasia and alveolar capillary dysplasia with misalignment of the pulmonary veins (ACDMPV), a lethal neonatal disease associated with *FOXF1* variants [28]. Emerging evidence shows that developmental lung disorders are phenotypically heterogeneous. ACDMPV was recently reported in older infants with seemingly precapillary PH, suggesting that *FOXF1*-related disease has a broader clinical spectrum than initially thought [29, 30]. *TBX4* variants were reported in a neonate presenting with lethal AD [15], one with lethal congenital alveolar dysplasia and one with an undefined alveolar growth abnormality and survival beyond 8 months of age [16]. Our pathology findings, with a broader range of age and clinical manifestations, shed light on the pathogenesis of PH in *TBX4* mutants, even though we cannot exclude a selection bias because the biopsies were obtained on a clinical basis without unified criteria. This study confirms that various developmental abnormalities affecting alveolar, interstitial and vascular structures underlie *TBX4*-associated PH. These features imply compromised growth of pulmonary endoderm severely affecting airway/alveolar development, and mesenchymal maldevelopment, reflected by hypertensive remodelling of pulmonary arteries, prominent IBA and other findings of interstitial disease. Our longitudinal observation suggests that these pathological fetal processes continue after birth and progress with age in certain cases, with gradual vascular and lymphatic remodelling and development of the collateral circulation, leading to progressive PH and end-stage lung disease in childhood or young adulthood.

Imaging studies suggest a combination of lung hypoplasia and alveolar dysfunction associated with the neonatal presentation, and progressive bronchial and interstitial changes developing over time in certain cases. The combination of prominent interstitial lung disease and PH in a subset of patients (cases 2, 12, 13, 18 and 19) suggests that mechanisms related to the lung disease, perhaps including chronic hypoxia, may be a contributing factor. However, RHC data suggest severe pulmonary vascular disease in most cases regardless of parenchymal disease, with earlier age at diagnosis and more severe functional values compared to a reference paediatric PAH cohort (the REVEAL cohort [31]). Lack of response to vasoreactivity testing has also been described in children with *BMPR2* mutations compared to those without [32], suggesting that genetic forms of PH have distinct vascular pathophysiology.

Kerstjens-Frederikse *et al.* [10] first described *TBX4* mutations in 30% of a cohort of children diagnosed with idiopathic/familial PAH and SPS, as opposed to only 2.5% of a control adult cohort. Zhu *et al.* [11] calculated a 7.7% prevalence of *TBX4*-related disease in a larger cohort of paediatric PAH. Levy *et al.* [12] also estimated a 7.5% *TBX4* mutation prevalence in 3 out of 40 infants with PAH. Eyries *et al.* [8] found a *TBX4* variant prevalence of one out of 36 (2.8%) and four out of 168 (2.4%) in French children and adults with PAH, respectively, which was lower than the prevalence of a *BMPR2* variant of 19.4% and 14.3%, respectively, in that cohort. A lower frequency of *TBX4* variants has been detected in adult-onset PAH than in paediatric-onset PAH, with an overall mutation frequency estimated at 1.5% (25 out of 1633 cases) [8, 10, 11, 33, 34]. Overall, the lack of standardised inclusion and diagnostic criteria among centres in this paediatric series precludes any inference on prevalence or comparison with *BMP*-related and other PAH genes.

TBX4 variants are associated with multiple anomalies, consistent with disruption of key developmental processes beyond the lung. Not all phenotypic features have the same expressivity. Whereas SPS has a high penetrance, that of PH appears lower [10]. This selective penetrance may putatively depend on the variant itself and its effect on protein dosage and function. However, phenotypical heterogeneity between relatives with a common variant, some having SPS alone and others having a combination of SPS and PH, suggests that *TBX4*-related PH is not purely monogenic, and that multiple innate and environmental factors may be at play, similarly to what is observed in other genetic forms of PH [6]. In *BMPR2*-related disease, PAH penetrance is only 20%, and secondary factors modulate expressivity and disease progression [6]. We observed a 2:1 female prevalence, presumably attributable to selective wastage of male fetuses or an abnormal primary sex ratio. Such disparity was observed in adult PH prior to the identification of causative genes [35], and confirmed in large registries [36]. The role of sex-dependent hormonal factors [37] and modifier genes [38] was subsequently demonstrated in *BMPR2*-related PAH, accounting for female predominance. Putatively, similar mechanisms also exist for *TBX4*.

The severity of PH does not necessarily correlate with the predicted level of protein expression, as observed in *FOXF1*-related ACDMPV [39]. Some CNV cases with complete *TBX4* haploinsufficiency have less severe PH than others with less gene-disruptive SNVs in our and other series [10], suggesting either a dominant-negative effect or interactions with genetic or environmental factors, which makes genetic-based prognosis challenging.

There was a greater incidence of developmental disability among our patients with CNVs (cases 1–6) than those with SNVs (cases 7–19), suggesting a role for neighbouring genes, although postnatal factors such as

PPHN or ECMO cannot be excluded in the CNV group. Comparing our cases with previously published 17q22–q23.2 deletions (supplementary table S3), deletions involving the contiguous *TBX2* and *TBX4* loci more frequently resulted in PH and congenital heart defects than did those sparing these two genes, suggesting a major role for these two genes in the cardiovascular components of the syndrome, whereas developmental disability had a homogeneous prevalence regardless of *TBX2/4* involvement, suggesting again a role for other neighbouring genes.

TBX2 contributes to airway growth and branching [40] and to endocardial cushion formation, critical in the pathogenesis of septal defects [41]. *TBX2* missense mutations were identified in individuals with cardiac septal defects, developmental delays and skeletal anomalies, but no PH [42]. We can speculate that, in the complex pathogenesis of congenital heart defect, *TBX4* and *TBX2* play significant yet distinct roles as causative or modifier genes, with *TBX4* contributing to PH onset and severity in this disease group. Conversely, developmental delay, hearing loss and skeletal defects were equally represented independently of *TBX2/TBX4* involvement, suggesting multiple gene interactions in the pathogenesis of non-cardiovascular anomalies.

Although *TBX4* was initially identified as a critical actor in hind limb development [43], it is highly expressed in developing lung mesenchyme [44], with a highly conserved *TBX4* enhancer sequence regulating its spatiotemporal expression [45]. Homozygous *TBX4* mutant mouse embryos die at embryonic day 10.5 from defective allantois formation and placental insufficiency [46], and conditional lung mesenchymal *TBX4* reduction leads to impaired lung development [44]. *TBX4* interacts with fibroblast growth factor 10 (*FGF10*), an essential regulator of the limb and lung bud growth and airway branching [47], which may account for combined lower limb/pulmonary phenotype in *TBX4*-associated disease. The *FGF10* pathway also regulates epithelial expression of thyroid transcription factor 1 (TTF1, encoded by the *NKX2.1* gene), a key factor in alveolar development and surfactant synthesis [48], which may contribute to neonatal respiratory symptoms in *TBX4* mutants.

Limitations of this study include a recruitment bias towards paediatric and neonatal forms of *TBX4*-linked PH; a lack of standardised inclusion criteria that precludes estimating the prevalence of *TBX4* variants among infants affected with PPHN, infantile/paediatric PH and congenital heart defects; and variable timing of follow-up precluding outcome comparisons.

In summary, this study confirms that *TBX4* variants underlie a complex variety of developmental lung disorders, resulting in a spectrum of clinical manifestations including PPHN, neonatal hypoxic respiratory failure, interstitial lung disease and chronic/progressive paediatric PH, often associated with multisystem anomalies. The variability and complexity of the phenotype and its potential overlap with other PH-associated gene defects warrant thorough molecular genetic testing, involving *TBX4*-inclusive diagnostic panels combined with CNV microarrays, in order to capture both small and large variants. The biphasic evolution we describe, characterised by hypoxic respiratory failure at birth followed by later-onset PH, suggests that infants with severe PPHN, especially if idiopathic, should undergo an appropriate echocardiography follow-up during infancy and early childhood, and should be tested for *TBX4* variants when positive and/or in the presence of suggestive features such as congenital heart defects, foot anomalies and SPS. Larger cohort- and population-based studies are needed to better delineate genotype–phenotype correlations and determine future diagnostic and therapeutic strategies.

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Conflict of interest: C. Galambos has nothing to disclose. M.P. Mullen has acted as a site principal investigator on trials sponsored by United Therapeutics, Actelion, Ikaria and GSK, and received travel support from Actelion, outside the submitted work. J.T. Shieh has nothing to disclose. N. Schwerk has nothing to disclose. M.J. Kielt has nothing to disclose. N. Ullmann has nothing to disclose. R. Boldrini has nothing to disclose. I. Stucin-Gantar has nothing to disclose. C. Haass has nothing to disclose. M. Bansal has nothing to disclose. P.B. Agrawal has nothing to disclose. J. Johnson has nothing to disclose. D. Peca has nothing to disclose. C. Surace has nothing to disclose. R. Cutrera has nothing to disclose. M.W. Pauciulo has nothing to disclose. W.C. Nichols has nothing to disclose. M. Griese has nothing to disclose. D. Ivy has contracts (through the University of Colorado School of Medicine) with Actelion, Bayer, Lilly and United Therapeutics for consultancy and research studies. S.H. Abman has nothing to disclose. E.D. Austin has nothing to disclose. O. Danhaive has nothing to disclose.

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