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Early View

Original article

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Title:

Phenotype characterization of *TBX4* mutation and deletion carriers with neonatal and pediatric pulmonary hypertension

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Authors' contribution:

OD and SHA designed the study and supervised the project; OD, CG, EA and MPM co-wrote the manuscript, CG performed the pathology studies in collaboration with JJ and RB; JTS performed the genetic analysis with the contribution of DP, CC, PBA and WCN; MPM and DI contributed to the cardiology and hemodynamics analysis; EA, MJK, MG and SHA contributed to the pulmonology analysis; NS, NU, RC, ISG, CH and MB contributed to clinical data.

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Summary message:

TBX4 mutations and deletions are associated with abnormal distal lung development, persistent pulmonary hypertension of the newborn and pediatric pulmonary hypertension, multiple congenital anomalies and developmental disabilities.

Abstract:

Rare variants in the T-Box transcription factor 4 gene (*TBX4*) have been recently recognized as an emerging cause of pediatric 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. *V*ariants included 6 17q23 deletions encompassing the entire *TBX4* locus and neighboring 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 2 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 remodeling. *TBX4* mutations and 17q23 deletions underlie a new form of developmental lung disease manifesting with severe, often biphasic pulmonary hypertension at birth and/or later in infancy and childhood, often associated with skeletal anomalies, cardiac defects neurodevelopmental disability and other anomalies.

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], leading 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 cases (FPAH) and a significant subset of idiopathic cases (IPAH). Mutations in the bone morphogenetic protein (BMP) receptor type 2 (*BMPR2)* gene and other BMP-associated genes are found in approximately 70% of FPAH cases, and 20% of IPAH 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 pediatric 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 etiologies causing persistent elevation of pulmonary vascular resistance and failure to transition from fetal to postnatal circulatory pattern. PPHN is more common than pediatric PAH, with an incidence of 0.18%, 20% of which 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 pediatric pulmonary hypertension, we collected data from 19 pediatric cases 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 centers (Table S1) on the basis of PH initially diagnosed by right heart catheterization (RHC) in 7 cases or echocardiography in 12 cases (table 2) during infancy or childhood and the presence of a *TBX4* rare variant identified via clinical or research testing. Small nucleotide variants (SNV) were identified by next generation sequencing (NGS) either from certified clinical laboratories or custom research panels, or by Sanger sequencing (Table S1). For missense variants, functional impact on protein structure was assessed by PolyPhen-2 and Combined Annotation Dependent Depletion v 1.3 (CADD) [17]. Minor allele frequency (<0.05) was checked searching the Exome Aggregation Consortium (ExAC) database [18]. Variants were compared to the ClinVar [19] and ClinGen [20] databases. Copy number variations (CNV) 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 (Table S1). Clinically obtained lung tissue, when available, was re-analyzed by a single pathologist (CG). This study was conducted in compliance with local institutional review boards.

Results

Genotype characterization (Figure 1, Table 1)

18 different heterozygous variants were identified in the 19 subjects (including 2 siblings) and consisted of 6 CNVs involving the 17q23.2 locus and 12 *TBX4* SNVs (Table 1). The CNVs comprised two sizes of approximately 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 #1,3,4,5) is a recurrent 17q23.1q23.2 deletion due to segmental duplications previously described [23] and reported in ClinGen. The larger CNV (cases #2,6) has only been reported once in ClinVar. The SNVs are novel. Among these, 10 are likely-gene-disrupting variants, including frameshift indel (#7-11), premature stop-gain (#12-14), and canonical splice site variants (#15, 16), and 3 are missense variants affecting the T-Box DNA binding domain consensus (#17-19) (Figure 1B). The 2 nonsense and 2 of the frameshift variants located downstream of the T-Box domain, in absence of experimental data demonstrating their gene disrupting effect, were classified as likely pathogenic. The 3 missense mutations were considered likely pathogenic on the basis of the Polyphen-2 and/or CADD scores 0.85-1 and ≥10-20 respectively, conservation of the aminoacid 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 8 variants in which inheritance was determinable, 3 (37%) were de novo and 5 (62%) were familial, with carrier siblings affected with SPS (#18) or determined previously to have PAH (#13/14), and carrier mothers affected with SPS (#12,15), PAH (#15) or asymptomatic (#16). Although the number and nature of tested genes varied from center to center (table S1), no

BMPR2, FOXF1 or other PH-related pathogenic gene variants could be found in any tested subject.

Clinical phenotype and outcomes (Table 2)

All infants were born term or late preterm (median 40.0 weeks; interquartile range (IQR) 38.0), with a female/male ratio of 2.16:1, similar for CNVs and SNVs (tables 2 and 4). Median birthweight was normal for gestational age (median 3,075g; IQR 2,450), although 3 newborns were small for gestational age (birthweight z-score < -1.28) [24]. 11 required invasive respiratory support. The most frequent presentation was PPHN in 10/19 subjects (53%), severe in 8 cases (oxygenation index >25 or need for ECMO); 4 neonates (10%) presented with transient respiratory distress without PH; the remaining 5 had an uneventful neonatal course. All infants survived their NICU course and were discharged home at a median age of 37 days (range 7–180), 6 with home oxygen and 2 with sildenafil. In 2 subjects 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 respectively; in the remaining 8 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 hypoxemia or for cardiorespiratory symptoms (see table 2). These subjects were diagnosed with PH (median age, 1.5 years; IQR 0.17). The duration of follow-up at the time of this report is variable between subjects, this study being retrospective (median, 10.0 years; IQR 4.7). 3 patients evolved to end-stage lung disease despite multiple vasodilator therapies: 1 died at 29 years and 2 underwent heart-lung transplantation at 18 or 11 years of age. 11 patients continue to have chronic PH at

their last evaluation, despite the use of multiple PH-targeted therapies in 7 subjects, 1 patient with a single PH-targeted therapy, and 3 children treated with supplemental oxygen alone. PH had resolved at the last follow-up (#14,15,16: 3 months-10 years) in the remaining 3 patients, 1 of whom was medication-free whereas 2 subjects remain on single vasodilator therapy. PH had resolved at the last follow-up (#14,15,16: 3 months-10 years) in the remaining 3 patients, 1 of which was medication-free and 2 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, neurosensorial deficits and muscular tone anomalies.

Cardiac imaging and hemodynamics (Table 3)

7 of the infants were assessed by echocardiography alone, while 12 underwent at least one RHC during their course. 10 subjects (53%) had systemic or supra-systemic RVSP in the neonatal period. 8 had patent ductus arteriosus (PDA), 4 of which persisted beyond the neonatal period. Two of these had left-to right shunting at initial PH diagnosis, one surgically closed at age 4 (#4), and one hemodynamically insignificant (#5). Two (#5 and 11) had right-to-left shunting at the diagnostic RHC persisting at follow up. An atrial septal defect (ASD) was present in 8 (42%). In the 12 subjects in whom it was performed (median age 1.5 years; IQR 0.17), cardiac catheterization demonstrated high mean pulmonary artery pressures (mPAP) (60.0 mmHg; IQR 57.5) and pulmonary vascular resistance indices (PVRi) (median 16.6 Wood units; IQR 10.7); however, only 6/12 subjects met all criteria for a strict diagnosis of PAH based on American Thoracic Society guidelines [25]. In the 6 subjects with serial RHCs, mPAP and PVRI values

resulted equally elevated or increased at follow-up (data not shown). Among the 10 subjects who underwent acute vasoreactivity testing, 8 (80%) failed to show a decrease in mPAP of at least 10mmHg to <40mmHg [25]. 6 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 subject (not shown).

Phenotypic characteristics and variant type (Table 4):

Table 4 compares the clinical, functional features between the 6 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% vs. 33%, p 0.029), in line with others' findings [8]. We also observed a trend to greater RHC functional severity in SNV carriers, although this may reflect an older age at first catheterization in that group. 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% vs. 0%, p = 0.02), and a similar trend for the presence of PH (57% vs. 17%, p = 0.16) for *TBX2/TBX4*-inclusive deletions.

Imaging studies

Thoracic images could be only collected in a subset of cases (Figure 2, Table S2). Neonatal chest radiograms (XR, n=5) showed lung hypoplasia (Figure 2A), air leaks and/or ground-glass

opacities; in infancy and early childhood (n=4), XR showed a pattern of septal thickening with multifocal areas of dysventilation, bronchial thickening and ground-glass opacities (figure 2B). 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 2 C-F).

Lung histopathology (Figures 3, Table 5)

Pathologic material was available for 7 patients, and histologic features of lung development and vessel remodeling were analyzed semi-quantitively. All samples showed diffuse alveolar growth abnormality and variable degree of PA wall remodeling with or without fibrointimal proliferation. No plexiform lesions or vessel necrosis was noted. In patients who had severe symptoms at early age and underwent biopsy in the neonatal period (Cases #1,7,12,13; Fig. 3, A-I*)* the histology showed severe disruption of distal lung development characterized 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 (PIG) [26], and three had patchy interstitial fibrosis. All had evidence of pulmonary arterial hypertensive remodeling. Back to back bronchiolar profiles were seen in 2 cases and one showed the presence of bronchial vessel recruitment including intrapulmonary bronchopulmonary anastomoses (IBA). Overall, these structural changes point to severe disruption of all compartment 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; Fig J-R) showed evidence of recruited bronchial vascular system, including intrapulmonary bronchopulmonary anastomoses and expanded bronchial veins and capillaries, in addition to alveolar simplification and PA remodeling. Features of airway remodeling and functional compromise were variably present characterized by airway wall thickening, increased number of intra-alveolar macrophages and multinucleated giant cells with cholesterol crystals (not shown).

A longitudinal histologic analysis was possible in patient 10 (biopsy at 2 y, and transplant at 18 y). The most striking histologic findings included the progression of compromised airway/alveolar growth characterized 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 maldevelopment. Evolving interstitial thickening with fibrosis, IBA recruitment and development of interstitial capillary proliferation was also noted.

Discussion

TBX4 variant carriers are at risk for abnormal distal lung development, PPHN, pediatric-onset PH, multiple congenital anomalies including congenital heart defects and a typical foot malformation, and developmental disabilities. The majority of our subjects (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 as well be classified as Group 3 (pulmonary hypertension 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 etiologies in our retrospective series we are using the term PH (vs PAH) for *TBX4* associated vascular disease.

Diffuse developmental lung disorders (DLD) are rare diseases related to aberrations in primary mechanisms of lung airway and vascular development, and include such diagnoses as AD, congenital alveolar dysplasia (CAD) and alveolar capillary dysplasia with misalignment of the pulmonary veins (ACDMPV), a lethal neonatal disease associated with *FOXF1* variants [27]. Emerging evidences show that DLDs 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 [28,29]. *TBX4* variants were reported in a neonate presenting with lethal AD [15], one with lethal CAD 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 a light on the pathogenesis of pulmonary hypertension in *TBX4* mutants, even though we cannot exclude a selection bias, as 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 remodeling of pulmonary arteries, prominent intrapulmonary bronchial anastomotic vessels (IBA), and other findings of interstitial disease. Our longitudinal observation suggests that these pathological fetal processes continue after birth and progresses with age in certain cases, with gradual vascular and lymphatic remodeling 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 disfunction 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 (#2,12,13,18,19) 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 pediatric PAH cohort (the REVEAL cohort [30]). Lack of response to vasoreactivity testing had been also described in children with *BMPR2* mutations compared to those without [31], suggesting that genetic forms of PH have distinct vascular pathophysiology.

Kerstjens-Frederikse first described *TBX4* mutations in 30% of a cohort of children labeled as I/FPAH and SPS, as opposed to only 2.5% of a control adult cohort [10]. Zhu calculated a 7.7% prevalence of *TBX4*-related disease in a larger cohort of pediatric PAH [11]. Levy also estimated a 7.5% *TBX4* mutation prevalence in 3/40 infants with PAH [12]. Eyries found a *TBX4* variant prevalence of 1/36 (2.8%) and 4/168 (2.4%) in French PAH children and adults respectively, below 19.4% and 14.3% for *BMPR2* in that cohort [8]. Overall, *TBX4* variants were detected with lower frequency in adult- than in pediatric-onset PAH, with an overall mutation frequency estimated at 1.5% (25/1633 cases) [8,10,11,32,33]. The lack of standardized inclusion and diagnostic criteria among centers in this pediatric 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 lung. Not all phenotypic features have the same expressivity. Whereas SPS has a high penetrance, 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 being affected with SPS alone and others combining 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 pulmonary hypertension

prior to the identification of causative genes [34], and confirmed in large registries [35]. The role of gender-dependent hormonal factors [36] and modifier genes [37] was subsequently demonstrated in *BMPR2*-relared 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 [38]. Some CNV cases with complete *TBX4* haploinsufficiency have less severe pulmonary hypertension 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.

Our patients with CNVs (#1-6) showed a greater incidence of developmental disability than those with SNVs (#7-19), suggesting a role for neighboring 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 (Table S3), PH and congenital heart defects resulted more frequent in the deletions involving the contiguous *TBX2* and *TBX4* loci than in those sparing these 2 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 neighboring genes.

TBX2 contributes to airway growth and branching [39] and to endocardial cushions formation, critical in the pathogenesis of septal defects [40]. *TBX2* missense mutations were identified in individuals with cardiac septal defects, developmental delays and skeletal anomalies, but no PH [41]. We can speculate that, in the complex pathogenesis of CHD, *TBX4* and *TBX2* play significant yet distinct role as causative or modifier genes, *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 [42], it is highly expressed in developing lung mesenchyme [43], with a highly conserved *TBX4* enhancer sequence regulating its spatio-temporal expression [44]. Homozygous *TBX4* mutant mouse embryos die at E10.5 from defective allantois formation and placental insufficiency [45], and conditional lung mesenchymal *TBX4* reduction leads to impaired lung development [43]. *TBX4* interacts with fibroblast growth factor 10 (*FGF10)*, an essential regulator of the limb and lung buds growth and airway branching [46], 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 [47], which may contribute to neonatal respiratory symptoms in *TBX4* mutants.

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

In summary, this study confirms that *TBX4* variants underlie a complex DLD, resulting in a spectrum of clinical manifestations including PPHN, neonatal hypoxic respiratory failure, interstitial lung disease, chronic/progressive pediatric 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, characterized 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 CHD, foot anomaly and SPS. Larger cohort and population-based studies are needed to better delineate genotype-phenotype correlations and determine future diagnostic and therapeutic strategies.

Figure legends

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 organization, peptidase activity and *TGFβ* signaling, ubiquitous with high expression in lung [48]), *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 [41]), *PPM1D* (protein phosphatase that regulates the DNA damage response pathway, mostly expressed in brain and associated with developmental disability in human [49], *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. UCSC Genome Browser (GRCH37/Hg19) [50].

B: Mutation positions in TBX4 protein.

The TBX4 protein is represented in grey, with the T-Box domain in pink. The 3 missense variants (yellow) are located in the T-Box domain. 3 of the frameshift variants (purple) are located either

upstream or inside the T-Box domain, while 2 are located downstream. The 2 nonsense mutations (green) are located downstream.

Figure 2. Lung imaging.

Case #1 (A): Chest XRay on day 1 with hyperlucent, hypoplastic lungs with poor vascular markings. Case #19 (B,C): Chest XR at age 3 years showing ground-glass opacities and bronchial thickening (B); chest CT at age 10 showing septal and bronchial wall thickening, honeycombing and diffuse nodules (C). Case #10 (D,E,F): chest CT at age 8 years (D) showing diffuse ground glass opacity and septal thickening, enlarged pulmonary arteries, and scattered emphysema. At age 18 years (E,F), markedly increased interstitial fibrosis with areas of central mosaic density in upper and lower lobes; small cystic areas, some bronchiectasis; some areas of lungs appear less involved; a parenchymal bulla is present in the lingula.

Figure 3. Representative histopathology images

Early biopsies obtained in the neonatal period show markedly underdeveloped respiratory lobules (A, B; 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 widened by the presence of immature, pale mesenchymal cells (D and E, arrows: 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 remodeling 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 characterized by large, dilated and simplified alveoli (examples are labelled with asterisks on K). Marked vascular changes including pulmonary arterial hypertensive remodeling (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), marked muscular hypertrophy of lymphatic vessels (arrows on M; H+E stain). Pathologic remodeling of 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 are of bone formation (arrows on Q; H+E stain). In one case (10b) marked pleural smooth muscle muscularization is noted (arrow on R; H+E stain).

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Table 1. 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	Neurologic and psychomotor deficits
1	CNV	17q23.1q23.2(58,078,171-60,316,749)x1	Gene deletion haploinsufficiency	Heterozygous De novo	n/a	Pathogenic (In 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 (in 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 (In ClinGen)	Joint contractures, foot anomaly	ASD	Mild developmental delay, hearing loss, 2-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 (In 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 (In 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*44)	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 apnea	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 (in Clinvar)	Small patella syndrome, 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 (in Clinvar)	Foot anomaly	PDA	No
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, Interstitial lung disease	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, interstitial lung disease	no
14							No	Transient PFO and PDA, interstitial lung disease	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 (in 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 (2 siblings carrying p.(Leu186Arg), with SPS	ExAC AF: 0 CADD : 28.8 P2 : 1.000	Likely pathogenic (in 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

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.

Abbreviations : ExAC: Exome Aggregation Consortium ; AF: allele frequency; CADD: Combined Annotation Dependent Depletion ; P2: Polyphen2; ACMG: American College of Medical Genetics ; AA: aminoacid; SPS : small patella syndrome ; PH : pulmonary hypertension ; ASD : atrial septal defect ; PDA : patent ductus arteriosus ;PFO : patent foramen ovale ; ADHD : attention deficit hyperactivity disorder. M: mother. SPS: small patella syndrome; ILD: interstitial lung disease.

Table 2. Clinical characteristics

case	Gender, ethnicity	Neonatal course							Postnatal PH course				
		GA (weeks) BW (grams) Z score	Clinical presentation	ECMO (days)	iNO (days)	MV (days)	home (days)	meds	Initial diagnosis		tissue sample	cardiovascular meds	Outcome Age at follow-up/death
1	Male Caucasian	36 2,000 (z -1.70)	PPHN (ref) RDS	Yes (12)	Yes (35)	Yes (45)	Yes (60)	O2 PDE5	E	3 months: hypoxemia 4 m: RSV infection, HRF, ECMO	4 m biopsy	O2, PDE5i,	5 months : death from hypoxic respiratory failure
2	Male Caucasian	38 3,090 (z -0.15)	Omphalocele Transient tachypnea	No	No	Yes	Yes (17)	No	E	5 months: hypoxemia	-	O2	4 years : stable mild PH, ILD
3	Female Caucasian	36 2,790 (z 0.42)	PPHN	No	Yes (6)	Yes (6)	Yes (41)	O2	E	2 months: hypoxemia	-	PDE5i, ERA	10 years : stable mild PH
4	Female	40	Normal	No	No	No	Yes		C	4 years: intermittent hypoxemia. 5 years: PDA closure Lack of follow-up until age 18.	-	O2, PDE5i, treprostinil	29 years : death from refractory pulmonary hypertension
5	Female	40	PPHN Pneumothorax	Yes (10)	Yes	Yes	Yes (45)	No	E	2 ½ months – hypoxemia,	-	O2, PDE5i, treprostrinil, epoprostenol, CCB	15 years : stable PH
6	Female Caucasian	39 1/7 3.136 (z -0.27)	RDS	No	No	No	Yes (12)	O2	C	6 months – hypoxemia, dyspnea	-	ERA, PDE5i, O2	11 years ; severe PH
7	Female Caucasian	37 4/7 weeks 1,890 g (z -2.34)	PPHN	No	Yes (1)	Yes (13)	Yes (180)	O2	E	1,5 month : hypoxemia, dyspnea	3 m biopsy	O2, iNO, PDE5i, digoxin,	8 months : death, pulmonary hypertension crisis during surgery (Meckel)
8	Female Caucasian	40 weeks	Normal	No	No	No	Yes	No	C	3 years – dyspnea, fatigability, syncope	-	O2, SQ treprostinil, PGE5i	12 years : severe PH
9	Female Caucasian	40 weeks maternal methamphetamine use	PPHN MAS	Yes (5)	Yes	Yes (15)	Yes (30)	No	C	2.5 years – hypoxemia, syncope	-	O2, PDE5i, ERA, SQ treprostinil	7 years : severe PH
10	Male Caucasian	40 weeks 3,490 g (z -0.17)	PPHN	Yes (2)	Yes (2)	Yes (5)	Yes (30)	O2	C	18 months - hypoxemia	2 y biopsy 18 y explant	O2, ERA, PDE5i, CCB	18 years : end-stage PH, heart-lung transplantation
11	Female	40 weeks	Normal	No	No	No	Yes	none	C	8 years – PDA R>L	-	ERA	
12	Female Caucasian	39 1/7 weeks 3,450 g (z 0.13)	PPHN (OI: 40) RDS Pneumothorax	No	Yes	Yes (11)	Yes (64)	O2	E	2.5 years : hypoxemia, chILD	2 m biopsy	O2, PDE5i, ERA,	11 years : End-stage chILD, heart-lung transplantation
13	Female Caucasian	40 3/7 weeks 3,510 g (z 0.04)	PPHN (OI: 78) RDS	No	Yes (7)	Yes (10)	Yes (27)	O2	E	1 month - chILD;	1.5 m biopsy	O2	4 years : Severe chILD mild PH

14	Male Caucasian	40 3/7 weeks 3,820 g (z 0.33)	PPHN (OI: 57) RDS	No	Yes (5)	Yes (5)	Yes (14)	No	E	1 month – persistent tachypnea	-	PDE5i	3 months : chILD, no pulmonary hypertension (short follow-up)
15	Female Caucasian	39 weeks 2,950 g (z -0.62)	RDS Pneumothorax	No	No	No	Yes (15)		E	5 months – persistent chILD following RSV infection	-	none	7 months : bronchiolitis 10 years : chILD, no pulmonary hypertension
16	Female Caucasian	40 weeks	RDS Pneumothorax	No	No	No	Yes (7)	No	E	1 month – persistent tachypnea	-	PDE5i	10 years : no residual PH
17	Male Caucasian	38 weeks 2,040 g (z -2.65)	Normal	No	No	No	Yes	No	C	12 years - hypoxemia during knee surgery;	-	O2, PDE5i, ERA, IV treprostinil	21 years : moderate PH
18	Male Caucasian	36 5/7 weeks 2,450 g (z -1.00)	PPHN (OI: 58) RDS	No	Yes (6)	Yes (9)	Yes (92)	O2	E	7 years – chILD	7 y biopsy	O2, PDE5i, ERA	9 years : moderate PH
19	Female, Caucasian	40 weeks 3,075 g (z -0.71)	Normal	No	No	No	Yes	No	E	5 months : chILD	7 y biopsy	O2	10 years :ch ILD, moderate PH

Abbreviations: GA : gestational age ; BW : birth weight ; HRF: hypoxic respiratory failure; ECMO : extracorporeal membrane oxygenation ; iNO : inhaled nitric oxide ; MV : mechanical ventilation ; d/c : discharge ; Meds : medications ; PPHN : persistent pulmonary hypertension of the newborn ; RDS : respiratory distress syndrome ; chILD: childhood interstitial lung disease; OI : oxygenation index ; (E) : echocardiography ; (C) : cardiac catheterization ; PDE5i : phosphodiesterase 5 inhibitor ; O2 : oxygen therapy ; ERA : endothelin receptor antagonist ; CCB: calcium channel blocker; PH : pulmonary hypertension ; RSV : respiratory syncytial virus infection.

PH was defined by a mean pulmonary artery pressure >25mmHg >3 months of age—each of these children had a pulmonary artery wedge pressure <15mmHg and pulmonary vascular resistance index >3 WU.m^2 consistent with pulmonary arterial hypertension (PAH), requiring right heart catheterization (RHC) for accurate, quantitative diagnosis, although Doppler echocardiography provide reliable qualitative data with the benefit of less invasiveness [25]. The echocardiography criteria for PPHN were: estimated right ventricle systolic pressure 2/3 equal to greater than systemic pressure by direction and velocity of ductus arteriosus flow and/or two-dimensional interventricular septum position and/or peak tricuspid regurgitant (TR) jet velocity [51]. Childhood Interstitial lung disease 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 (tachypnea, adventitious sounds, retractions, digital clubbing, failure to thrive, or respiratory failure); (3) hypoxemia; and (4) diffuse abnormalities on a chest radiograph or computed tomography scan, after exclusion of the common diseases that can cause DLD as the primary diagnosis [52].

Table 3. Echocardiography and catheterization data at initial diagnosis.

Case	Original echocardiography		Heart defects	Original right heart catheterization data														
				Hemodynamics at baseline								Hemodynamics on max O2/iNO vasodilation						
	Age	estimated RVSP		Age	MPAP	MSP	PCWP	PVRi	RA	CI	Qp/Qs	MPAP	PCWP	PVRi	RA	CI	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**	

Abbreviations and measurement units: Age (years) ; MPAP : mean pulmonary artery pressure (mmHg) ; MSP : main systemic pressure (mmHg) ; PCWP : post-capillary wedge pressure (mmHg) ; PVRI : pulmonary vascular resistance index ; RA : right atrium pressure (mmHg) ; CI : cardiac index (L/min/M2); Qp/Qs pulmonary-to-systemic blood flow ratio ; RVSP: right ventricle systolic pressure (mmHg). ASD: atrial septal defect; PDA: patent ductus arteriosus beyond neonatal period; VSD: ventricular septal defect. n/a: not available. IQR: interquartile range.

Maximum vasodilation testing was performed by exposure to FiO2 100% and iNO 40ppm; responsiveness was defined as a decrease in mPAP of at least 10 mmHg to achieve values below 40 mmHg with a normal or increase in CO and a decrease or no change in PVR/SVR ratio [25].

* Echocardiography report could not be retrieved, but mild PH was reported in medical record, prior to PDA closure at age 5. The infant was subsequently lost to follow-up and untreated until age 18, when right heart catheterization revealed very elevated MPAP and PRV with intracardiac R>L shunting.

Table 4. Clinical and functional differences in subjects with chromosomal deletions vs. small nucleotide variants.

Clinical and functional features		All Subjects (n=19)	Subjects with CNV (n=6)	Subjects with SNV (n=13)	P value #
Biometrics:	Female, number (%)	13 (68%)	4 (67%)	9/13 (69%)	1
	Birth weight (grams): median (IQR) *	3,075 (2,450)	2,754 (523)	2,896 (706)	0.36
	Gestational age (weeks): median (IQR)	40.0 (38.0)	38.5 (36.5)	40.0 (39.0)	0.12
	Birth weight z-score: median (IQR) *	-0.27 (-1.00)	0.42 (0.90)	0.77 (1.06)	0.57
Presentation:	PPHN	10 (53%)	3/6 (50%)	7/13 (54%)	1
	Age at PH diagnosis, years: median (IQR)	2.69 (3.56)	0.92 (1.38)	3.45 (3.92)	0.16
	Death or transplant	5 (26%)	2/6 (33%)	3/13 (23%)	1
Associated anomalies:	CHD **	7 (39%)	5 (83%)	5 (42%)	0.15
	Toe anomaly	13 (68%)	4/4 (100%)	6/9 (67%)	0.49
	Developmental disability	17 (89%)	5/5 (100%)	4/12 (33%)	**0.029**
Right heart catheterization:	n performed	12	4	8	
	MPAP, mmHg: mean (SD)	67.8 (27.1)	56.0 (33.5)	76.3 (21.9)	0.23
	CI: mean (SD)	3.3 (0.9)	3.5 (1.0)	3.1 (0.8)	0.49
	PVRI, WU: mean (SD)	23.2 (18.7)	18.4 (19.4)	26.7 (18.9)	0.47
	Vasodilator responsiveness $	2 (16%)	1 (25%)	1 (14%)	1

Abbreviations: IQR: interquartile range; PPHN: persistent pulmonary hypertension of the newborn; PH: pulmonary hypertension; SD: standard deviation; CHD: congenital heart defect; MPAP: mean pulmonary arterial pressure; CI: cardiac index; PVRI: pulmonary vascular resistance index; WU: Wood units; CNV: copy number variant; SNV: small nucleotide variant.

* Birth weight only available for 13 subjects. ** CHD was not determined in one patient due the presence of PDA and PFO at <1month of age, not re-tested subsequently. $ Vasodilator Responsiveness categorized according to Revised Barst Criteria [30]. # P value for comparison of the group with CNVs versus Nucleotide Variants; T-test for numerical values, Fisher test for categorical values.

Table 5 Semiquantitative analysis of histologic features contrasted to outcome and age at biopsy.

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	4m	death at 5 m	diffuse	diffuse	diffuse	none	focal	moderate	absent	absent
#7	3m	death at 8 m	diffuse	diffuse	patchy	patchy	absent	moderate	moderate	absent
#12	2m	txplt at 11 y	diffuse	patchy	absent	patchy	multifocal	moderate	absent	present
#13	1,5m	severe ILD at 4y	diffuse	patchy	diffuse	patchy	absent	moderate	absent	absent
#10a	2 y	txplt at 18 y	diffuse	absent	absent	absent	absent	moderate	absent	present
#10b	2 y	txplt at 18 y	diffuse	diffuse	absent	diffuse	multifocal	moderate	absent	present
#18	7y	moderate PH at 9 y	diffuse	diffuse	absent	patchy	absent	moderate	absent	present
#19	7y	moderate PH at 10 y	diffuse	absent	absent	absent	absent	moderate	moderate	present

Semiquantitative analysis of histologic features of 7 cases contrasted with time of biopsy and outcome. The first four cases (#1,7,12,13) had biopsies at neonatal period and showed grimmer prognosis, while samples of the last 3 cases (#10,18,19) were obtained at childhood with better outcome. Case #10 had biopsy at age 2 and transplant at age 18 allowing longitudinal assessment of evolving histologic features (see details in text) (patchy< 50% of parenchyma, diffuse>50% of parenchyma, focal, 3 foci, multifocal>3 foci, moderate PA wall remodeling: PA muscle wall thickness estimated 40-70% of diameter)

Abbreviations: PA: pulmonary artery, txplt: lung transplant , PIG: pulmonary interstitial glycogenosis, PH: pulmonary hypertension, m: months, y: years

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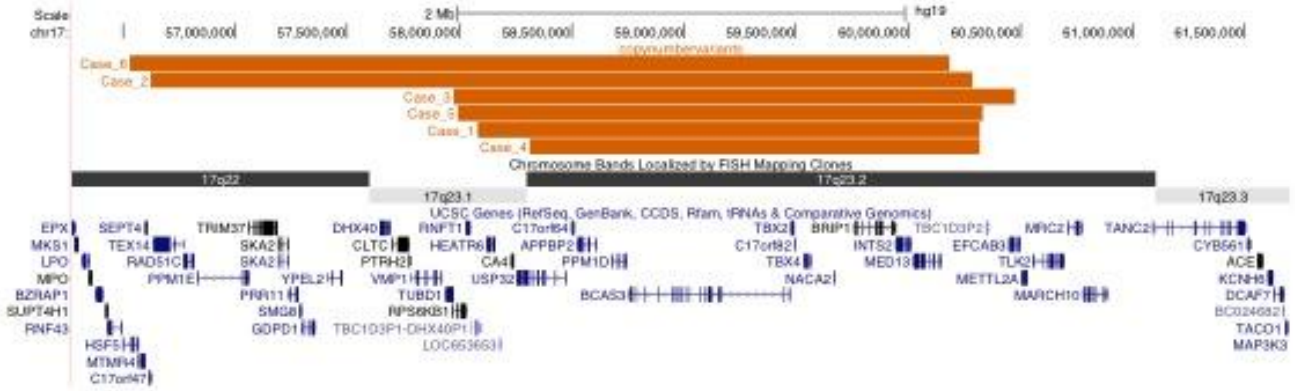
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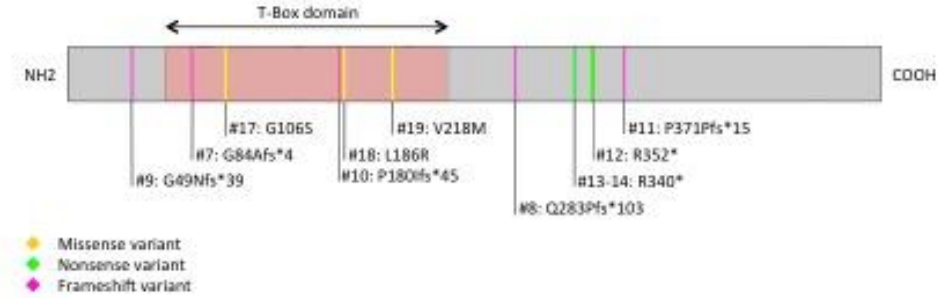
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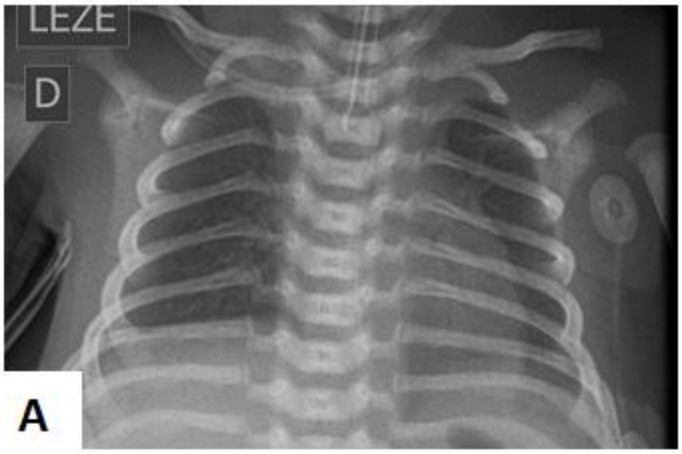
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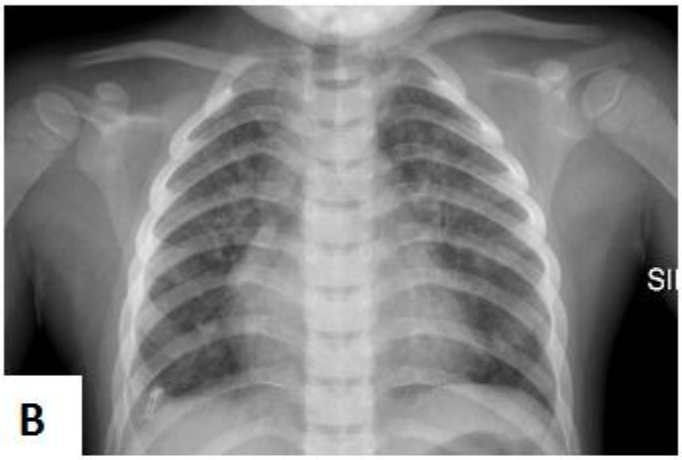

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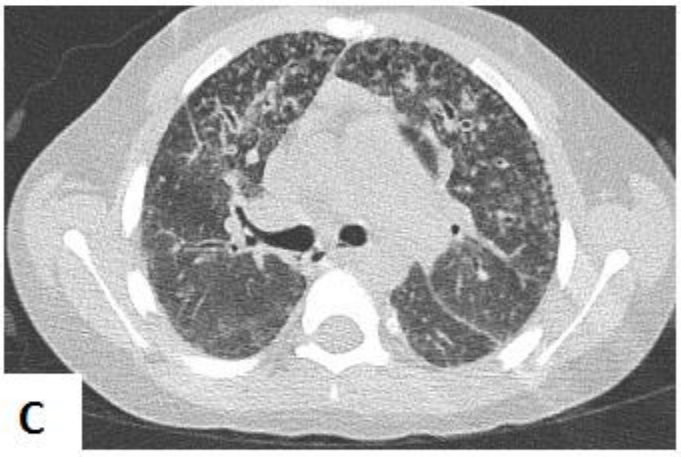

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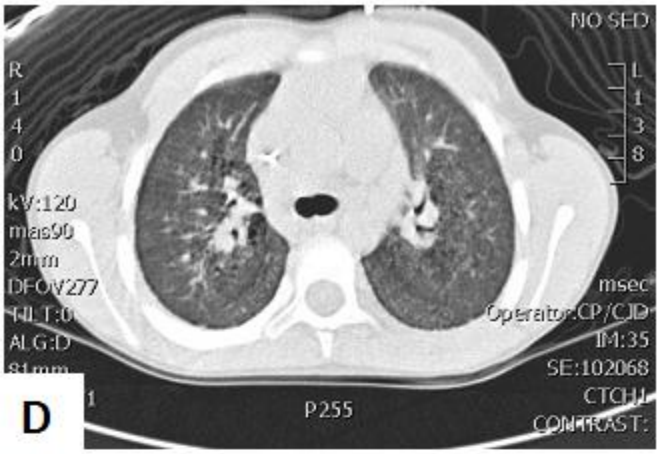
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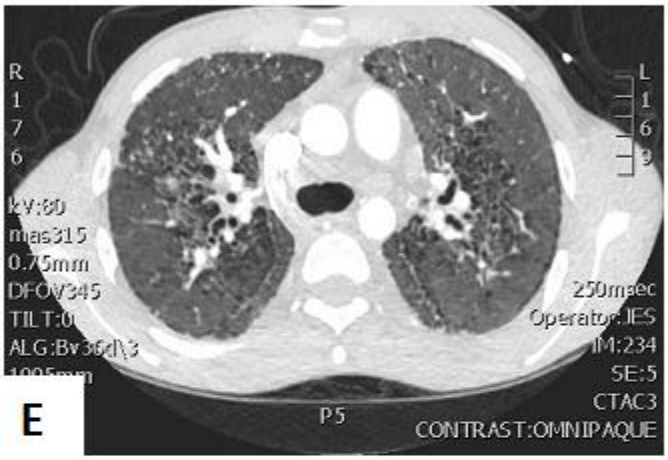
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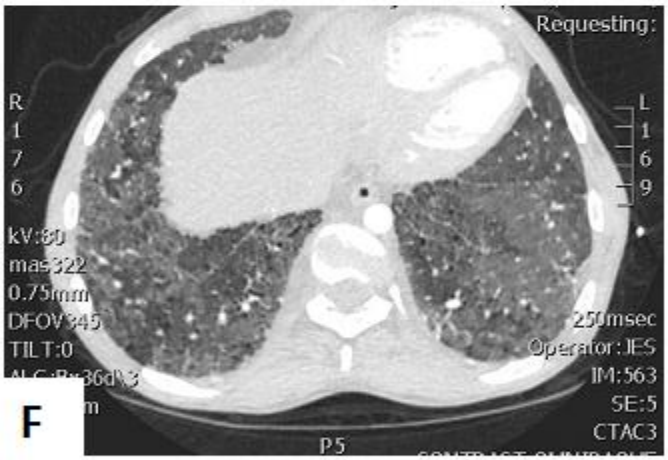
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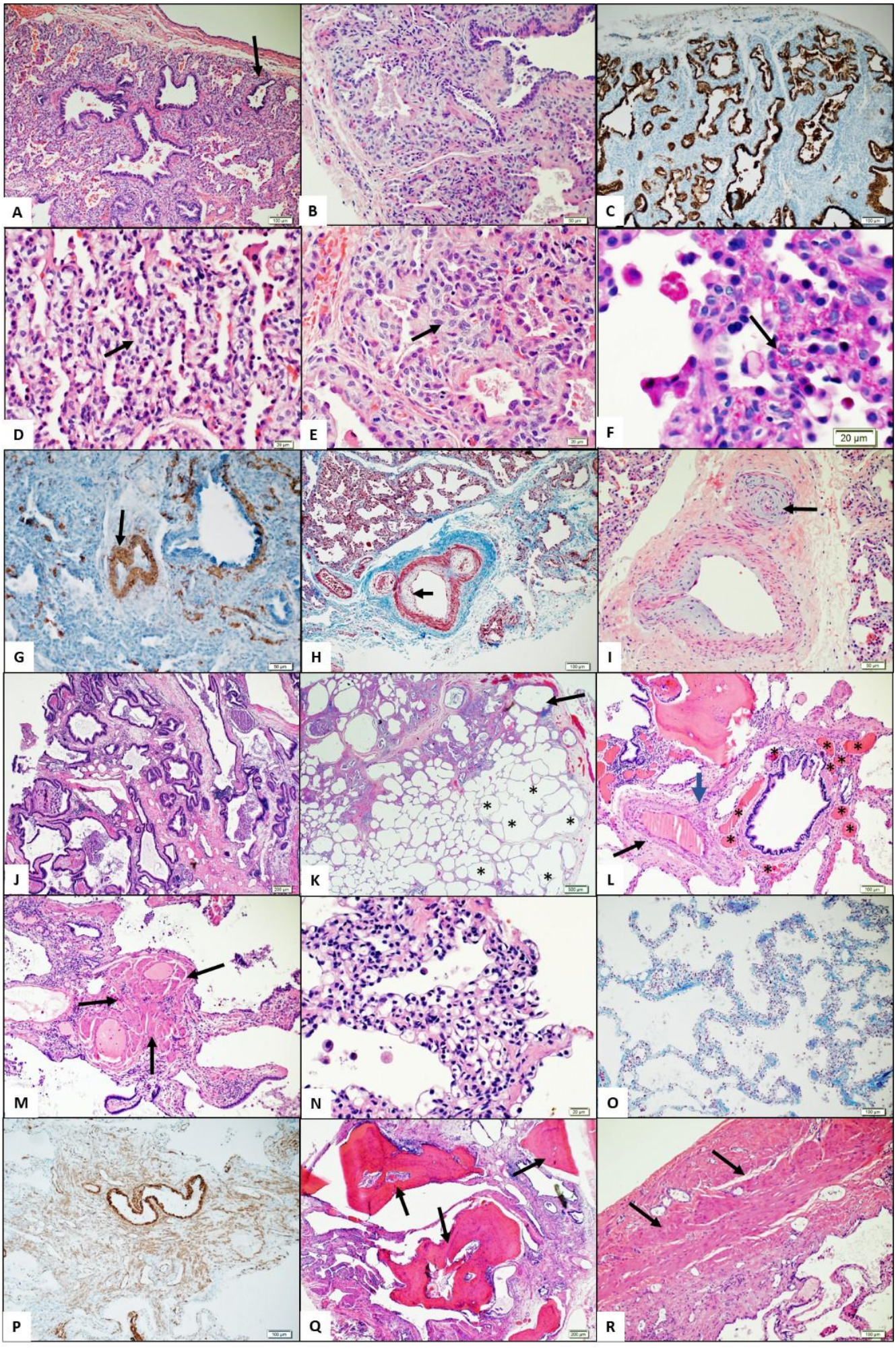
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Phenotype characterization of *TBX4* mutation and deletion carriers with neonatal and pediatric pulmonary hypertension.

Supplementary material.

Table S1.

Case #	Origin of testing	NGS panel	CNV array	Sanger sequencing	Other findings	Family testing
1, 7, 13, 15, 19	Bambino Gesù Children's Hospital, Rome	*ABCA3, SFTPB, SFTPC, SFTPA1, SFTPA2, SFTPD, NKX2.1, ENG, CAV1, BMPR2, SMAD1, SMAD5, SMAD9, ACVRL1, TBX4, FOXF1, MEOX2, CSF2RB, CBLN2, CRHR1, CRHBP, PPARG* *	GCH array	#7,13,15,19 : TBX4	no	#1,7: CGH trio analysis #15 : TBX4 Sanger in parents
4,5,11	Boston Children's Hospital	no	#4, 5 : CGH array	#11	#4 : Xp22.31 duplication*	no
12,14,18	Munich University	*ACVRL1, BMPR1B, BMPR2, CAV1, EIF2AK4, ENG, GDF2, KCNA5, KCNK3, SMAD1, SMAD4, SMAD9, TBX4***	no	#12, 14, 18	n/a	#12, 18 : confirmatory TBX4 Sanger sequencing in mother and maternal grandmother (SPS affected)
2, 8, 9, 16, 17	Children's Hospital Colorado	#8,16,17: *BMPR2, ALK1, ENG, CAV1, KCNK3, EIF2AK4, SMAD 9* #9: *BMPR2, ALK1, ENG, CAV1, KCNK3, EIF2AK4, SMAD 9, ABCC8, KCNA5, SNAD4, GDF2, TBX4* ***	#2 : SNP array	#8, 16, 17	#9 : Del(3)(q26.2-q26.32) (inheritance not confirmed)	#2 : Trio FISH analysis
#3, 10	Vanderbilt University Hospital	#10: *BMPR2, ALK1, ENG* ****	#3 : CGH array	#10	no	no
#6	University of Iowa Hospital	Not performed	ChiP array	no	no	no

Cases were selected from the following registries and centers : the Pediatric Pulmonary Hypertension Network (PPHNet) [s1] (cases #2,8,9,16,17), Childhood Interstitial Lung Disease – Europe consortium (chILD-EU) [s2] (cases #12,13,14,18), Bambino Gesù Children's Hospital, Rome, Italy (1,7,15,19), Boston Children's Hospital, Boston, MA (cases #4,5,11), Vanderbilt University Children's Hospital, Nashville, TN (cases # 3,10) and Iowa Children's Hospital, Iowa city, IA (case #6). Genetic testing: * Custom research panel target; ** Center for Human Genetic, Munich University diagnostic panel; ***

National Institute of Health Pulmonary Arterial Hypertension Biobank reserach panel, former and current versions ; ****Commercial pulmonary hypertension diagnostic panel.

Table S2: initial and subsequent chest imaging.

	Chest radiogram	Chest high-resolution computed tomography	
		Early (first year of life)	Late (1-18 years)
1	1 day : decreased expansion, decreased vascular markings.		
2			1 year : mild interlobular septal thickening and ground glass opacities in the lower lobes, juxtapleural nodules in upper and lower lobes.
3	3 years : mild, generalized hyperinflation ; moderate, parahilar, peribronchial wall thickening.		9 years : normal
7	1 day : decreased expansion, diffuse groud glass opacity, right tension pneumothorax,	20 days : diffuse areas of hyperinflation and dysventilation, ground-glass opacities.	
8		1 month : nonspecific lower lobes septal thickening, patchy areas of atelectasis ands hyperinflation.	
9	.		3 years : RV and pulmonary artery enlargement, Multiple small nodules in right upper lobe with patchy ground glass opacities, peripheral septal thickening
10			18 years : multifocal densities with cystic changes. Central emphysema and fibrosis ; right ventricular hypertrophy, enlarged pulmonary artery and veins ; mediastinal/hilar lymphadenopathy.
12	1 day : decreased expansion, left tension pneumothorax.		9 years : Patchy ground glass opacities, septal thickening, consolidation, mosaic perfusion, regional hyperinflation and air trapping
13	1 day : bilateral pneumothorax, diffuse ground glass opacities, interlobular thickening, regional hyperinflation.	4 weeks : mild diffuse ground glass opacities	2.5 years : severe diffuse patchy ground glass and consolidations
14	1 day : right pneumothorax, diffuse ground-glass opacities	12 days : prominent PA, very discrete diffuse ground glass	
15	2 years : Minimal perilar bronchial thickening		9 years : mild bronchial collapse, expiratory air trapping; no significant interstitial lung disease.
16			9 years : small diffuse opacities (possible PCH), mild peribronchial thickening, enlarged pulmonary arteries.
17			13 years : mild central bronchial wall thickening, patchy air trapping and lower lobe atelectasis, lobar nodular opacities.
18	1 month : diffuse ground glass opacities, interlobular thickening, consolidations, hyperinflated areals .	5 months: bilateral diffuse ground glass opacities, interlobular thickening, consolidations, hyperinflated areals	7 years : irregular emphysema (mosaic like pattern) septal thickenings, consolidations
19	3 years : bilateral diffuse thickening of bronchial walls prevalent in basal lungs (3 yrs)		10 years : interstitial thickening, centrilobular nodules (« tree in bud » pattern), diffuse bronchiectasis, initial honeycombing.

Table S3. Phenotypic spectrum in published 17q23 deletions including the current cohort.

Studies	Tech	n	Sex	TBX2/TBX4	PAH	CHD	HL	DD	facial	skel	Remarks
S3	Mic	1	F	-	(+)*	-	+	+	+	-	Hydrocephalus, * secondary PAH, comfort care
S4	Mic	1	M	-	-	-	-	+	-	-	Epilepsy, microcephaly
S5	Mic	1	M	-	-		+	+	+	+	TEF, microcephaly, hypothyroidism
S6	Mic	1	F	-	-	-	+	+	+	+	
S7	Kar	1	F	-	-	-	-	+	+	+	TEF
S8	Kar	1	F	-	-	-	-	+	+	+	Epilepsy
S9	Kar	1	M	+	-	-	-	+	+	+	Craniosynostosis
S10	Kar	1	M	+	-	-	unk	+	+	+	TEF, VSD, PS, death 3 mo
S11	Kar	1	F	+	+*	+	unk	unk	+	+	* death at 17 days ; PAH changes at autopsy
S12	Kar	1	M	+	-	-	+	+	+	+	
S13	Kar	1	M	+	+*	+	unk	unk	+	unk	TEF, VSD, * likely PAH, death 4 mo
S14	Mic	1	F	+	+	-	+	+	+	+	Microcephaly
S15	Mic	2	F	+	-	-	+	+	-	-	
			M	+	-	-	+	+	-	-	
S16	Mic	7	F	+	-	+	+	+	+	+	
			F	+	-	+	-	+	+	+	Esotropia
			M	+	+	-	-	-	+	+	PPHN
			F	+	+	+	+	-	-	+	
			F	+	-	+	-	unk	+	+	Cutis aplasia
			F	+	-	-	-	+	+	+	
			F	+	+	+	-	+	+	+	
This study	Mic	6	M	+	+	-	-	unk	-	-	Hypothyroidism, cortisol deficiency, death 5 mo
			M	+	+	+	-	-	-	-	Omphalocoele, seizures, nystagmus
			F	+	+	+	-	+	+	+	
			F	+	+	+	-	-	+	+	
			F	+	+	+	-	+	+	+	
			F	+	+	+	+	+	+	+	Microcephaly, esotropia
Statistics :											
Total (n)		27	17F (63%)	21/27 (78%)	13/27 (48%)	11/27 (40%)	10/24 (42%)	19/23 (83%)	21/27 (78%)	20/26 (77%)	
TBX2/TBX4 -		6			1/6 (17%)	0/6 (0%)	3/6 (50%)	6/6 (100%)	5/6 (83%)	4/6 (67%)	
TBX2/TBX4 +		21			12/21 (57%)	12/21 (57%)	7/18 (39%)	13/17 (76%)	16/21 (76%)	16/20 (80%)	
P value					0.16	0.02	0.66	0.28	1	0.59	

Legend : Tech : testing technique ; Mic : chromosomal microarray ; Kar : karyotype ; unk : unknown ; PAH pulmonary arterial hypertension ; CHD : congenital heart disease (patent ductus arteriosus, atrial septal defect) ; HL hearing loss ; DD developmental delay ; digits - abnormal toes and/or fingers ; facial – facial dysmorphisms ; skel – other skeletal anomalies ; TEF tracheo-esophageal fistula ; VSD ventricular septal defect. Statistics : n : number of positive subjects over total tested per category; TBX2/TBX4 - : deletions exclusive of the TBX2/TBX4 loci ; TBX2/TBX4 + : deletions inclusive of the TBX2/TBX4 loci. P values determined by Fisher exact test.

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