The Genetic Epidemiology of Pediatric Pulmonary Arterial Hypertension

Meindina G. Haarman, MD[1], Wilhelmina S. Kerstjens-Frederikse, MD, PhD[2], Theresia R. Vissia-Kazemier, RN, MANP[1], Karel T. N. Breeman, MD[1], Wim Timens, MD, PhD[3], Yvonne J. Vos, PhD[2], Marc T. R. Roofthooft, MD, PhD[1], Hans L. Hillege, MD, PhD[4,5], and Rolf M. F. Berger, MD, PhD[1]

Objective To describe the prevalence of pulmonary arterial hypertension (PAH)-associated gene mutations, and other genetic characteristics in a national cohort of children with PAH from the Dutch National registry and to explore genotype-phenotype associations and outcomes.

Study design Children (n = 70) diagnosed with idiopathic PAH, heritable PAH, PAH associated with congenital heart disease with coincidental shunt (PAH-congenital heart disease group 3), PAH after closure of a cardiac shunt (PAH-congenital heart disease group 4), or PAH associated with other noncardiac conditions were enrolled. Targeted next-generation sequencing was performed on PAH-associated genes (*BMPR2*, *ACVRL1*, *EIF2AK4*, *CAV1*, *ENG*, *KCNK3*, *SMAD9,* and *TBX4*). Also, children were tested for specific genetic disorders in case of clinical suspicion. Additionally, children were tested for copy number variations.

Results Nineteen children (27%) had a PAH-associated gene mutation/variant: *BMPR2* n = 7, *TBX4* n = 8, *ACVRL1* n = 1, *KCNK3* n = 1, and *EIF2AK4* n = 2. Twelve children (17%) had a genetic disorder with an established association with PAH (including trisomy 21 and cobalamin C deficiency). In another 16 children (23%), genetic disorders without an established association with PAH were identified (including Noonan syndrome, Beals syndrome, and various copy number variations). Survival rates differed between groups and was most favorable in *TBX4* variant carriers.

Conclusions Children with PAH show a high prevalence of genetic disorders, not restricted to established PAH-associated genes. Genetic architecture could play a role in risk-stratified care management in pediatric PAH. *(J Pediatr 2020;225:65-73)*.

A number of gene mutations have been identified that are associated with pulmonary arterial hypertension (PAH), including bone morphogenetic protein receptor type 2 (*BMPR2*), T-box 4 (*TBX4*), activin receptor-like kinase 1 (*ACVRL1*), endoglin (*ENG*), potassium channel subfamily K member 3 (*KCNK3*), eukaryotic translation initiation factor 2-alpha kinase 4 (*EIF2AK4*), caveolin-1 (*CAV1*), and *SMAD9*.[1-5]

In 80% of familial PAH cases and in 10%-21% of the sporadic patients (both pediatric and adult) a mutation in the *BMPR2* gene is identified.[4,6-8] In contrast with the rather dominant role of *BMPR2* mutations in adults, in children more genetic diversity has been reported. Previously, *TBX4* variants (either mutations or copy number variations [CNVs]) were identified to be associated with unexplained childhood PAH in 21% of the cases.[1] Levy et al found *TBX4* variants to account for 7.5% of pediatric patients with PAH, where *BMPR2* and *ACVRL1* mutations were present in 12.5% and 10%, respectively.[9] Zhu et al further confirmed the association between *TBX4* and pediatric PAH in a large cohort of children with PAH.[10] In adults with PAH, the prevalence of *TBX4* variants seems to be lower, with a reported frequency of occurrence of 2%-3%; nevertheless, it remains the most frequently reported mutated gene after *BMPR2*.[11]

Further, children with PAH have been reported to frequently present with concomitant genetic or syndromic conditions that may or may not have an established association with PAH.[7]

ACTA2	Alpha-actin-2	*MMACHC*	Methylmalonic aciduria and homocystinuria type C protein
ACVRL1	Activin receptor-like kinase 1	PAH	Pulmonary arterial hypertension
BMPR2	Bone morphogenetic protein receptor type 2	PH	Pulmonary hypertension
CblC	Cobalamin C	PVOD	Pulmonary veno-occlusive disease
CHD	Congenital heart disease	*TBX4*	T-box 4
CNV	Copy number variation	*VHL*	Von Hippel Lindau
EIF2AK4	Eukaryotic translation initiation factor 2-alpha kinase 4	WES	Whole exome sequencing
JAG1	Jagged1	WHO-FC	World Health Organization Functional Class
KCNK3	Potassium channel subfamily K member 3		

From the [1]Department of Pediatric Cardiology, Center for Congenital Heart Diseases, Beatrix Children's Hospital; [2]Department of Genetics, University of Groningen; [3]Department of Pathology and Medical Biology; [4]Department of Epidemiology; and [5]Department of Cardiology, University Medical Center Groningen, Groningen, the Netherlands

Supported by the Sebald Fund. The authors declare no conflicts of interest.

https://doi.org/10.1016/j.jpeds.2020.05.051

This study aimed to describe the epidemiology of genetic disorders in a Dutch national cohort of children with PAH, divided into 3 groups: mutations and variants in PAH-associated genes, other genetic disorders with an established association with PAH, and concomitant genetic disorders without an established association with PAH. Furthermore, genotype-phenotype associations concerning clinical presentation and outcome were explored.

Methods

In the Netherlands, all pediatric patients with suspected PAH are referred to the University Medical Center Groningen, the National Referral Center for Pulmonary Hypertension in Childhood. All patients are prospectively followed according to a standardized protocol and included in a national registry. Ethical approval for this ongoing registry was obtained from the Medical Ethics Review Board (METc 2008.009) and written informed consent from the patients (and/or their guardians) is given at enrollment.

Patients

Children with a diagnosis of idiopathic or heritable PAH, PAH associated with congenital heart disease (CHD) and coincidental shunt or after closure of a cardiac shunt (PAH-CHD groups 3 and 4 according to the most recent clinical classification of pulmonary hypertension [PH] of 2019), pulmonary veno-occlusive disease (PVOD), or PAH associated with other noncardiac conditions referred between 2003 and 2018, were included.[12-14] Children with PAH-CHD group 3 and 4 were included in this study since clinical course, including (transplant-free) survival rates in these patients have been reported to be similar to that of patients with idiopathic PAH and genetic susceptibility has been suggested in these patients.[8,15,16] Children with PAH-CHD group 1 and 2 were excluded from this study, because genetic analyses were not performed routinely in these patients.

PAH diagnosis was defined as mean pulmonary arterial pressure of ≥25 mm Hg, pulmonary vascular resistance index of ≥3 Wood units·m^2, and a mean pulmonary capillary wedge pressure of ≤15 mm Hg confirmed by right heart catheterization, or in case of clinical instability with echocardiography only (n = 7). World Health Organization Functional Class (WHO-FC), N-Terminal pro-B-Type natriuretic peptide, and hemodynamic parameters were collected at the time of diagnosis. (Heart-)lung transplantation or death were defined as outcome events.

Genetic Analysis

In the Dutch National referral center, all children are assessed by a clinical geneticist and genetic counseling and testing is offered, as evolved over time (**Figure 1**; available at www.jpeds.com). Chromosomal abnormalities (CNVs: deletions and duplications) are investigated with single nucleotide polymorphism array or array comparative genomic hybridization. From 2003 onward, this testing was combined with Sanger sequencing and multiplex ligation-dependent probe amplification, to detect small intragenic deletions in the *BMPR2* gene. In 2014 a panel of 7 PAH-associated genes (*BMPR2*, *ACVRL1*, *CAV1*, *ENG*, *KCNK3*, *SMAD9*, and *TBX4*) was introduced and expanded in 2015 with the *EIF2AK4* gene, using targeted next-generation sequencing, still combined with the multiplex ligation-dependent probe amplification test for *BMPR2*. In 2017 whole exome sequencing (WES) was introduced to screen for mutations/variants in the 8 PAH-associated genes. Variants were classified according to standardized guidelines based on Richards et al by using Alamut software and predictions of the effect on the protein by scale-invariant feature transform, Polymorphism Phenotyping, Grantham score, MutationTaster, Align GVGD, and PhyloP.[17] A diagnosis of heritable PAH was made in the case of familial PAH or when genetic testing revealed a PAH-associated gene mutation in a child with unexplained PAH.

Patients diagnosed with PAH before the introduction of this PAH-associated gene panel and still alive were retrospectively screened on PAH-associated genes.

The presence of specific genetic disorders, such as trisomy 21 (associated with increased risk for PH), mutations in von Hippel Lindau (*VHL*) gene (causing familial erythrocytosis), in methylmalonic aciduria and homocystinuria type C protein (*MMACHC)* gene (causing cobalamin C [CbIC] deficiency), or in alpha-actin-2 (*ACTA2*) gene (multisystemic smooth muscle dysfunction syndrome) have been previously reported to be associated with the development of PAH.[18-21] Children were screened for these mutations in cases of suspected clinical diagnosis. For this study, we designated 3 groups of genetic disorders: (1) mutations/variants in PAH-associated genes, (2) genetic disorders with an established association with PAH, and (3) genetic anomalies without an established association with PAH. In case a mutation/variant in group 1 or 2 was identified, additional diagnostic testing for other PAH-associated gene mutations/variants was not standard.

Statistical Analyses

Data are presented as median (IQR) or frequencies (percentage). The patient characteristics groups of children with different genetic architecture were compared using Kruskal-Wallis or χ^2 test when appropriate. Kaplan-Meier survival curves with log-rank testing were used to study differences in survival between patient groups that included ≥4 patients.

Results

Between 2003 and 2018, 80 patients with PAH meeting the inclusion criteria for this study were identified. In 10 patients (idiopathic PAH n = 5, PAH-CHD group 3/4 n = 4, PAH

associated with a portosystemic shunt n = 1), no genetic testing was performed owing to either rapid death (n = 5) or unavailability for retrospective testing (late death, transition to adult center, or denied consent [n = 5]). In the remaining 70 children, genetic testing was performed.

In 19 children, a PAH-associated gene mutation was identified. Twelve children were diagnosed with a genetic disorder with an established association with PAH. Additional specific PAH-associated gene testing in these 12 children is shown in **Figure 2** (available at www.jpeds.com). Of the 39 children in whom no PAH-associated gene mutation or genetic disorder with an established association with PAH was identified, all were screened for *BMPR2* mutations, except for 1 child with Noonan syndrome. In 36 of these children, this testing was combined with the PAH gene panel screening. In 3 children (without a diagnosis of PVOD), the screening panel did not include *EIF2AK4*.

Of the 70 children tested, 40 children were diagnosed with isolated PAH: 19 with idiopathic PAH, 16 with heritable PAH, and an additional 5 children with a final diagnosis of PVOD, histopathologically confirmed in lung tissue, collected either at lung transplantation or post mortem. One of these histologic PVOD diagnoses was made in a *TBX4* variant carrier, initially diagnosed as heritable PAH. Of the patients diagnosed with heritable PAH, only 1 child, a *BMPR2* mutation carrier, had a family history of PAH. Fourteen children were diagnosed with PAH-CHD group 3, 6 children with PAH-CHD group 4, 1 child with connective tissue disease, and 2 children with a portosystemic shunt. Additionally, 5 children presented with PAH associated with CbIC deficiency and 2 brothers with PAH associated with familial erythrocytosis (**Table I**). Clinical and hemodynamic characteristics of the patients are shown in **Table II**.

PAH-Associated Gene Mutations/Variants

Of the 70 children tested, 19 (27%) had a PAH-associated gene mutation/variant (*BMPR2* mutation n = 7, *TBX4* variant n = 8, *KCNK3* mutation n = 1 [classified as likely pathogenic], *ACVRL1* n = 1 [classified as variance of unknown significance], and *EIF2AK4* mutation n = 2).

One child had a homozygous mutation in *EIF2AK4* and 1 child had 2 heterozygous mutations in *EIF2AK4*: a pathogenic frameshift mutation and a missense mutation that is classified as a variance of unknown significance (patient 17 and patient 18, respectively, in **Table III** (available at www.jpeds.com)). In both children, the diagnosis of PVOD was histopathologically confirmed in lung tissue collected at transplantation or autopsy (**Figure 3**, A and B; available at www.jpeds.com; patient 18 in **Table III**). Two of 8 children with a *TBX4* variant showed signs of interstitial lung disease on a chest computed tomography scan. One child (patient 15, **Table III**) was initially diagnosed with heritable PAH, but autopsy disclosed a histopathologic diagnosis of PVOD (with patchy distribution) (**Figure 3**, E and F). In the other child (patient 12, **Table III**), histopathology after lung transplantation disclosed a "difficult to classify interstitial (fibrotic) lung disease" (**Figure 3**, C and D), showing some capillary proliferation, but no venous occlusion and only limited iron deposition.[22] The remaining 6 children with a *TBX4* variant showed no signs of parenchymal lung disease on either a computed tomography scan (n = 3) or chest radiograph (n = 3). In 6 of the 8 patients with a *TBX4* variant (75%) signs of small patella syndrome were found.

Other Concomitant Genetic Disorders

In 12 children (17%), we identified a genetic disorder with an established association with PAH: 5 children with an *MMACHC* mutation and CbIC deficiency, 2 brothers with

Table I. Distribution of PAH-associated gene mutations/variants (including *BMPR2*, *TBX4*, *KCNK3*, *EIF2AK4*, *ACVRL1*), genetic disorders with an established association with PAH (including *MMACHC*, *VHL*, *ACTA2*, trisomy 21, *JAG1*), and genetic disorders without an established association with PAH (including *PTPN11*, *FBN2*, and various CNVs)

	Isolated PAH (n = 40)			PAH-CHD (n = 20)					
Genes	Idiopathic PAH (n = 19)	Heritable PAH (n = 16)	PVOD (n = 5)	Group 3 (n = 14)	Group 4 (n = 6)	PAH-CTD (n = 1)	PAH associated with a portosystemic shunt (n = 2)	PAH associated with CbIC deficiency (n = 5)	PAH associated with familial erythrocytosis (n = 2)
BMPR2	–	7	–	–	–	–	–	–	–
TBX4	–	7	1*	–	–	–	–	–	–
KCNK3	–	1	–	–	–	–	–	–	–
EIF2AK4	–	–	2	–	–	–	–	–	–
ACVRL1	–	1	–	–	–	–	–	–	–
MMACHC	–	–	–	–	–	–	–	5	–
VHL	–	–	–	–	–	–	–	–	2
ACTA2	–	–	–	1	–	–	–	–	–
Trisomy 21	–	–	–	1	2	–	–	–	–
JAG1	–	–	–	–	–	–	1	–	–
PTPN11	2	–	–	–	–	–	–	–	–
FBN2	1	–	–	–	–	–	–	–	–
CNV	5	2	1	7	2	–	–	1	–

CTD, connective tissue disease; *FBN2*, fibrillin 2; *PTPN11*, protein-tyrosine phosphatase, nonreceptor-type 11.
*In 1 patient diagnosed as *TBX4*-associated heritable PAH, autopsy showed histopathologic features of PVOD.

Table II. Patient characteristics stratified for the different PAH groups at time of diagnosis

Characteristics	n	Full patient cohort (n = 70)	n	Idiopathic PAH (n = 19)	n	Heritable PAH with *TBX4* variant (n = 7)	n	Heritable PAH with *BMPR2* mutation (n = 7)	n	PVOD (n = 5)	n	PAH-CHD group 3 and 4 (n = 20)	n	PAH with *MMACHC* mutation and CbIC deficiency (n = 5)
Female, n	70	38 (54)	19	10 (53)	7	3 (43)	7	6 (86)	5	1 (20)	20	15 (75)	5	1 (20)
Age at diagnosis, y	70	7.2 (2.6-13.4)	19	9.8 (4.6-13.7)	7	2.8 (2.2-15.4)	7	14.0 (7.0-15.8)	5	7.1 (3.7-10.9)	20	5.1 (1.2-9.5)	5	6.4 (1.9-9.8)
Follow-up, y	70	3.3 (1.1-10.4)	19	2.8 (1.4-5.2)	7	14.2 (2.7-17.4)	7	7.3 (1.6-10.4)	5	0.8 (0.0-6.3)	20	5.5 (1.8-10.6)	5	0.2 (0.0-3.8)
Death or (H)LTx	70	34 (49)	19	9 (47)	7	1 (14)	7	6 (86)	5	5 (100)	20	8 (40)	5	4 (80)
WHO-FC	69		18		7		7		5		20		5	
I+II		24 (34)		8 (44)		2 (29)		0 (0)		0 (0)		11 (55)		1 (20)
III		26 (38)		5 (28)		5 (71)		3 (43)		1 (20)		8 (40)		0 (0)
IV		19		5 (28)		0 (0)		4 (57)		4 (80)		1 (5)		4 (80)
NT-proBNP, ng/l	57	953 (194-6530)	15	657 (178-8770)	3	179 (55-2748)	6	6926 (1481-20 107)	4	7684 (2836-18 249)	17	676 (203-1137)	5	1551 (145-55 478)
mRAP, mm Hg	63	5.0 (4.0-8.0)	17	5.0 (3.5-8.5)	7	4.0 (2.0-5.0)	6	6.0 (5.0-8.8)	4	10.0 (4.3-15.0)	20	5.5 (5.0-7.8)	3	5.0 (5.0-5.0)
mPAP, mm Hg	63	48 (34-65)	17	47 (33-67)	7	41 (26-72)	6	56 (48-59)	4	63 (58-72)	20	50 (35-67)	3	30 (26-30)
PVRi, WU • m^2	62	12.8 (6.5-23.3)	17	14.1 (6.6-21.9)	6	8.3 (4.2-26.5)	6	22.6 (10.1-28.1)	4	21.7 (13.2-40.3)	20	13.2 (6.4-20.6)	3	4.7 (4.2-8.3)
Cardiac index, L/min/m^2	62	3.1 (2.5-3.8)	17	3.0 (2.6-4.1)	6	3.4 (2.6-3.9)	6	2.3 (1.5-3.2)	4	3.1 (1.7-3.9)	20	3.1 (2.8-3.8)	3	4.4 (2.8-4.9)
Acute vasodilator responder*	62	7 (11)	17	1 (6)	6	2 (33)	6	0 (0)	4	1 (25)	20	3 (15)	3	0 (0)

Data presented as median (IQR) or frequencies (%).
(H)LTX, (heart) lung transplantation; *mRAP*, mean right atrial pressure; *mPAP*, mean pulmonary arterial pressure; *NT-proBNP*, N-Terminal pro-B-Type natriuretic peptide; *PVRi*, pulmonary vascular resistance index.
*According to Sitbon criteria.

a *VHL* mutation and familial erythrocytosis, and 1 child with an *ACTA2* mutation and multisystemic smooth muscle dysfunction syndrome and patent ductus arteriosus (classified as PAH-CHD group 3). Additionally, 3 children with PAH-CHD had Down syndrome (trisomy 21): 1 child with PAH-CHD group 3 and 2 children with PAH-CHD group 4. Finally, 1 child with Alagille syndrome and a jagged1 (*JAG1*) mutation had a portosystemic shunt.

Three children diagnosed with idiopathic PAH had a concomitant genetic disorder without an established association with PAH: Noonan syndrome (*PTPN11* mutation, n = 2), and Beals syndrome (*FBN2* mutation, n = 1).

Sixty of the 70 children included in the current study were additionally tested for CNVs with single nucleotide polymorphism array or array comparative genomic hybridization. Eighteen children (30%) showed CNVs, either deletions or duplications, not previously reported to have an established association with PAH. In 5 of these 18 children (28%), the CNV occurred together with a PAH-associated gene mutation or genetic disorder with an established association with PAH (*BMPR2* mutation n = 1, *EIF2AK4* mutation n = 1, *ACVRL1* mutation n = 1, *ACTA2* mutation n = 1, and *MMACHC* mutation n = 1) (**Table I** and **Table IV** [**Table IV** available at www.jpeds.com]), whereas in the remaining 13 children no PAH-associated gene mutation or genetic disorder with an established association with PAH was identified. Of the identified CNVs, 4 (22%) have previously been associated with non-PAH diseases, whereas in 14 children (78%) the identified CNV has not (yet) been related to any pathologic condition (**Table IV**).

In summary, we identified 19 children (27%) with an established PAH-associated gene mutation, and in 12 children (17%) other genetic disorders with an established association with PAH were identified. These disorders included *MMACHC*, *VHL*, *ACTA2*, and *JAG1* gene mutations and trisomy 21. Finally, in another 16 children (23%) we found CNVs (n = 13) or genetic syndromes (n = 3) without an established association with PAH. In 23 of the 70 children with PAH (33%), no genetic abnormalities were identified (**Table V**).

Clinical Disease and Outcome

At the time of diagnosis, the median age of the children was 7.2 years (IQR, 2.6-13.4 years) (54% female) (**Table II**). Overall, no statistically significant differences between different patient groups could be demonstrated, except for WHO-FC, follow-up time, and the number of events (death or lung transplantation). Children with PVOD or a CbIC deficiency most often presented with severe disease in WHO-FC III or IV, whereas those with a *TBX4* variant most often presented in WHO-FC II and III and patients with PAH-CHD most often presented in WHO-FC I and II (P = .033). The longest follow-up time with the lowest number of events was found in children with a *TBX4* variant, whereas children with PVOD or CbIC deficiency had shortest follow-up with the highest number of events. Transplant-free survival, unadjusted for clinical variables,

Table V. Genetic architecture in a national cohort of children with PAH

PAH-associated gene mutation 27%	Genetic disorder with an established association with PAH 17%	Genetic syndromes and CNVs without an established association with PAH 23%	No genetic abnormality 33%
n = 19	n = 12	n = 16	n = 23
BMPR2, n = 7	*MMACHC*, n = 5	Noonan syndrome, n = 2	
TBX4, n = 8	*VHL*, n = 2	Beals syndrome, n = 1	
EIF2AK4, n = 2	*ACTA2*, n = 1	CNV, n = 13	
KCNK3, n = 1	Down syndrome, n = 3		
ACVRL1, n = 1	Alagille syndrome associated with portosystemic shunt with a *JAG1* mutation, n = 1		

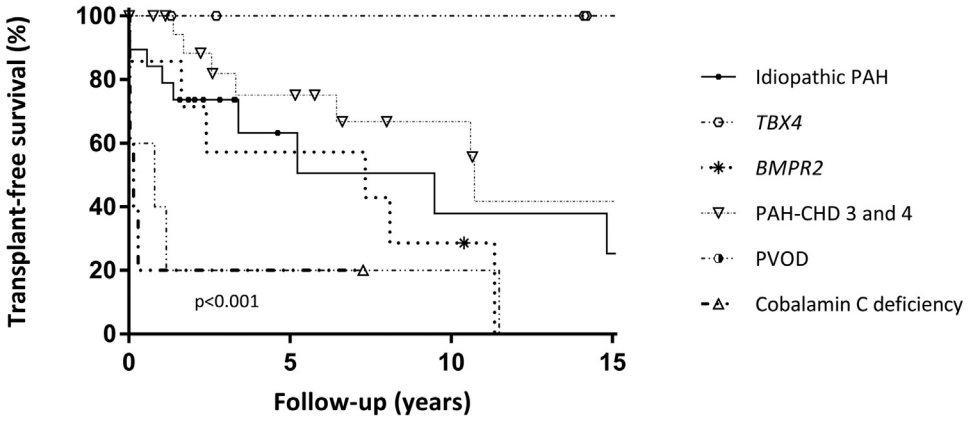
varied significantly between groups of children with different genetic architecture (Figure 4). Children with CbIC deficiency and children with PVOD had the worst unadjusted outcome with a median transplant-free survival of <1 year, whereas pediatric *TBX4* variant carriers showed the most favorable outcome.

Discussion

In this national cohort of children with PAH, we identified a PAH-associated gene mutation in 27% of the total cohort. An additional 17% of this pediatric cohort was found to have another genetic disorder with an established association with PAH. In another 23% of the children, we found genetic syndromes or CNVs without an established association with PAH. This study shows that children with PVOD and children with PAH associated with CbIC deficiency were diagnosed at the most advanced stage and had the worst outcomes, whereas pediatric *TBX4* variant carriers had better outcomes compared with other children with PAH, including those with a *BMPR2* mutation.

In the current study, the number of *BMPR2* gene mutations that were identified was in line with previous studies in children and adults with PAH.[9-11] In contrast with the observations in cohorts of adult patients with PAH, *BMPR2* was not the most commonly affected gene in this pediatric cohort.

TBX4 variants were the most frequent variants in this pediatric cohort, with a prevalence higher than that reported in adult cohorts.[1,11] The association between *TBX4* variants and childhood PAH was first recognized in 2013 and since then the enrichment of *TBX4* variants in pediatric PAH has been confirmed in other pediatric studies.[9,10] The clinical phenotype associated with *TBX4* variants has been recently recognized to expand beyond heritable PAH, including a spectrum of "developmental" or interstitial lung disorders and respiratory compromise that may present in newborns, associated with persistent PH of the newborn, but also during adulthood.[23-29] This expanding spectrum will complicate classification of such patients according to the clinical classification of PH as either heritable PAH or PH owing to lung disease. In light of the growing insight in the heterogeneous



Patients at risk, n				
Idiopathic PAH	19	5	3	2
TBX4	7	5	5	3
BMPR2	7	4	2	0
PAH-CHD group 3 and 4	20	11	6	3
PVOD	5	1	1	0
Cobalamin C deficiency	5	1	0	0

Figure 4. Transplant-free survival of children with PAH with different genetic backgrounds truncated at 15 years of follow-up.

phenotypes of human *TBX4* variants, the authors recommend a meticulous and focused diagnostic workup in patients with PH and a *TBX4* variant to be able to start the most appropriate treatment.[28,29] In the current study, 1 patient with a *TBX4* variant had a histopathologic diagnosis of PVOD, confirmed at autopsy. As far as we know, the concomitant occurrence of a *TBX4* mutation and PVOD has not been described before. Whether a *TBX4* gene malfunction may affect the pathophysiological pathway involved in the development of PVOD needs to be elucidated.

In the current cohort of pediatric PAH, 5 children (7%) were diagnosed with PVOD, a rare and lethal disease characterized by extensive and diffuse occlusion of pulmonary veins by neointimal fibrosis together with often segmental, focal capillary dilatation and/or congestion and occult alveolar hemorrhage.[30] Both homozygous and compound heterozygous *EIF2AK4* mutations have been associated with the development of PVOD.[5] The prevalence of *EIF2AK4* mutations has been reported in 25% of adults with sporadic PVOD.[5] Levy et al reported that 2 out of 3 pediatric patients (67%) with PVOD had a homozygous *EIF2AK4* mutation.[9] In the current cohort, in 2 out of 5 children with PVOD (40%) an *EIF2AK4* mutation was identified.

An *MMACHC* mutation associated with CbIC deficiency, renal thrombotic microangiopathy, and PAH was found in 5 children in this cohort (7%). Four of these children were reported previously.[20] All but 1 were diagnosed at an advanced stage of PAH, characterized by overt right heart failure and WHO-FC IV, and died shortly after diagnosis, despite support of vital functions, intensive PAH-targeted therapy, and hydroxycobalamin supplementation. The remaining patient was diagnosed early in the disease course, was treated for a prolonged time with parenteral hydroxycobalamin supplementation as well as PAH-targeted therapy, and eventually showed clinical improvement with normalization of pulmonary hemodynamics. This finding suggests a potentially beneficial effect of hydroxycobalamin supplementation in this CblC-associated PAH. Incorporating urine testing for (microscopic) hematuria and plasma levels of total homocysteine and methylmalonic acid in the standard diagnostic workup of children with PAH may enable early identification and treatment of these high-risk patients.[31]

Two Moroccan brothers had PAH associated with familial erythrocytosis caused by a homozygous *VHL* mutation.[32] Specific *VHL* gene mutations are associated with severe early onset childhood PAH owing to the dysregulation of the hypoxia-inducible factor pathway in these patients.[18] Because *VHL* mutations seem to be endemic in specific regions (ie, the Chuvash region and Croatia) testing for these gene mutations in certain circumstances may be warranted.[33,34]

One patient presented with PAH and persistent ductus arteriosus, associated with serious developmental cerebral and multiorgan disorders, including aortic and ductal aneurysm, and was found to have an *ACTA2* mutation, associated with the multisystemic smooth muscle cell dysfunction syndrome.[21] Knowledge of this syndrome and its association with PAH seems relevant to pediatricians and pediatric PH experts for the timely treatment of PAH and potential contraindications for surgical interventions, such as duct closure, owing to vascular fragility.[35]

Down syndrome was present in only 3 of the 70 patients (4%) in this population, which is substantially less frequent than previously reported in cohorts of children with PAH.[7,36-38] This discrepancy is most likely explained by the exclusion of children with PAH-CHD and large open shunts (PAH-CHD group 1 and 2 according to the latest clinical classification[12-14]) and of children with PH associated with respiratory diseases or hypoxia. In the complete Dutch national pediatric PAH cohort, the prevalence of trisomy 21 was 17%. The clinical phenotype of trisomy 21 is known to include persistent PH of the neonate, congenital cardiac shunts, developmental lung and airway diseases, obstructive breathing, gastroesophageal reflux, and recurrent airway infection, all well-recognized risk factors for the development of PH.[38] Consequently, an extensive diagnostic workup is required in children with trisomy 21 with PH to establish the exact nature of PH and to initiate the most appropriate treatment. The pathophysiologic link between trisomy 21 and intrinsic pulmonary vascular disease has not been elucidated yet. Both in human and animal models, trisomy 21 has been associated with increased pulmonary vascular expression of antiangiogenic factors.[39] Inhibition of angiogenesis has been suggested to lead to impaired development of pulmonary vasculature and airways, and the development of PH in these patients.[40,41]

Other genetic disorders found in this study were Noonan syndrome and Beals syndrome. The relation between these syndromes and PAH is not clear. Although several case studies have reported patients with a combination of Noonan syndrome, specifically with *RAF1* mutations, and P(A)H, a clear association between these 2 entities has not been established.[42,43]

In almost one-third of the 60 children tested with single nucleotide polymorphism array or array comparative genomic hybridization, a CNV was found, with the highest frequency in children with idiopathic PAH and PAH-CHD group 3. Children with PAH-CHD group 3 have cardiac defects that are regarded as not solely responsible for the PAH. It has been speculated that these children bear an increased susceptibility for the development of pulmonary vascular disease, so that a relatively mild hemodynamic "second hit" might induce pulmonary vascular disease in these patients. Today, such presumed susceptibility is not explained by the concomitant presence of any established PAH-associated gene. The presence of a *SOX17* variant has been suggested as a candidate risk gene in children with PAH after successful closure of a cardiac shunt correction (PAH-CHD, group 4).[44] The observed high occurrence of CNVs, especially in these groups of children, might be related to such an increased susceptibility. In 22% of the CNVs the affected regions included OMIM disease-related genes, but no associations of these CNVs with PAH have been previously recognized. Also, in the current study no

clustering of similar or overlapping CNV regions was found. Zhu et al recently showed using WES that *de novo* variants in novel genes were present in 19% of a cohort of children with idiopathic PAH.[10] Further studies into common CNVs might provide clues to guide further research in the mechanisms of PAH.

In these authors' opinion, genetic analysis of children with PAH should be performed only in combination with genetic counseling of the parents and—age appropriately—the child as well. The clinical consequences for the child, the possibilities and consequences of testing siblings or other relatives on carrier status, the benefits of early diagnosis and careful monitoring for a progressive fatal disease, and well-informed child-bearing decisions should be weighed together with parents and patient, against the high emotional stress of knowledge of carrier status, combined with uncertainties regarding penetrance of specific mutations in pediatrics. Appropriate genetic counseling is a prerequisite for genetic testing in pediatric PAH. Today, genetic testing will have direct implications for treatment in selected situations, such as confirmation of clinical syndromes, and identification of pathologic *EIF2AK4*, *MMACHC*, or *VHL* mutations. Also, information on a pathologic carrier status may help in early diagnosing other diseases (such as *ACVRL1* mutation and the emergence of hereditary hemorrhagic telangiectasia).[45] However, in the case of other PAH-associated gene mutations, the lack of sufficient data on genotype-phenotype relationships currently limits a directive role for carrier status in treatment decisions. The current study suggests different survival in patients with different genetic architecture with the worst survival in children with PVOD and PAH associated with CbIC deficiency and the most favorable survival in children with a *TBX4* variant. Similar to the observations in the current pediatric cohort, a better survival of *TBX4* variant carriers when compared with *BMPR2* mutation carriers has previously been shown in adults with PAH.[46] Although survival rates were not adjusted for clinical variables, the significant differences between patients groups in the current study suggests that genetic architecture could play a role in risk stratification of children newly diagnosed with PAH.[12,47] Further studies on genotype-phenotype relations, but in particular the relation between genotype and treatment response, will reveal whether genetic characterization will play a role in personalized treatment strategies in pediatric PAH.

This study aimed to describe genetic characteristics of a national cohort of children with PAH. Although we aimed for genetic analysis of the complete study cohort, 10 of 80 children were not screened for PAH-associated gene mutations, one-half of these owing to rapid death after diagnosis. This may have resulted in under-reporting of genetic disorders associated with severe PAH with worse survival. Because the routine use of WES for genetic screening was introduced only recently in our center, we could not explore novel genes in the study cohort. The majority of the parents of the children with CNVs of unknown significance was not tested, hampering the interpretation of the pathogenicity of these CNVs.

The number of genes associated with PAH is increasing, so genetic screening strategies in patients with PAH will also evolve continuously. WES trio-analyses allow for the identification of new genetic abnormalities associated with pediatric PAH, and may be applied retrospectively to individual patients in a diagnostic setting.[44,48-50] Costs for whole genome sequencing and RNA sequencing are decreasing rapidly and rapid analysis techniques are increasing. These techniques may be used in large cohorts of children with P(A)H in a research setting to also study noncoding DNA and epigenetic factors that may contribute to the disease. Family studies are needed to map the penetrance of the different PAH-associated mutations.[51,52]

Specific pediatric-genetic studies will provide clues for the identification of pathogenetic mechanisms leading to PAH. Mapping the different genotypes of PAH needs to go hand in hand with meticulous clinical phenotyping to allow for the exploration of specific genotype-phenotype associations that eventually may lead to optimization and individualization of treatment strategies in pediatric PAH.

This study shows a high prevalence of genetic disorders in pediatric PAH, including PAH-associated gene mutations and other genetic disorders with an established association with PAH, including *MMACHC*, *VHL*, *ACTA2*, and *JAG1* gene mutations and trisomy 21. Furthermore, a substantial proportion of children had genetic anomalies currently without an established association with PAH. Only 23% of the children with PAH in this national cohort showed no genetic anomaly. Transplant-free survival differed between patient groups with different genetic backgrounds and future studies are needed to understand whether this should be incorporated in risk stratification. ■

Submitted for publication Feb 24, 2020; last revision received Apr 27, 2020; accepted May 26, 2020.

Reprint requests: Meindina G. Haarman, MD, Department of Pediatric Cardiology, Center for Congenital Heart Diseases, Beatrix Children's Hospital, University Medical Center Groningen, PO Box 30 001, 9700 RB Groningen, the Netherlands. E-mail: m.g.haarman@umcg.nl

Data Statement

Data sharing statement available at www.jpeds.com.

References

1. Kerstjens-Frederikse WS, Bongers EMHF, Roofthooft MTR, Leter EM, Douwes MJ, van Dijk A, et al. TBX4 mutations (small patella syndrome) are associated with childhood-onset pulmonary arterial hypertension. J Med Genet 2013;50:500-6.
2. Austin ED, Ma L, LeDuc C, Rosenzweig EB, Borczuk A, Phillips JA, et al. Whole exome sequencing to identify a novel gene (Caveolin-1) associated with human pulmonary arterial hypertension. Circ Cardiovasc Genet 2012;5:336-43.
3. Harrison RE, Berger R, Haworth SG, Tulloh R, Mache CJ, Morrell NW, et al. Transforming growth factor-β receptor mutations and pulmonary arterial hypertension in childhood. Circulation 2005;111:435-41.
4. Rosenzweig EB, Morse JH, Knowles JA, Chada KK, Khan AM, Roberts KE, et al. Clinical implications of determining BMPR2 mutation

status in a large cohort of children and adults with pulmonary arterial hypertension. J Heart Lung Transplant 2008;27:668-74.
5. Eyries M, Montani D, Girerd B, Perret C, Leroy A, Lonjou C, et al. EIF2AK4 mutations cause pulmonary veno-occlusive disease, a recessive form of pulmonary hypertension. Nat Genet 2014;46:65-9.
6. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, et al. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 2000;67:737-44.
7. Van Loon RLE, Roofthooft MTR, van Osch-Gevers M, Delhaas T, Strengers JLM, Blom NA, et al. Clinical characterization of pediatric pulmonary hypertension: complex presentation and diagnosis. J Pediatr 2009;155:176-82.e1.
8. Van Loon RLE, Roofthooft MTR, Hillege HL, Ten Harkel ADJ, Van Osch-Gevers M, Delhaas T, et al. Pediatric pulmonary hypertension in the Netherlands: epidemiology and characterization during the period 1991 to 2005. Circulation 2011;124:1755-64.
9. Levy M, Eyries M, Szezepanski I, Ladouceur M, Nadaud S, Bonnet D, et al. Genetic analyses in a cohort of children with pulmonary hypertension. Eur Respir J 2016;48:1118-26.
10. Zhu N, Gonzaga-Jauregui C, Welch CL, Ma L, Qi H, King AK, et al. Exome sequencing in children with pulmonary arterial hypertension demonstrates differences compared with adults. Circ Genomic Precis Med 2018;11:e001887.
11. Eyries M, Montani D, Nadaud S, Girerd B, Levy M, Bourdin A, et al. Widening the landscape of heritable pulmonary hypertension mutations in paediatric and adult cases. Eur Respir J 2018;53:1801371.
12. Rosenzweig EB, Abman SH, Adatia I, Beghetti M, Bonnet D, Haworth S, et al. Paediatric pulmonary arterial hypertension: updates on definition, classification, diagnostics and management. Eur Respir J 2019;53:1801916.
13. Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, et al. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. Eur Respir J 2019;53:1801913.
14. Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, et al. Updated clinical classification of pulmonary hypertension. J Am Coll Cardiol 2013;62(25 Suppl):D34-41.
15. Mañn MJDC, Rotés AS, Ogando AR, Soto AM, Jiménez MQ, Camacho JLG, et al. Assessing pulmonary hypertensive vascular disease in childhood data from the Spanish registry. Am J Respir Crit Care Med 2014;190:1421-9.
16. Roberts KE, McElroy JJ, Wong WPK, Yen E, Widlitz A, Barst RJ, et al. BMPR2 mutations in pulmonary arterial hypertension with congenital heart disease. Eur Respir J 2004;24:371-4.
17. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-24.
18. Caravita S, Deboeck G, Vachiery J-L, Naeije R. Pulmonary arterial hypertension associated with a von Hippel-Lindau gene mutation. J Heart Lung Transplant 2016;35:1138-9.
19. Smith TG, Brooks JT, Balanos GM, Lappin TR, Layton DM, Leedham DL, et al. Mutation of von Hippel-Lindau tumour suppressor and human cardiopulmonary physiology. PLoS Med 2006;3:1178-86.
20. Kömhoff M, Roofthooft MT, Westra D, Teertstra TK, Losito A, Van de Kar NCAJ, et al. Combined pulmonary hypertension and renal thrombotic microangiopathy in cobalamin C deficiency. Pediatrics 2013;132:e540-4.
21. Milewicz DM, Østergaard JR, Ala-Kokko LM, Khan N, Grange DK, Mendoza-Londono R, et al. De novo ACTA2 mutation causes a novel syndrome of multisystemic smooth muscle dysfunction. Am J Med Genet A 2010;152A:2437-43.
22. Kurland G, Deterding RR, Hagood JS, Young LR, Brody AS, Castile RG, et al. An official American Thoracic Society clinical practice guideline: classification, evaluation, and management of childhood interstitial lung disease in infancy. Am J Respir Crit Care Med 2013;188:376-94.
23. Galambos C, Mullen MP, Shieh JT, Schwerk N, Kielt MJ, Ullmann N, et al. Phenotype characterisation of TBX4 mutation and deletion carriers with neonatal and pediatric pulmonary hypertension. Eur Respir J 2019:1801965.
24. Maurac A, Lardenois É, Eyries M, Ghigna MR, Petit I, Montani D, et al. T-box protein 4 mutation causing pulmonary arterial hypertension and lung disease. Eur Respir J 2019:1900388.
25. Karolak JA, Vincent M, Stankiewicz P, Gambin T, Cogné B, Pichon O, et al. Complex compound inheritance of lethal lung developmental disorders due to disruption of the TBX-FGF pathway. Am J Hum Genet 2019;104:213-28.
26. Szafranski P, Coban-Akdemir ZH, Rupps R, Grazioli S, Wensley D, Jhangiani SN, et al. Phenotypic expansion of TBX4 mutations to include acinar dysplasia of the lungs. Am J Med Genet Part A 2016;170:2440-4.
27. Suhrie K, Pajor NM, Ahlfeld SK, Dawson DB, Dufendach KR, Kitzmiller JA, et al. Neonatal lung disease associated with TBX4 mutations. J Pediatr 2019;206:286-92.e1.
28. Haarman MG, Kerstjens-Frederikse WS, Berger RMF. The ever-expanding phenotypical spectrum of human TBX4 mutations: from toe to lung. Eur Respir J 2019;54:1901504.
29. Haarman MG, Kerstjens-Frederikse WS, Berger RMF. TBX4 variants and pulmonary diseases. Curr Opin Pulm Med 2020;26:277-84.
30. Mandel J, Mark EJ, Hales CA. Pulmonary veno-occlusive disease. Am J Respir Crit Care Med 2000;162:1964-73.
31. Huemer M, Diodato D, Schwahn B, Schiff M, Bandeira A, Benoist JF, et al. Guidelines for diagnosis and management of the cobalamin-related remethylation disorders cblC, cblD, cblE, cblF, cblG, cblJ and MTHFR deficiency. J Inherit Metab Dis 2017;40:21-48.
32. Bartels M, Van der Zalm MM, Van Oirschot BA, Lee FS, Giles RH, Kruip MJHA, et al. Novel homozygous mutation of the internal translation initiation start site of VHL is exclusively associated with erythrocytosis: indications for distinct functional roles of von Hippel-Lindau tumor suppressor isoforms. Hum Mutat 2015;36:1039-42.
33. Tomasic NL, Piterkova L, Huff C, Bilic E, Yoon D, Miasnikova GY, et al. The phenotype of polycythemia due to Croatian homozygous VHL (571C>G:H191D) mutation is different from that of Chuvash polycythemia (VHL 598C>T:R200W). Haematologica 2013;98:560-7.
34. Sarangi S, Lanikova L, Kapralova K, Acharya S, Swierczek S, Lipton JM, et al. The homozygous VHL(D126N) missense mutation is associated with dramatically elevated erythropoietin levels, consequent polycythemia, and early onset severe pulmonary hypertension. Pediatr Blood Cancer 2014;61:2104-6.
35. Meuwissen MEC, Lequin MH, Bindels-de Heus K, Bruggenwirth HT, Knapen MFCM, Dalinghaus M, et al. ACTA2 mutation with childhood cardiovascular, autonomic and brain anomalies and severe outcome. Am J Med Genet Part A 2013;161:1376-80.
36. Berger RMF, Beghetti M, Humpl T, Raskob GE, Ivy DD, Jing ZC, et al. Clinical features of paediatric pulmonary hypertension: a registry study. Lancet 2012;379:537-46.
37. Espinola-Zavaleta N, Soto ME, Romero-Gonzalez A, Gómez-Puente LDC, Muñoz-Castellanos L, Gopal AS, et al. Prevalence of congenital heart disease and pulmonary hypertension in down's syndrome: an echocardiographic study. J Cardiovasc Ultrasound 2015;23:72-7.
38. Bush D, Galambos C, Ivy DD, Abman SH, Wolter-Warmerdam K, Hickey F. Clinical characteristics and risk factors for developing pulmonary hypertension in children with Down syndrome. J Pediatr 2018;202:212-9.e2.
39. Galambos C, Minic AD, Bush D, Nguyen D, Dodson B, Seedorf G, et al. Increased lung expression of anti-angiogenic factors in Down syndrome: potential role in abnormal lung vascular growth and the risk for pulmonary hypertension. PLoS One 2016;11:1-15.
40. Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF, et al. Inhibition of angiogenesis decreases alveolarization in the developing rat lung. Am J Physiol Lung Cell Mol Physiol 2000;279:600-7.
41. Le Cras TD, Markham NE, Tuder RM, Voelkel NF, Abman SH. Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure. Am J Physiol Lung Cell Mol Physiol 2002;283:555-62.
42. Hopper RK, Feinstein JA, Manning MA, Benitz W, Hudgins L. Neonatal pulmonary arterial hypertension and Noonan syndrome: two fatal cases with a specific RAF1 mutation. Am J Med Genet Part A 2015;167:882-5.

43. Tinker A, Uren N, Schofield J. Severe pulmonary hypertension in Ullrich-Noonan syndrome. Br Heart J 1989;62:74-7.
44. Zhu N, Welch CL, Wang J, Allen PM, Gonzaga-Jauregui C, Ma L, et al. Rare variants in SOX17 are associated with pulmonary arterial hypertension with congenital heart disease. Genome Med 2018;10:1-11.
45. Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I, et al. Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. N Engl J Med 2001;345:325-34.
46. Navas P, Tenorio J, Quezada CA, Barrios E, Gordo G, Arias P, et al. Molecular analysis of BMPR2, TBX4, and KCNK3 and genotype-phenotype correlations in Spanish patients and families with idiopathic and hereditary pulmonary arterial hypertension. Rev Española Cardiol (English Ed) 2016;69:1011-9.
47. Haarman MG, Douwes JM, Ploegstra M-J, Roofthooft MTR, Vissia-Kazemier TR, Hillege HL, et al. The clinical value of proposed risk stratification tools in pediatric pulmonary arterial hypertension. Am J Respir Crit Care Med 2019;200:1312-5.
48. Gräf S, Haimel M, Morrell NW, Hadinnapola C, Southgate L, Li W, et al. Identification of rare sequence variation underlying heritable pulmonary arterial hypertension. Nat Commun 2018;9:1416.
49. Zhu N, Pauciulo MW, Welch CL, Lutz KA, Coleman AW, Gonzaga-Jauregui C, et al. Novel risk genes and mechanisms implicated by exome sequencing of 2572 individuals with pulmonary arterial hypertension. Genome Med 2019;11:1-16.
50. Hodgson J, Swietlik EM, Morrell NW, Hadinnapola C, Nikolic I, Wharton J, et al. Characterization of GDF2 mutations and levels of BMP9 and BMP10 in pulmonary arterial hypertension. Am J Respir Crit Care Med 2020;201:575-85.
51. McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JPA, et al. Genome-wide association studies for complex traits: consensus, uncertainty and challenges. Nat Rev Genet 2008;9:356-69.
52. Ackers-Johnson M, Tan WLW, Foo RSY. Following hearts, one cell at a time: recent applications of single-cell RNA sequencing to the understanding of heart disease. Nat Commun 2018;9:8-11.

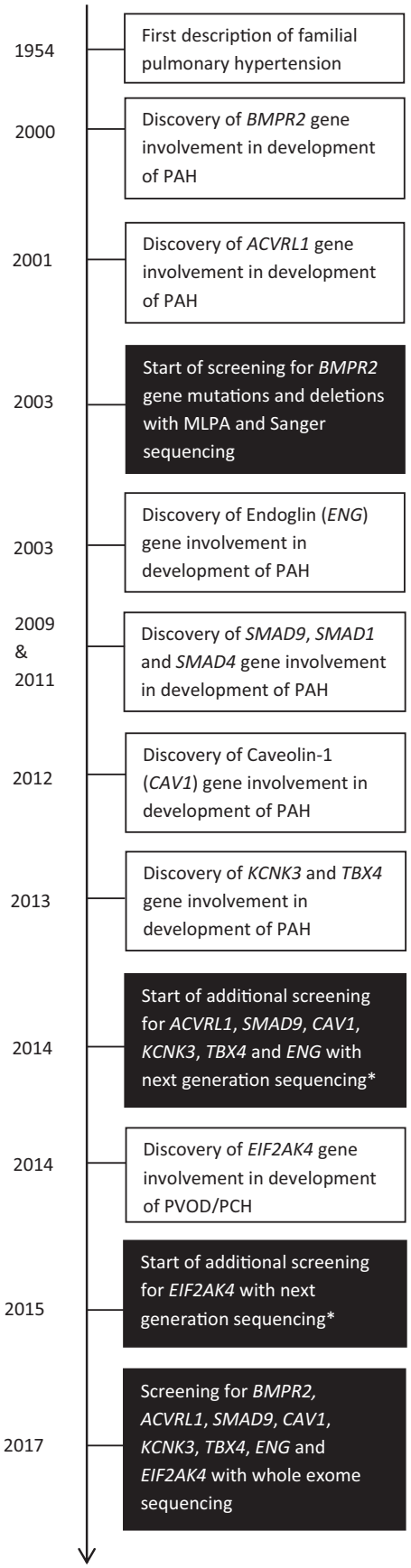


Figure 1. Timeline of the discovery of genes contributing to PAH (*whites boxes*) and the implementation of structural genetic screening for PAH-associated genes in the Dutch National Referral Center (*black boxes*). *Patients diagnosed with PAH before the introduction of this PAH-associated gene panel and still alive were retrospectively screened with WES. *PCH*, pulmonary capillary hemangiomatosis; *MLPA*, multiplex ligation-dependent probe amplification.

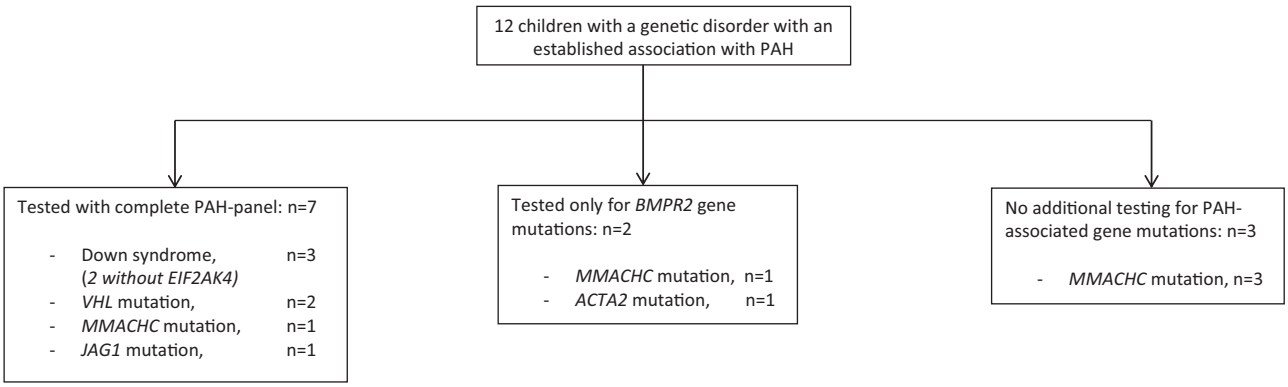


Figure 2. Testing on PAH-associated gene mutations in children with a genetic disorder with an established association with PAH.

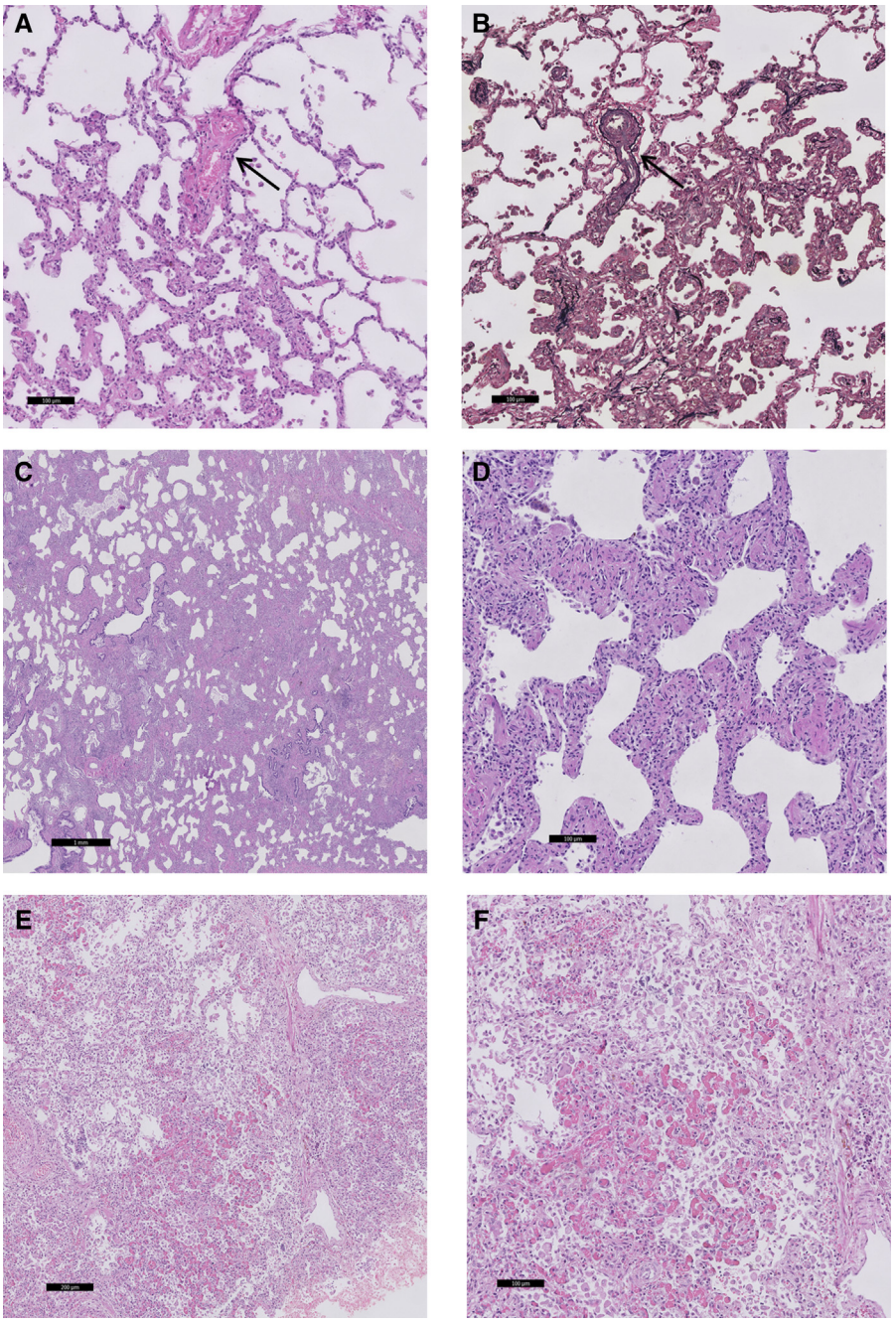


Figure 3. Histopathologic assessment in 3 patients. **A,** Lung parenchyma of patient with 2 heterozygous mutations in *EIF2AK4* with PVOD with at the left side thickened alveolar septa caused by capillary widening and congestion (formerly called capillary hemangiomatosis), sharply demarcated from the apposed normal alveolar septa. In the central part (*arrow*) a partially obstructed venule (stain: hematoxylin and eosin). **B,** Elastin stain of the same area in particular clearly showing obstructed venule (both bars = 100 micron). **C,** Lung parenchyma of patient with *TBX4* mutation with difficult to classify interstitial lung disease with mainly fibrotic nonspecific interstitial pneumonia (NSIP) pattern with also metaplastic smooth muscle proliferation (hematoxylin and eosin; bar = 1 mm). **D,** Larger magnification (bar = 100 micron). **E,** Lung parenchyma of patient with *TBX4* mutation with PVOD with in the middle and upper part thickened alveolar septa caused by capillary widening and pronounced congestion with red blood cells (formerly called capillary hemangiomatosis); (stain: hematoxylin and eosin; bar = 200 micron). **F,** Larger magnification (bar = 100 micron).

Table III. (Likely) pathogenic mutations and CNVs identified in children with PAH

Pt	Gene	Mutation category	Nucleotide change	Amino acid change	Pathology	Genebank accession number	Mutation previously reported in
1	*BMPR2*	Nonsense	c.47G>A	p.(Trp16*)	Heritable PAH	NM_001204.6	–
2	*BMPR2*	Frameshift	c.399delT	p.(Pro134Leufs*18)	Heritable PAH	NM_001204.6	–
3	*BMPR2*	Intragenic deletion	c.530-?_c.621+?del	p.?	Heritable PAH	NM_001204.6	Aldred et al, Hum Mutat, 2006
4	*BMPR2*	Missense	c.1471C>T	p.(Arg491Trp)	Heritable PAH	NM_001204.6	Deng et al, Am J Hum Genet, 2000; Dewachter et al, Eur Respir J, 2009
5	*BMPR2*	Nonsense	c.2695C>T	p.(Arg899*)	Heritable PAH	NM_001204.6	–
6	*BMPR2*	Unknown	–	–	Heritable PAH	–	–
7	*BMPR2*	Frameshift	c.941_945delATCTT	p.(Tyr314Serfs*11)	Heritable PAH	NM_001204.6	–
8	*TBX4*	Deletion	17q23.2 (55,654,379-57,679,097)	–	Heritable PAH	–	Kerstjens-Frederikse et al, J Med Genet, 2013
9	*TBX4*	Deletion	17q23.21q23.2 de novo	–	Heritable PAH	–	–
10	*TBX4*	Deletion	17q23.2q23.3(RP11-332h18->RP11-156L14)x1	–	Heritable PAH	–	Kerstjens-Frederikse et al, J Med Genet, 2013
11	*TBX4*	Deletion	17q23.1q23.2 de novo	–	Heritable PAH	–	Kerstjens-Frederikse et al, J Med Genet, 2013
12	*TBX4*	Frameshift	c.355dupA	p.(Ile119Asnfs*6)	Heritable PAH	NM_018488.3	Kerstjens-Frederikse et al, J Med Genet, 2013
13	*TBX4*	Missense	c.401G>C	p.(Trp134Ser)	Heritable PAH	NM_018488.3	–
14	*TBX4*	Frameshift	c.1164dupC	p.(Arg389Glnfs*30)	Heritable PAH	NM_018488.3	Kerstjens-Frederikse et al, J Med Genet, 2013
15	*TBX4**	Missense	c.1145A>C	p.(Tyr382Ser)	Heritable PAH	NM_018488.2	Kerstjens-Frederikse et al, J Med Genet, 2013
16	*KCNK3*	Missense	c.616G>T	p.(Val206Leu)	Heritable PAH	NM_002246.2	–
17	*EIF2AK4*	Frameshift	c.1739dupA (homozygous)	p.(Arg581Glufs*9)	PVOD	–	–
18	*EIF2AK4*	Frameshift Missense	c.4205dup; c.2968C>T†	p.(Ser1403Lysfs*45); p.(Pro990Ser)	PVOD	NM_001013703.3	–
19	*ACVRL1*	Missense	c.511G>A‡	p.(Asp171Asn)	Heritable PAH	NM_000020.2	–

*Patient diagnosed as *TBX4*-associated heritable PAH, whereas autopsy showed a histopathologic diagnosis of PVOD.
†c.4205dup p.(Ser1403Lysfs*45 is pathogenic, c.2968C>T p.(Pro990Ser) is a variant of unknown significance.
‡c.511G>A p.(Asp171Asn) is a variant of unknown significance.

Table IV. CNVs and variants with unknown significance

PAH groups	n	Abnormality array CGH/SNP-array
Idiopathic PAH/heritable PAH	7	1. arr[hg19] 3q13.33q21.1(120,205,270-121,936,796)x1 (in combination with *BMPR2* mutation) *parents not tested* 2. arr CGH 3p12.3(75,622,607-75,804,387)x1 (in combination with *ACTA2* mutation) *parents not tested* 3. arr[hg19] 9q22.1(90,543,679-90,960,845)x3, and 9q22.31(94,568,831-95,122,101)x3 *not present in mother; father not tested* 4. arr[hg19] 9q22.1(90,543,679-90,960,845)x3, and 9q22.31(94,568,831-95,142,500)x3 (in combination with *ACVRL1* mutation) *parents not tested* 5. arr CGH duplication of a sub part of 7q21.3 *parents not tested* 6. arr CGH 8p23.2 (7 oligo's)x3 *parents not tested* 7. arr 6q12(67,006,745-67,489,490)x1, and 11p15.4(7,012,268-8,074,827)x3 *parents not tested*
PAH-CHD group 3	7	1. arr[hg19] Xp22.31(6,454,369-8,138,035)x1, and 20p12.3(8,093,416-8,579,037)x3 *parents not tested* Gene on this location (20p12.3): *PLCB1*. Has been described in relation with WPW and ADHD (DOI: 10.1002/ajmg.a.35701) 2. arr[GRCh37] 14q32.33(104884787_106339625)x1 *parents not tested* 3. arr[GRCh37] 11p14.2p14.1(26996699_27240343)x3 *pat* 4. arr CGH 16q24.1(84,500,030-85,260,589)x3 *pat* 5. arr[GRCh37] 18p11.32q12.2(0_33540595)x3 dn. Genes on this location: *TAF4B*, *DTNA*. Has been described in association with ASDs and aortic coarctation (DOI: 10.1002/9780470015902.a0025246 and DOI: 10.1016/j.gene.2012.12.001) 18q21.2(48598295_52648984)x3 dn, Gene on this location: *SMAD4*. Associated with structural heart diseases (DOI: 10.1002/9780470015902.a0025246). 18q23(77706676_78077248)x3 dn Gene on this location: *TXNL4A*. Is possibly associated with structural heart diseases. (DOI: 10.1002/9780470015902.a0025246) 6. arr [hg19] (8)x2∼3 7. arr CGH 17q25.1(RP11-91m1->RP11-91o17)x3 *mat*
PAH-CHD group 4	2	1. arr CGH 2q24.1(158,038,869-158,403,190)x1, and 7q31.32q33(123,235,288-136,121,540)x1 *parents not tested* Genes on this location (7q31.32q33): *IRF5*, *CCDC136*, *CPA5*. One patient reported with an ASD and a 7q31.32q33 deletion. (DOI: 10.1016/j.ejmg.2010.10.012) 2. arr CGH 11q22.3(106,986,448-107,409,919)x3 *parents not tested*
PVOD	1	1. arr[hg19] 4p14(38,835,879-39,134,967)x3, and 15q11.2(21,903,815-23,464,839)x3 (in combination with *EIF2AK4* mutation) Genes on this location (15q11.2): *POTEB*, *OR4M2*, *GOLGA6L1* Associated with an increased risk of developmental disorders, learning problems, craniofacial dysmorphias, autism and epilepsy. (DOI: 10.1007/s00439-011-0970-4 and DOI: 10.1097/DBP.0b013e31826052ae)
PAH with *MMACHC* mutation and CblC deficiency	1	1. arr[hg19] Xp22.31(6,640,543-6,981,714)x3, and Xp22.31(7,935,381-8,138,035)x3 (in combination with *MMACHC* mutation) *parents not tested*

ADHD, attention deficit hyperactivity disorder; *ASD*, atrial septal defect; *CGH*, comparative genomic hybridization; *SNP*, single nucleotide polymorphism; *WPW*, Wolff-Parkinson-White syndrome.