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The genetic epidemiology of pediatric pulmonary arterial hypertension

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Abstract

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 correlations and outcome.

Study design: Seventy children diagnosed with idiopathic PAH (IPAH), heritable PAH (HPAH), PAH associated with congenital heart disease (CHD) with coincidental shunt (PAH-CHD group 3), PAH after closure of a cardiac shunt (PAH-CHD group 4), or PAH associated with other non-cardiac 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 (CNVs).

Results: Nineteen children (27%) had a PAH-associated gene mutation/variant: *BMPR2* n=7, *TBX4* n=8, *ACVRL1* n=1, *KCNK3* n=1, *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 CNVs). 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.

Word count: 226

List of abbreviations:

PAH: pulmonary arterial hypertension; HPAH: heritable PAH; IPAH: idiopathic PAH; CHD: congenital heart disease; WHO-FC: World Health Organization Functional Class; CNV: copy number variation; SNP array: single nucleotide polymorphism array; array CGH: array comparative genomic hybridization; *BMP2*: bone morphogenetic protein receptor type 2; *ACVRL1*: activin receptor-like kinase 1; *TBX4*: T-box 4; *EIF2AK4*: eukaryotic translation initiation factor 2-alpha kinase 4; *KCNK3*: potassium channel subfamily K member 3; PVOD: pulmonary veno-occlusive disease; NT-proBNP: N-Terminal pro-B-Type natriuretic peptide; *MMACHC*: methylmalonic aciduria and homocystinuria type C protein; *VHL*: von Hippel Lindau; *ACTA2*: alpha-actin-2; *JAG1*: jagged1; CblC: cobalamin C.

INTRODUCTION

In recent years a number of gene mutations have been identified to be associated with pulmonary arterial hypertension (PAH), including *BMPR2*, *TBX4*, *ACVRL1*, *ENG*, *KNCK3*, *EIF2AK4*, *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 to 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 lower, with a reported frequency of occurrence of 2-3%, nevertheless still 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

This study aimed to describe the epidemiology of genetic disorders in a Dutch national cohort of children with PAH, divided into three 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 correlations 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 (IPAH/HPAH), PAH associated with congenital heart disease (CHD) and coincidental shunt or after closure of a cardiac shunt (PAH-CHD group 3 and 4 according to the most recent clinical classification of pulmonary hypertension (PH) of 2019^{12–14}), pulmonary veno-occlusive disease (PVOD), or PAH associated with other non-cardiac conditions referred between 2003 and 2018, were included. 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 IPAH patients 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 ≥ 25 mmHg, pulmonary vascular resistance index ≥ 3 Wood units·m², and mean pulmonary capillary wedge pressure ≤ 15 mmHg confirmed by right heart catheterization (RHC), 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 (NT-proBNP), 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; online).

Chromosomal abnormalities (CNVs: deletions and duplications) are investigated with single nucleotide polymorphism (SNP) array or array comparative genomic hybridization (CGH).

From 2003 this was combined with Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA), to detect small intragenic deletions in the *BMPR2* gene. In 2014 a panel of seven 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 MLPA-test for *BMPR2*. In 2017 whole exome sequencing (WES) was introduced to screen for mutations/variants in the eight 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 (SIFT), Polymorphism Phenotyping (PolyPhen), Grantham score, MutationTaster, Align GVGD, and PhyloP.¹⁷ A diagnosis of HPAH was made in 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

methylnalonic aciduria and homocystinuria type C protein (*MMACHC*) gene (causing cobalamin C [CblC] 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 case of suspected clinical diagnosis. For this study, we designed three 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.

Statistics

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 Chi-squared test when appropriate. Kaplan-Meier survival curves with log-rank testing were used to study differences in survival between patient groups that included four or more patients.

RESULTS

Genetic testing

Between 2003 and 2018 eighty patients with PAH meeting the inclusion criteria for this study, were identified. In ten patients (IPAH n=5, PAH-CHD group 3/4 n=4, PAH associated with a porto-systemic shunt n=1) no genetic testing was performed, due 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 twelve children is shown in Figure 2 (online). Of the 39 children, in whom no PAH-associated gene mutation or a genetic disorder with an established association with PAH was identified, all were screened for *BMPR2* mutations except for one child with Noonan syndrome. In 36 of these children this was combined with the PAH gene panel screening. In three children (without a diagnosis of PVOD) the screening panel did not include *EIF2AK4*.

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

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 [VUS]), *EIF2AK4* mutation n=2). One child had a homozygous mutation in *EIF2AK4* and one child had two heterozygous mutations in *EIF2AK4*: a pathogenic frameshift mutation and a missense mutation that is classified as VUS (patient 17 and patient 18, respectively in Supplementary Table 1). In both children the diagnosis of PVOD was histopathologically confirmed in lung tissue collected at transplantation or autopsy (Figure 3A/B; online; patient 18 in Supplementary Table 1). Two out of eight children with a *TBX4* variant showed signs of interstitial lung disease on chest CT. One child (patient 15, Supplementary Table 1) was initially diagnosed as HPAH, but autopsy disclosed a histopathological diagnosis of PVOD (with patchy distribution) (Figure 3E/F; online). In the other child (patient 12, Supplementary Table 1) histopathology after lung transplantation disclosed “difficult to classify interstitial (fibrotic) lung disease” (Figure 3C/D; online), showing some capillary proliferation, but no venous occlusion and only limited iron deposition.²² The remaining six children with a *TBX4* variant showed no signs of parenchymal lung disease on either CT-scan (n=3) or chest X-ray (n=3). In six of the eight patients with a *TBX4* variant (75%) signs of small patella syndrome were found.

Other concomitant genetic disorders

In twelve children (17%) we identified a genetic disorder with an established association with PAH: five children with an *MMACHC* mutation and CblC deficiency, two brothers with a *VHL* mutation and familial erythrocytosis, and one child with an *ACTA2* mutation and multisystemic smooth muscle dysfunction syndrome and patent ductus arteriosus (classified

as PAH-CHD group 3). Additionally, three children with PAH-CHD had Down syndrome (trisomy 21): one child with PAH-CHD group 3 and two children with PAH-CHD group 4. Finally, one child with Alagille syndrome and a jagged1 (*JAG1*) mutation had a porto-systemic shunt.

Three children diagnosed with IPAH 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 seventy children included in the current study were additionally tested for CNVs with SNP array or array CGH. Eighteen children (30%) showed CNVs, either deletions or duplications, not previously reported to have an established association with PAH. In five 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 1 and Supplementary Table 2), 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, four (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 (Supplementary Table 2).

In summary, we identified 19 children (27%) with an established PAH-associated gene mutation, while in twelve 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 out of 70 children with PAH (33%) no genetic abnormalities were identified (Table 3).

Clinical disease and outcome

At time of diagnosis, median age of the children was 7.2 years (IQR 2.6-13.4) (54% female) (Table 2). Overall no statistical significant differences between different patient groups could be demonstrated, except for WHO-FC, follow-up time and the number of events (death or LTx). 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 ($p=0.033$). Longest follow-up time with the lowest number of events was found in children with *TBX4* variants, whereas children with PVDO or CbIC deficiency had shortest follow-up with the highest number of events. Transplant-free survival, unadjusted for clinical variables, 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 less than one 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 showed 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 most advanced stage and had the worst outcome, whereas pediatric *TBX4* variant carriers had better outcome compared with other PAH-children, also 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 to the observations in cohorts of adult PAH patients, *BMPR2* was not the most common affected gene in this pediatric cohort.

TBX4 variants were the most frequent variants in this pediatric PAH 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 HPAH, including a spectrum of “developmental” or interstitial lung disorders and respiratory compromise that may present in newborns, associated with persistent pulmonary hypertension of the newborn, but also during adulthood.²³⁻²⁹ This expanding spectrum will complicate classification of such patients according to the clinical classification of pulmonary hypertension as either HPAH or PH due to lung disease. In the light of the growing insight in the heterogeneous phenotypes of human *TBX4* variants, the authors recommend a meticulous and focused diagnostic work-up in patients with PH and a *TBX4* variant in order to be able to start the most appropriate treatment.^{28,29} In the current study, one 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, five children (7%) were diagnosed PVOD, a rare and lethal disease characterized by extensive and diffuse occlusion of pulmonary veins by neo-intimal 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 25% in adults with sporadic PVOD.⁵ Levy et al. reported that two out of three pediatric patients (67%) with PVOD had a homozygous *EIF2AK4* mutation.⁹ In the current cohort in two out of five children with PVOD (40%) an *EIF2AK4* mutation was identified.

An *MMACHC* mutation associated with CblC deficiency, renal thrombotic microangiopathy and PAH was found in five children in this cohort (7%). Four of these children were reported previously.²⁰ All but one were diagnosed at an advanced stage of PAH, characterized by overt right heart failure and WHO-FC IV, and died shortly after diagnosis, despite the 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 parental hydroxycobalamin supplementation, on top of PAH-targeted therapy, and eventually showed clinical improvement with normalization of pulmonary hemodynamics. This suggests a 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 work-up of children with PAH can 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 due to the dysregulation of the hypoxia-inducible factor (HIF) pathway in these patients.¹⁸ As *VHL* mutations seem to be endemic in specific regions (i.e. Chuvash region and Croatia) testing for these gene mutations on specific indications seems warranted.^{33,34}

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

Down syndrome was present in only three out of 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 work-up is required in children with trisomy 21 with PH in order to establish the exact nature of PH and to install most appropriate treatment.

The pathophysiological 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 anti-angiogenic 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, but a clear association between these two entities has not been established yet.^{42,43}

In almost a third of the 60 children tested with SNP array or array CGH a CNV was found, with the highest frequency in children with IPAH and PAH-CHD group 3. Children with PAH-CHD group 3 have cardiac defects that are regarded not solely responsible for the PAH. It has been speculated that these children bare 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. Recently, 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 In analogy, 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% in a cohort of children with IPAH.¹⁰

Further studies into common CNVs might provide clues to guide further research in the mechanisms of PAH.

In the 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 itself.

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, well-informed procreation decisions should be weighed together with parents and patient, against the high emotional stress of knowledge on 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 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 *TBX4* variants. 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.

Limitations

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, ten out of 80 children were not screened for PAH-associated gene mutations, half of these due to rapid death after diagnosis. This may have resulted in under-reporting of genetic disorders associated with severe PAH with worse survival. Since 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.

Future recommendations

The number of genes associated with PAH is increasing continuously, so genetic screening strategies in patients with PAH will also evolve continuously, where WES trio-analyses allow for the identification of new genetic abnormalities associated with pediatric PAH, also in retrospect in the individual patient 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 can be used in large cohorts of children with P(A)H in a research

setting to also study non-coding 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 need to go hand in hand with meticulous clinical phenotyping in order to allow for the exploration of specific genotype-phenotype correlations, that eventually may lead to optimization and individualization of treatment strategies in pediatric PAH.

CONCLUSION

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 background and future studies are needed whether this should be incorporated in risk stratification.

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FIGURES

Figure 1 (online). 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 whole exome sequencing.

PAH: pulmonary arterial hypertension; PVOD: pulmonary veno-occlusive disease; PCH: pulmonary capillary hemangiomatosis; *BMPR2*: bone morphogenetic protein receptor type 2; *ACVRL1*: activin receptor-like kinase 1; *TBX4*: T-box 4; *EIF2AK4*: eukaryotic translation initiation factor 2-alpha kinase 4; *KCNK3*: potassium channel subfamily K member 3.

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

PAH: pulmonary arterial hypertension; *BMPR2*: bone morphogenetic protein receptor type 2; *ACVRL1*: activin receptor-like kinase 1; *TBX4*: T-box 4; *EIF2AK4*: eukaryotic translation initiation factor 2-alpha kinase 4; *KCNK3*: potassium channel subfamily K member 3; *MMACHC*: methylmalonic aciduria and homocystinuria type C protein; *VHL*: von Hippel Lindau; *ACTA2*: alpha-actin-2; *JAG1*: jagged1.

Figure 3 (online). Histopathological assessment in three patients.

Figure 3A, B

A: Lung parenchyma of patient with two heterozygous mutations in *EIF2AK4* with pulmonary venous occlusive disease (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 (hematoxylin and eosin). B: Elastin stain of the same area in particular clearly showing obstructed venule (both bar= 100 micron).

Figure 3C, D

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= 1mm). D: Larger magnification (bar = 100 micron).

Figure 3E, F

E: Lung parenchyma of patient with *TBX4* mutation with pulmonary venous occlusive disease (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); (hematoxylin and eosin; bar= 200 micron). F: Larger magnification (bar = 100 micron).

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

Table 1. Distribution of PAH-associated gene mutations/variants (including *BMPPR2*, *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)		PAH-CTD (n=1)	PAH associated with a porto-systemic shunt (n=2)	PAH associated with CbIC deficiency (n=5)	PAH associated with familial erythrocytosis (n=2)
	IPAH (n=19)	HPAH (n=16)	PVOD (n=5)	Group 3 (n=14)	Group 4 (n=6)				
<i>BMPPR2</i>	-	7	-	-	-	-	-	-	-
<i>TBX4</i>	-	7	1*	-	-	-	-	-	-
<i>KCNK3</i>	-	1	-	-	-	-	-	-	-
<i>EIF2AK4</i>	-	-	2	-	-	-	-	-	-
<i>ACVRL1</i>	-	1	-	-	-	-	-	-	-
<i>MMACHC</i>	-	-	-	-	-	-	-	5	-
<i>VHL</i>	-	-	-	-	-	-	-	-	2
<i>ACTA2</i>	-	-	-	1	-	-	-	-	-

Table 2. Patient characteristics stratified for the different PAH groups at time of diagnosis

	n	Full patient cohort (n=70)	n	IPAH (n=19)	n	HPAH with <i>TBX4</i> variant (n=7)	n	HPAH with <i>BMPR2</i> mutation (n=7)	n	PVOD (n=5)	n	PAH-CHD group 3 and 4 (n=20)	n	PAH with <i>MMACHC</i> mutation and CblC 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, yrs	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, yrs	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 (1481-20107)	4	7684 (2836-18249)	17	676 (203-1137)	5	1551 (145-55478)

mRAP, mmHg	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, mmHg	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)
CI, 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 (interquartile range) or frequencies (percentage). PAH: pulmonary arterial hypertension; HPAH: heritable PAH; IPAH: idiopathic PAH; CHD: congenital heart disease; PVOD: pulmonary veno-occlusive disease; *MMACHC*: methylmalonic aciduria and homocystinuria type C protein; CbIC: cobalamin C; (H)LTX: (heart) lung transplantation; WHO-FC: World Health Organization Functional Class; NT-proBNP: N-Terminal pro-B-Type natriuretic peptide; mRAP: mean right atrial pressure; mPAP: mean pulmonary arterial pressure; PVRi: pulmonary vascular resistance index; CI: cardiac index.

* According to Sitbon criteria.

Table 3. Genetic architecture in a national cohort of children with PAH

<i>PAH-associated gene mutation</i>	<i>Genetic disorder with an established association with PAH</i>	<i>Genetic syndromes and CNVs without an established association with PAH</i>	<i>No genetic abnormality</i>
27%	17%	23%	33%
N=19 <ul style="list-style-type: none"> - <i>BMPR2</i>, n=7 - <i>TBX4</i>, n=8 - <i>EIF2AK4</i>, n=2 - <i>KCNK3</i>, n=1 - <i>ACVRL1</i>, n=1 	N=12 <ul style="list-style-type: none"> - <i>MMACHC</i>, n=5 - <i>VHL</i>, n=2 - <i>ACTA2</i>, n=1 - Down syndrome, n=3 - Alagille syndrome associated with porto-systemic shunt with a <i>JAG1</i> mutation, n=1 	N=16 <ul style="list-style-type: none"> - Noonan syndrome, n=2 - Beals syndrome, n=1 - CNV, n=13 	N=23
PAH: pulmonary arterial hypertension; <i>BMPR2</i> : bone morphogenetic protein receptor type 2; <i>ACVRL1</i> : activin receptor-like kinase 1; <i>TBX4</i> : T-box 4; <i>EIF2AK4</i> : eukaryotic translation initiation factor 2-alpha kinase 4; <i>KCNK3</i> : potassium channel subfamily K member 3; <i>MMACHC</i> : methylmalonic aciduria and homocystinuria type C protein; <i>VHL</i> : von Hippel Lindau; <i>ACTA2</i> : alpha-actin-2; <i>JAG1</i> : jagged1; CNV: copy number variation.			

Figure 1. Timeline of the discovery of genes contributing to PAH (whites boxes) and the implementation of structural genetic screening for PAH genes in the Dutch National Referral Center (black boxes).

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

Patients at risk, n					
IPAH	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	

SUPPLEMENTAL MATERIAL

Supplementary Table 1. (Likely) pathogenic mutations and copy number variations identified in children with pulmonary arterial hypertension

Pt	Gene	Mutation category	Nucleotide change	Amino Acid change	Pathology	Genebank accession number	Mutation previously reported in
1	<i>BMPR2</i>	Nonsense	c.47G>A	p.(Trp16*)	HPAH	NM_001204.6	
2	<i>BMPR2</i>	Frameshift	c.399delT	p.((Pro134Leufs*18)	HPAH	NM_001204.6	
3	<i>BMPR2</i>	Intragenic deletion	c.530-?_c.621+?del	p.?	HPAH	NM_001204.6	Aldred et al. 2006, Hum Mut , Mutation in Brief #869 online
4	<i>BMPR2</i>	Missense	c.1471C>T	p.(Arg491Trp)	HPAH	NM_001204.6	(Deng et al., Am. J. Hum. Genet., 2000; Dewachter et al., Eur. Respir. J., 2009)
5	<i>BMPR2</i>	Nonsense	c.2695C>T	p.(Arg899*)	HPAH	NM_001204.6	
6	<i>BMPR2</i>	Unknown			HPAH		
7	<i>BMPR2</i>	Frameshift	c.941_945delATCTT	p.(Tyr314Serfs*11)	HPAH	NM_001204.6	
8	<i>TBX4</i>	Deletion	17q23.2 (55,654,379-57,679,097)	-	HPAH		Kerstjens-Frederikse et al 2013, J Med Genet. 2013 Aug;50(8):500-6)
9	<i>TBX4</i>	Deletion	17q23.21q23.2 de novo	-	HPAH		
10	<i>TBX4</i>	Deletion	17q23.2q23.3(RP11-332h18->RP11-156L14)x1	-	HPAH		Kerstjens-Frederikse et al 2013, J Med Genet. 2013 Aug;50(8):500-6)
11	<i>TBX4</i>	Deletion	17q23.1q23.2 de novo	-	HPAH		Kerstjens-Frederikse et al 2013, J Med Genet. 2013 Aug;50(8):500-6)
12	<i>TBX4</i>	Frameshift	c.355dupA	p.(Ile119Asnfs*6)	HPAH	NM_018488.3	Kerstjens-Frederikse et al 2013, J Med Genet. 2013 Aug;50(8):500-6)

13	<i>TBX4</i>	Missense	c.401G>C	p.(Trp134Ser)	HPAH	NM_018488.3	
14	<i>TBX4</i>	Frameshift	c.1164dupC	p.(Arg389Glnfs*30)	HPAH	NM_018488.3	Kerstjens-Frederikse et al 2013, J Med Genet. 2013 Aug;50(8):500-6)
15	<i>TBX4*</i>	Missense	c.1145A>C	p.(Tyr382Ser)	HPAH	NM_018488.2	Kerstjens-Frederikse et al 2013, J Med Genet. 2013 Aug;50(8):500-6)
16	<i>KCNK3</i>	Missense	c.616G>T	p.(Val206Leu)	HPAH	NM_002246.2	
17	<i>EIF2AK4</i>	Frameshift	c.1739dupA (homozygous)	p.(Arg581Glnfs*9)	PVOD		
18	<i>EIF2AK4</i>	Frameshift Missense	c.4205dup ;c.2968C>T ⁽¹⁾	p.Ser1403Lysfs*45;p. Pro990Ser	PVOD	NM_001013703.3	
19	<i>ACVRL1</i>	Missense	c.511G>A ⁽²⁾	p.(Asp171Asn)	HPAH	NM_000020.2	

* patient diagnosed as *TBX4*-associated HPAH, where autopsy showed a histopathological diagnosis of pulmonary veno-occlusive disease.

⁽¹⁾ c.4205dup p.(Ser1403Lysfs*45 is pathogenic, c.2968C>T p.(Pro990Ser) is a variant of unknown significance

⁽²⁾ c.511G>A p.(Asp171Asn) is a variant of unknown significance

PAH: pulmonary arterial hypertension; HPAH: heritable PAH; PVOD: pulmonary veno-occlusive disease; *BMP2*: bone morphogenetic protein receptor type II; *TBX4*: T-Box 4; *KCNK3*: potassium channel subfamily K member 3; *EIF2AK4*: eukaryotic translation initiation factor 2-alpha kinase 4; *ACVRL1*: activin receptor-like kinase 1.

Supplementary Table 2. Copy number variations and variants with unknown significance (VUS)

PAH-group	N	Abnormality array CGH/SNP-array
IPAH/HPAH	7	<ol style="list-style-type: none"> 1. arr[hg19] 3q13.33q21.1(120,205,270-121,936,796)x1 (in combination with <i>BMPR2</i> mutation) <i>parents not tested</i> 2. arr CGH 3p12.3(75,622,607-75,804,387)x1 (in combination with <i>ACTA2</i> mutation) <i>parents not tested</i> 3. arr[hg19] 9q22.1(90,543,679-90,960,845)x3, and 9q22.31(94,568,831-95,122,101)x3 <i>not present in mother; father not tested</i> 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 <i>ACVRL1</i> mutation) <i>parents not tested</i> 5. arr CGH duplication of a sub part of 7q21.3 <i>parents not tested</i> 6. arr CGH 8p23.2 (7 oligo's)x3 <i>parents not tested</i> 7. arr 6q12(67,006,745-67,489,490)x1, and 11p15.4(7,012,268-8,074,827)x3 <i>parents not tested</i>
PAH-CHD group 3	7	<ol style="list-style-type: none"> 1. arr[hg19] Xp22.31(6,454,369-8,138,035)x1, and 20p12.3(8,093,416-8,579,037)x3 <i>parents not tested</i> Gene on this location (20p12.3): <i>PLCB1</i>. Has been described in relation with WPW and ADHD (DOI: 10.1002/ajmg.a.35701) 2. arr[GRCh37] 14q32.33(104884787_106339625)x1 <i>parents not tested</i> 3. arr[GRCh37] 11p14.2p14.1(26996699_27240343)x3 <i>pat</i> 4. arr CGH 16q24.1(84,500,030-85,260,589)x3 <i>pat</i> 5. arr[GRCh37] 18p11.32q12.2(0_33540595)x3 dn. Genes on this location: <i>TAF4B</i>, <i>DTNA</i>. 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: <i>SMAD4</i>. Associated with structural heart diseases (DOI: 10.1002/9780470015902.a0025246). 18q23(77706676_78077248)x3 dn Gene on this location: <i>TXNL4A</i>. 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 <i>mat</i>
PAH-CHD group 4	2	<ol style="list-style-type: none"> 1. arr cgh 2q24.1(158,038,869-158,403,190)x1, and 7q31.32q33(123,235,288-136,121,540)x1 <i>parents not tested</i> Genes on this location (7q31.32q33): <i>IRF5</i>, <i>CCDC136</i>, <i>CPA5</i>. 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 <i>parents not tested</i>
PVOD	1	<ol style="list-style-type: none"> 1. arr[hg19] 4p14(38,835,879-39,134,967)x3, and 15q11.2(21,903,815-23,464,839)x3 (in combination with <i>EIF2AK4</i> mutation) Genes on this location (15q11.2): <i>POTEB</i>, <i>OR4M2</i>, <i>GOLGA6L1</i> 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 <i>MMACHC</i> mutation and CbIC deficiency	1	<ol style="list-style-type: none"> 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 <i>MMACHC</i> mutation) <i>parents not tested</i>

PAH: pulmonary arterial hypertension; HPAH: heritable PAH; IPAH: idiopathic PAH; PVOD: pulmonary veno-occlusive disease; CHD: congenital heart disease; CblC: cobalamin C; ASD: atrial septal defect; *BMPR2*: bone morphogenetic protein receptor type II; *EIF2AK4*: eukaryotic translation initiation factor 2-alpha kinase 4; *ACTA2*: alpha-actin-2; *ACVRL1*: activin receptor-like kinase 1; *MMACHC*: methylmalonic aciduria and homocystinuria type C protein; WPW: Wolff-Parkinson-White syndrome; ADHD: attention deficit hyperactivity disorder.