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ORIGINAL ARTICLE

Genetic testing and blood biomarkers in paediatric pulmonary hypertension. Expert consensus statement on the diagnosis and treatment of paediatric pulmonary hypertension. The European Paediatric Pulmonary Vascular Disease Network, endorsed by ISHLT and DGPK

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ABSTRACT

Childhood-onset pulmonary arterial hypertension (PAH) is considered complex and multifactorial, with relatively poor estimates of the natural history of the disease. Strategies allowing earlier detection, establishment of disease aetiology together with more accurate and sensitive biomarkers could enable better estimates of prognosis and individualise therapeutic strategies. Evidence is accumulating that genetic defects play an important role in the pathogenesis of idiopathic and hereditary forms of PAH. Altogether nine genes have been reported so far to be associated with childhood onset PAH suggesting that comprehensive multigene diagnostics can be useful in the assessment. Identification of disease-causing mutations allows estimates of prognosis and forms the most effective way for risk stratification in the family. In addition to genetic determinants the analysis of blood biomarkers are increasingly used in clinical practice to evaluate disease severity and treatment responses. As in genetic diagnostics, a multiplex approach can be helpful, as a single biomarker for PAH is unlikely to meet all requirements. This consensus statement reviews the current evidence for the use of genetic diagnostics and use of blood biomarkers in the assessment of paediatric patients with PAH.

INTRODUCTION

During the last decade, advances in genomic technologies have accelerated genetic research and broadened our understanding of basic human biology. Using genomics approaches to study well-characterised patient cohorts has revealed the molecular genetic background of many human diseases. Fast accumulation of knowledge on clinically relevant genomic variants has started to reshape clinical practices in several fields of medicine. These include hereditary cardiovascular diseases such as cardiomyopathies and channelopathies, where thousands of mutations in over 100 genes have been found. In these disorders the use of genetic testing is becoming a standard procedure in the diagnostic workup. Several international guidelines supporting the use of genetic testing have been published.[1] It has been shown that these approaches can save costs by enabling rational follow-up focused on family members with an identified risk.[2] Although genetic testing has not been widely applied to patients with familial pulmonary arterial hypertension (HPAH, hereditary forms of PAH) or idiopathic PAH (IPAH), there is increasing evidence for its usefulness in the diagnostic workup. Other strategies to individualise patients' treatments and follow-up care involve the use of blood biomarkers. Biomarkers are suggested as potential clinical end points that can optimise the assessment and treatment of patients. This consensus statement reviews the current evidence for the use of genetic diagnostics and blood biomarkers in the assessment of paediatric patients with pulmonary arterial hypertension (PAH) with respect to diagnosis, prognosis and treatment.

METHODS

The recommendations given in table 1 relate to the grading system currently suggested by the European Society of Cardiology and the American Heart Association, and was based on paediatric data only (class, level of evidence). The grading and voting process within the writing group is outlined in the executive summary[31] of this online supplement. Computerised searches of the PubMed/MEDLINE bibliographic database from 1990 to January 2015 were conducted. The developer searched using the terms 'genetics of pulmonary hypertension', 'paediatric pulmonary hypertension', 'genetic diagnostics in pulmonary hypertension', 'genetics of childhood-onset pulmonary hypertension', 'biomarkers of pulmonary hypertension', 'BNP and children', 'uric acid and children', 'circulating endothelial cells and children', 'biomarkers in children with pulmonary hypertension', 'circulating endothelial cells and pulmonary hypertension', 'BNP and pulmonary hypertension', 'uric acid and pulmonary hypertension'.

Genetics of PAH

To date, there are at least eight genes associated with HPAH and IPAH (table 2). From these genes, bone morphogenetic protein receptor 2 (*BMPR2*),

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Table 1 Recommendations on the use of genetic testing and biomarkers in children with pulmonary hypertension

Genetics/genetic testing/blood biomarker	COR	LOE
Genetic counselling is recommended for families with children suffering from severe genetic disorder. This includes children with IPAH or HPAH[17–19]	I	B
Families of patients with syndromes associated with PAH should be educated on the symptoms of PAH and recommended to seek clinical evaluation if the child should develop symptoms of PAH	I	C
It is recommended to screen asymptomatic PAH mutation carriers, also children, with serial echocardiograms, and potentially other non-invasive studies	I	C
Genetic testing for PAH-associated genes such as *ACVRL1, BMPR2, CAV1, ENG and KCNK3* can be useful for children with PAH of unknown cause to allow definition of aetiology, estimation of prognosis and identification of family members at risk, and to inform family planning	IIa	C
Genetic testing of first-degree relatives of an index patient with PAH and a known disease-causing mutation can be useful for risk stratification and rationalising surveillance	IIa	C
Genetic testing for mutations in EIF2AK4 gene should be considered for children with suspicion of PVOD to allow for definition of aetiology and identification of family members at risk, and to inform family planning	IIa	C
Family members of patients with HPAH who develop new cardiorespiratory symptoms should be evaluated immediately for PAH	I	C
Comprehensive NGS panels targeting all known genetics in PAH can be considered to maximise diagnostic efficacy and increase cost-effectiveness in genetic diagnostics	IIb	C
Genetic testing for PAH-associated genes can be considered for patients with CHD and 'out of proportion' PAH (eg, PAH with small atrial shunt)	IIb	C
Measurement of natriuretic peptides BNP or NT-proBNP is recommended to evaluate disease severity, progression and treatment response in patients with PH[20–22]	I	B
It is uncertain whether analysis of uric acid levels can be used to evaluate disease severity	IIb	C
Analysis of circulating endothelial cells can be useful to stratify operative risk, to evaluate for progression of disease and/or response to therapy in children with PAH	IIa	B

ACVRL1 (ALK1) activin-like kinase-type 1; BMPR2, bone morphogenetic protein receptor 2; BNP, brain natriuretic peptide; CAV1, caveolin 1; CHD, congenital heart disease; COR, class of recommendation; EIF2AK4, eukaryotic translation initiation factor 2-α kinase 4; ENG, endoglin; HPAH, hereditary pulmonary hypertension; IPAH, idiopathic pulmonary arterial hypertension; KCNK3, potassium channel subfamily K, member 3; LOE, level of evidence; NGS, next generation sequencing; NT-proBNP, N-terminal fragment of probrain natriuretic peptide; PH, pulmonary hypertension; PVOD, pulmonary veno-occlusive disease; Definitions for COR and LOE classes can be found in our executive summary table 1 (LINK).

is the major gene associated with HPAH and IPAH. Today over 300 *BMPR2* mutations have been identified in patients with PAH. BMPR2 mutations are estimated to account for approximately 75% of patients with HPAH and up to 25% of IPAH cases.[3] The penetrance of the disease associated with *BMPR2* mutations has originally been considered low. These observations have been questioned as disease manifestations can take 75 years and phenotypical variability exists. Supporting evidence for higher penetrance was established through the Vanderbilt Pulmonary Hypertension Registry where overall penetrance was 27% (female 42% and the male 14%).[4] Over 40% penetrance and high proportion of mutation positive individuals among patients with HPAH demonstrates that PAH is a genetic disease comparable with other forms of genetic diseases, i.e., cardiomyopathies and channelopathies. *BMPR2* mutations are associated with childhood-onset PAH (table 2).[5 6]

PAH associated with hereditary haemorrhagic telangiectasia (HHT) has been identified in multiple studies to associate with pathogenic mutations in the *ACVRL1* gene and in some occasions with Endoglin (*ENG*), a type III TGF-β receptor.[7] *ACVRL1* mutations can also be identified in patients with PAH with no features of HHT. *SMAD9* mutations have been suggested as a genetic cause of PAH although existing evidence is

Table 2 Genes associated with childhood-onset pulmonary hypertension

Gene	Form of PH	Affecting children	High evidence of pathogenicity
ACVRL1 (ALK1)	IPAH, HPAH	✓	✓
BMPR1B	IPAH	✓	
BMPR2	IPAH, HPAH	✓	✓
CAV1	IPAH, HPAH	✓	✓
EIF2AK4	PVOD	✓	✓
ENG	IPAH	✓	
KCNK3	IPAH, HPAH	✓	✓
NOTCH3	IPAH	✓	
SMAD9	IPAH	✓	

High evidence of pathogenicity requires evidence for positive family segregation between genotype and phenotype or multiple reports supporting causative role in human disease together with additional evidence such as functional studies.
ACVRL1 (ALK1) activin-like kinase-type 1; BMPR1B, bone morphogenetic protein receptor 1B; BMPR2, bone morphogenetic protein receptor 2; CAV1, caveolin 1; EIF2AK4, eukaryotic translation initiation factor 2-α kinase 4; ENG, Endoglin; HPAH, hereditary pulmonary hypertension; IPAH, idiopathic pulmonary arterial hypertension; KCNK3, potassium channel subfamily K, member 3; NOTCH3, neurogenic locus notch homologue protein 3; PH, pulmonary hypertension; PVOD, pulmonary veno-occlusive disease; SMAD9, Smad family member 9.

mainly based on experimental data derived from functional in vitro and animal studies.[8] Two missense mutations in another bone morphogenetic protein (BMP) signaling associated gene, *BMPR1B,* were identified in children with IPAH.[9] An exome sequencing analysis of a three-generation family with multiple family members presenting with PAH revealed *CAV1* as a novel gene associated with PAH. Another exome sequencing study on a family with multiple affected individuals revealed a missense mutation in a potassium channel gene *KCNK3*.[10] A recent report suggested that mutations in the *NOTCH3* gene could also cause childhood-onset IPAH.[11] A recent breakthrough in pulmonary veno-occlusive disease (PVOD) has revealed that mutations in the *EIF2AK4* gene play a major role in hereditary forms of PVOD.[12] Genetic testing for mutations in *EIF2AK4* gene should be considered for children with suspicion of PVOD. To date the evidence for causality in human IPAH and HPAH is considered significant for the following genes: *ACVRL1, BMPR2, CAV1, ENG* and *KCNK3,* whereas the role of *BMPR1B, NOTCH3* and *SMAD9* is still unconfirmed (table 2).

There is evidence for genotype-phenotype correlations in PAH. Multiple studies from different registries have implicated that carriers of a *BMPR2* mutation, irrespective of family history, develop PAH at a younger age than mutation negative individuals.[13–16] Furthermore, patients with the hereditary form of PAH developed higher mean pulmonary arterial pressure (PAP), lower cardiac index, higher pulmonary vascular resistance (PVR), and were more likely to be treated with parenteral prostanoid therapy or lung transplant when compared with patients with idiopathic disease. Both children and adults with PAH in association with a *BMPR2* mutation are less likely to respond to vasodilators and are unlikely to benefit from treatment with calcium channel blockers. Symptomatic patients with HPAH carrying *ACVRL1* mutations, most without HHT, have an earlier age of disease onset and show more rapid disease progression than patients with *BMPR2* mutations.[16] A recent study on 54 children with IPAH and HPAH revealed that 25 individuals (46%) were positive for either *BMPR2* (18 patients; 33%) or *ACVRL1* (7 patients; 13%) mutations.[6] In this study the 5-year survival was significantly lower in patients tested positive for *BMPR2* and *ACVRL1* mutations. The study outcome correlated well with previous studies showing earlier onset and more severe phenotype in mutation positive patients. These studies suggest that patients with PAH carrying the *BMPR2* or *ACVRL1* mutation should be considered for more aggressive treatment strategies.

PAH is a rare complication in many genetic disorders such as Down syndrome (trisomy 21) where PAH can be caused by the high incidence of left-to-right shunt, upper airway obstructions and sleep apnoea. Other disorders where PAH has been described are congenital heart disease (CHD), 22q11 deletion syndrome (DiGeorge), VACTERL syndrome, CHARGE syndrome, Scimitar syndrome, Noonan syndrome, chromosomal anomalies associated with congenital diaphragmatic hernia, Cantu syndrome, autoimmune polyendocrine syndrome, mitochondrial disorders including mitochondrial encephalopathy lactic acidosis and stroke-like episodes, Gaucher disease, and glycogen storage diseases (GSDI and GSDIII). Establishing the underlying molecular genetic defect in the majority of these syndromes can be beneficial in determining the exact diagnosis and estimating prognosis, clinical follow-up as well as treatment strategies. This recommendation does not focus on the molecular genetics of these syndromes nor does it recommend testing of PAH-associated genes in these patients.

Genetic testing in PAH

Considering the possible hereditary nature of PAH, identification of a child with idiopathic PAH will initiate concerns of disease susceptibility in other family members, including the parents. Clinical evaluation of patient and family members will not necessarily establish the aetiology of IPAH and cannot define their risk to develop PAH later in life. The latter, like in other hereditary diseases, causes significant distress for the family. Identification of the genetic background enables effective family member testing and risk stratification among relatives. Considering the relatively high penetrance, especially among women, identification of the causative mutation allows effective clinical monitoring of those individuals at increased risk for the disease. Similarly, negative testing in family members following the molecular diagnosis for an index patient reduces stress burden in family members and allows discontinuation of future clinical monitoring in these individuals. This approach could therefore enable the establishment of cost-effective monitoring and follow-up programmes.[2] Follow-up strategies for mutation carrier relatives have to be evaluated individually based on the age and sex of the family member, and existing family history and disease characteristics.

Genetic counselling is recommended for families with children suffering from a genetic disorder. Genetic counselling has to elucidate to the patient and family the rationale for genetic testing and describe the expected yield of genetic testing. Counselling also includes addressing the complex issues of incomplete penetrance and the possibility of finding variants that currently are classified as variants of unknown significance. All variant classification scenarios in genetic testing have to be clear for the referring physician.[17] Different end points may affect the future surveillance of family members. It is also recommended to discuss concerns about genetic discrimination, and psychosocial issues of guilt and blame that accompany genetically based diseases. Although there have been concerns that predictive genetic testing in clinically unaffected children and adults could provoke anxiety and distress, current studies suggest instead that it can provide reassurance, lessen uncertainty and promote a sense of control.[18] Genetic counselling should be provided by a healthcare professional with experience in genetics and PAH. Determining the best age for testing the asymptomatic children has to be evaluated separately in every family based on the family history and family's requests after counselling. An algorithm for genetic testing of a child with IPAH or HPAH has been described in figure 1.

An important part of the counselling process is to establish an inclusive family history and determine the clinical features of the index patient. This allows comprehensive description of the disease phenotype and will facilitate interpretation of genetic test results. Any family history of HHT-like symptoms could point towards a mutation in the *ACVRL1* or *ENG* gene, whereas at the same time a negative family history of HHT, does not rule out the possibility of disease-causing mutation in the *ACVRL1* gene. On the contrary, suspicion of PVOD, especially if recessive inheritance is possible, suggests mutations in the *EIF2AK4* gene. Although *BMPR2* and *ACVRL1* are currently the major disease-causing genes in PAH, any patient with IPAH or HPAH could harbour a mutation in any of the other known PAH genes. As the number of disease-causing genes is expanding there is increasing rationale to select more effective high-throughput next generation sequencing (NGS) strategies instead of gene-specific direct sequencing (Sanger sequencing). NGS-based applications are already enabling more cost-effective, comprehensive and faster analysis of causative genes in diagnostic setting. As the field and

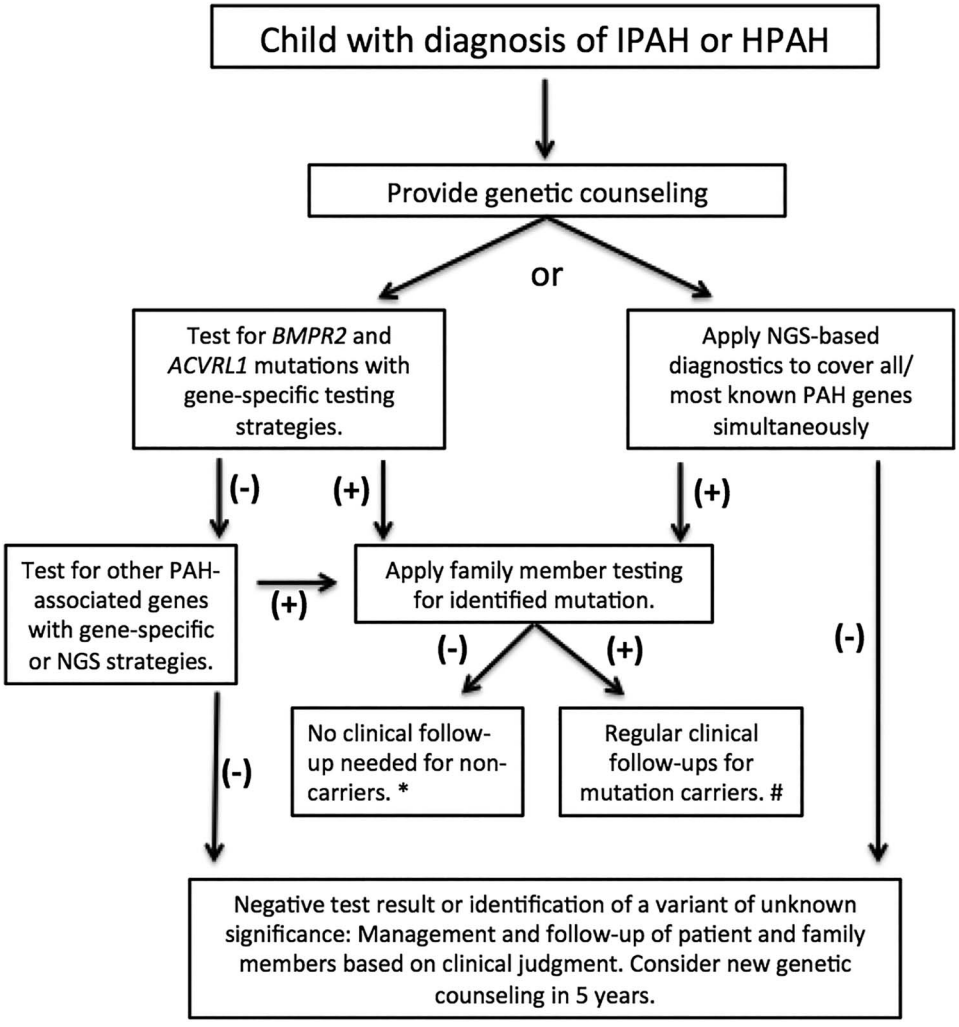

Figure 1 Algorithm for applying genetic testing for a child with IPAH or HPAH, and family members. (–) negative test result and (+) positive test result with identification of pathogenic or likely pathogenic mutation. If NGS-based comprehensive analysis of PAH genes is not available consider gene-specific testing for *BMPR2* and *ACVRL1* genes as first-line diagnostics. *If genetic test reveals a mutation classified as likely pathogenic consider new genetic counselling in 5 years to confirm classification and validity of applied follow-up strategy. # Follow-up strategies for mutation carrier relatives have to be evaluated individually based on the age and sex of the family member, and existing family history and disease characteristics. HPAH, hereditary pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; NGS, next generation sequencing; PAH, pulmonary arterial hypertension.

technologies are evolving rapidly there is increasing pressure for the referring physician to select optimal diagnostic tools to ensure high-end test quality and interpretation of test results. NGS strategies are already enabling the analysis of all nine genes mentioned in this statement with the same costs as single gene analysis using conventional technologies. Multigene strategies will provide simultaneous full analysis of *BMPR2* and *ACVRL1* and can include the analysis of other genes causative for PAH as well as potential new candidates. Adapting this strategy will allow for increasing diagnostic power but may also accumulate variant data impacting future diagnostics. Today's genetic diagnostics is increasingly based on public databases, which accumulate variant information from diagnostics and research. It is becoming a standard procedure in modern genetic diagnostics to publish variant data and classifications in public databases such as ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/).

The use of biomarkers in paediatric PAH

Existing and potential biomarkers in PAH relate to heart failure, inflammation, haemostasis, vascular remodeling and endothelial cell-smooth muscle cell dysfunctions. In current PAH guidelines, brain natriuretic peptide (BNP) and N-terminal fragment of pro-BNP (NT-proBNP) have been recommended.[19] Other prognostic markers such as growth differentiation factor-15, osteopontin and red cell distribution width, are considered emerging biomarkers of PAH. Anaemia, hypocapnia, elevated uric acid and C reactive protein levels are unspecific markers of disease severity. Certain chemokines, matrix metalloproteases and apelin have been linked to the vascular pathology in PAH and are considered as potential biomarkers. Circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) have received much attention as markers of disease activity but with controversial findings. Circulating microparticles and microRNAs have also been suggested. The majority of the studies in this field have been done with adults and the role of most candidate biomarkers in paediatric PAH is unknown. As in adults, the strongest evidence is on the use of BNP and NT-proBNP in monitoring disease severity in paediatric PAH. Biomarkers in children with PAH are likely to be impacted by additional factors, more so than in adult patients with PAH. These factors include degree of physical activity, age and/or developmental stage, gender differences, puberty and nutritional status. Although several promising markers have been identified over the past few years, the development of more specific markers, standardisation and prospective validation are warranted, also in paediatric PAH.

Natriuretic peptides and other plasma protein biomarkers

There is solid evidence in adult and paediatric patients with PAH that natriuretic peptides have prognostic value.[20–22] In these studies, elevated natriuretic peptides correlated with poor haemodynamics and increased mortality. The natriuretic peptides atrial natriuretic peptide (ANP) and BNP are synthesised in the atrial and the ventricular myocardium. These peptides

regulate natriuresis, diuresis and vasomotor tone. Atrial natriuretic peptide and BNP are elevated in association with pressure overload in order to reduce the workload of the heart and therefore have been found to be elevated in PAH. BNP is produced as a 108 amino acid prohormone, which is cleaved and secreted as a biological active sequence (BNP-32) and an inactive NT-proBNP peptide. BNP-32 has a short half-life of around 20 min and NT-proBNP a longer half-life of several hours, but both fragments are cleared by the kidney and influenced by renal function. A recent study elucidates that NT-proBNP levels correlated with haemodynamic parameters and outcome in PAH regardless of renal function.[23] Large variability of normal NT-proBNP levels at different ages during childhood has to be acknowledged when applied in paediatric PAH.[24]

Cardiac troponin T has also been shown as a significant biomarker in PAH correlating independently with mortality.[2] Evidence in paediatric population is lacking. Another study on children with CHD and PAH revealed elevated serum levels of soluble intracellular adhesion molecule 1.[3] In addition growth differentiation factor-15, galectin-3, endothelins and C reactive protein are promising as biomarkers in adult PAH, at least with respect to inflammation. A large number of other inflammatory proteins have also been suggested as candidates for biomarkers in PAH. As in genetics, the need for a comprehensive approach targeting large number of PAH-specific analytes is useful to optimise use of protein biomarkers in everyday clinical practice.

Uric acid

Uric acid is the final degradation product of purines and an endogenous free radical scavenger that is elevated following tissue ischaemia and hypoxia. Raised levels have been described in chronic obstructive pulmonary disease,[8] chronic heart failure[9] and cyanotic CHD.[10] This was underlined by reductions of uric acid levels in response to chronic vasodilator therapy. Potential confounding factors, however, include renal dysfunction and diuretic use and hence uric acid levels must be interpreted in the context of these factors. The role of uric acid in monitoring PAH severity and treatment responses may be useful although further studies are needed.[11 25]

Circulating endothelial cells

Circulating endothelial cells (CECs) have been established as non-invasive markers for vascular damage, remodeling and dysfunction. CECs have been measured in children with IPAH and PAH associated with CHD, before and after treatment.[26] Rising CEC counts predicted clinical deterioration, suggesting that CECs may be an important tool to evaluate prognosis.[26, 27] Another study reported elevated levels of CECs associated with reversible APAH-CHD in children.[27] These studies[26, 27] involved analysis of soluble biomarkers, and determined that markers of angiogenic cytokines, inflammation or endothelial microparticles were not able to predict PAH reversibility. These studies suggest that circulating CECs could be a potential biomarker in the assessment of paediatric PAH. On the other hand, some studies have failed to establish any correlation between CECs and PAH.[6 28] The lack of standards for cell isolation and characterisation methods as well as differences in the pathological mechanisms of the investigated patients may contribute to the existing discrepancies.

Conclusions

Emerging evidence is accumulating supporting the use of genetic testing and blood biomarkers in paediatric PAH to individualise diagnostics,[32] prognostics and therapy.[33] In addition, genetic testing supports efficient risk stratification in family members. As the number of genes and biomarkers associated with PAH is accumulating, we need more comprehensive diagnostic approaches in both fields. In addition to new diagnostic technologies, advanced experimental approaches and patient registries including multicentre approaches are needed, to strengthen current evidence and develop future recommendations.

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