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CASE REPORT

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Antenatal phenotype associated with *PAK2* pathogenic variants: bilateral pleural effusion as a warning sign

Louis Domenach[1*], Caroline Rooryck[1,2], Marine Legendre[1], Hanane Bouchghoul[3], Claire Beneteau[1] and Henri Margot[1*]

Abstract

Fetal pleural effusions can arise in various contexts with different prognosis. They have been reported in fetuses presenting with hereditary or acquired conditions. One particularly rare genetic disorder, known as Knobloch syndrome, seems to emerge as a potential new cause of fetal pleural effusions, associated with severe outcomes. Knobloch syndrome 1 can be caused by biallelic variants in *COL18A1*. It is primarily characterized by its ophthalmic features, including severe vitreoretinal degeneration with retinal detachment and macular abnormalities. Neurological defects such as encephalocele and developmental delay, along with skeletal and renal malformations, are also associated with the syndrome. The Knobloch syndrome 2 is caused by monoallelic variants in the kinase domain of *PAK2*. It is less described and seems to also be associated with cardiac and respiratory damage in addition to the Knobloch syndrome 1 phenotype. PAK2 is a ubiquitous protein with a major implication in regulation and remodeling of the cytoskeleton and numerous other cellular pathways. Knobloch-associated variants seem to cause a loss of the kinase function of the protein. Even if the ophthalmic defects are almost constant, *PAK2*-associated Knobloch syndrome has slightly different features from Knobloch syndrome 1 in which pulmonary and lymphatic damages are still unseen. In a prenatal trio exome sequencing, we identified a novel *de novo PAK2* missense variant, NM_002577.4:c.836 A > C, p.(Gln279Pro), classified as likely pathogenic in a 24 weeks of gestation fetus whose only sign was severe bilateral pleural effusion. From a literature review of patients, we recognize this sign as an important antenatal indicator of Knobloch syndrome 2, as it was the first sign identifiable in 2 out of 5 patients. This adds new evidence for the implication of this gene in fetal pleural effusions, with potentially severe outcomes.

Keywords Fetal pleural effusion, PAK2, Knobloch

*Correspondence:
Louis Domenach
louis.domenach@chu-bordeaux.fr
Henri Margot
henri.margot@hug.ch
[1]Service de Génétique Médicale, CHU de Bordeaux, Bordeaux F-33000, France
[2]Univ. Bordeaux, Génétique et Métabolisme (MRGM), INSERM U1211, Bordeaux F-33000, France
[3]Service de Gynécologie Obstétrique, CHU de Bordeaux, Bordeaux F-33000, France

Background

Fetal pleural effusion is fluid accumulation in the pleural space and occurs in 1:10,000 to 1:15,000 pregnancies [1]. Fetal pleural effusions can have numerous etiologies with very different prognoses. Pleural effusions have been reported in fetuses with genetic syndromes (both chromosomal and monogenic), ultrasound defects such as obstructive uropathy or thoracic cavity anomalies, immune or hematologic diseases (Rh or ABO incompatibility, α-thalassemia…), metabolic diseases, non-immune hydrops or even infections. A syndrome called Knobloch syndrome 2 (MIM #618458) involving retinal degeneration, encephalocele, potential neurodevelopmental delay and pulmonary damages is emerging as a new cause of fetal pleural effusions. It is caused by pathogenic variants in the kinase domain of *PAK2*.

p21 activated kinases, or PAKs, are a group of serine/threonine protein kinases that interact with the Rho GTPases Rac1 and Cdc42 [2]. They comprise two groups, of which group 1 is the most extensively studied and includes PAK1, PAK2 and PAK3. While PAK1 and PAK3 are mainly expressed in the brain, PAK2 is ubiquitously expressed [3]. They share 80% sequence similarity [4] and have a highly conserved structure: a N-terminal end containing autoregulatory domains with a GTPase binding domain (GBD) overlapping an autoinhibitory domain (AID), and a C-terminal end containing the kinase domain [5]. PAKs can autophosphorylate on various serine and threonine sites [5, 6] in order to maximize their kinase activity. Thr402 is the most important aminoacid in the kinase activation loop. To avoid constitutive PAK activation and to regulate their kinase activities, PAKs exist in a heterodimer basal state where the AID domain of one PAK molecule can bind to and inhibit the kinase domain of a second molecule [7]. This allows precise regulation of PAK activation by Rac1 and Cdc42. Binding of one of these GTPases to the GBD domain allows the dimers to dissociate, releasing the kinase domain and the autophosphorylation potential of the protein [8]. Once activated, PAKs have several effectors such as actin [9], myosin [10] and filamin [11] involved in cytoskeleton structure and remodeling, as well as other proteins involved in death signalling [12], gene transcription and cell cycle regulation [13] or other essential cellular pathways.

PAK2 has been shown to be essential for cell adhesion [14], cell survival [15], inflammatory [16] and immune regulation [17], myelination of the peripheral nervous system [18] or even megakaryocytopoiesis [19] among numerous other cellular mechanisms.

Although mutations in *PAK1* [20, 21] and *PAK3* have been shown to cause a neurodevelopmental phenotype (MIM #618158 and MIM #300588 respectively), we still lack a clear understanding of the phenotype associated with *PAK2* pathogenic variants and their functional impact. Two different phenotypes have been described. Some authors suggest loss-of-function variants in the *PAK2* gene are associated with neurological phenotypes [22–24], whereas missense variants located in the kinase domain cause Knobloch syndrome 2 [25–28]. The first description of this syndrome, called Knobloch syndrome 1 (MIM #267750), is linked to *COL18A1* and associates retinal degeneration with encephalocele. There is no respiratory involvement in these cases. While retinal degeneration appears to be almost constantly associated with *PAK2* kinase domain pathogenic variants, a respiratory phenotype also seems to emerge. Some interstitial damages and lymphatic dilatations were described in the first patients with Knobloch syndrome 2 but only one patient has been described with clear antenatal chylothorax [27] and neither pleural effusion nor chylothorax are mentioned in the OMIM phenotypic description (accessed 23/12/2024).

We report a new case of Knobloch syndrome diagnosed in a fetus presenting with severe pleural effusion associated with a *PAK2* likely pathogenic variant in the Kinase domain, adding new evidence for the implication of this gene in fetal pleural effusions with potentially severe respiratory outcomes. We also perform a brief literature review of all the *PAK2*-associated phenotypes to better understand the impact of *PAK2* pathogenic variants.

Case presentation

The mother was referred to the local multidisciplinary prenatal diagnosis center for the first time at 24 weeks of gestation (WG) after ultrasound showed the presence of a fetal bilateral pleural effusion and a moderate cephalic subcutaneous effusion. Fetal growth was normal and there were no other morphologic abnormalities except for a slight excess of amniotic fluid (Single Deepest Vertical Pocket = 70 mm).

It was the first pregnancy and family history was not informative. CMV and B19 parvovirus PCR, karyotype and array-CGH performed on amniotic fluid showed no abnormalities. Echocardiography ruled out cardiogenic involvement in this pleural effusion.

A fetal pleural drain was placed in the pleura at 26 + 6 WG due to the persistence of a large bilateral pleural effusion. This resulted in partial resolution of the effusion. Cytologic analysis of the pleural fluid sample showed an inflammatory fluid with a predominance of lymphocytes and a few macrophages, consistent with a chylothorax. Biochemical analysis showed no increase in triglycerides and no presence of chylomicrons, but the predominance of lymphocytes alone was sufficient to diagnose a congenital chylothorax.

Then a trio exome analysis was performed. Library was prepared by capture thanks to the SureSelect Human

All Exon V8 Agilent protocol. Each sample was then sequenced in 75 bp paired-end reads on an Illumina NextSeq550Dx sequencer. Alignment (GRCh38), annotation and filtering of variants were performed with Alissa Align and Call and Alissa Interpret (Agilent). Variants of interest sequences were visualized thanks to Alamut Visual Plus (Sophia Genetics). Maternal contamination was ruled out with the use of PowerPlex 16 System kit (Promega). Filtering and interpretation of variants were performed on the clinical exome (genes described in human pathology in the OMIM database).

Exome sequencing identified the *de novo* heterozygous likely pathogenic c.836 A > C, p.(Gln279Pro) variant in *PAK2* (NM_002577.4). No pathogenic or likely pathogenic variants were identified in other genes related to intra-uterine pleural effusions or any other genes of interest. The variant is located in the Kinase domain of the protein and cannot be found in gnomAD or any other population databases. It occurs in a highly intolerant position (Metadome 0.14) at a conserved position across species (phastCons 1.00, phyloP 8.80). Most of the in-silico pathogenicity predictors are in favor of a deleterious effect with a CADD phred score of 28,60, SIFT score 0, AlphaMissense 0,999, ClinPred 0,996, REVEL 0,63, Mistic 0,50.

The OMIM description of *PAK2*-associated Knobloch syndrome (MIM #618458) did not match our fetal phenotype. To ensure the variant's pathogenicity, we reviewed all *PAK2* pathogenic variants reported in the literature (Fig. 1) and their associated phenotypes (Table 1). A literature review was conducted in PubMed using the following search equation: (variant OR patient OR pleural)

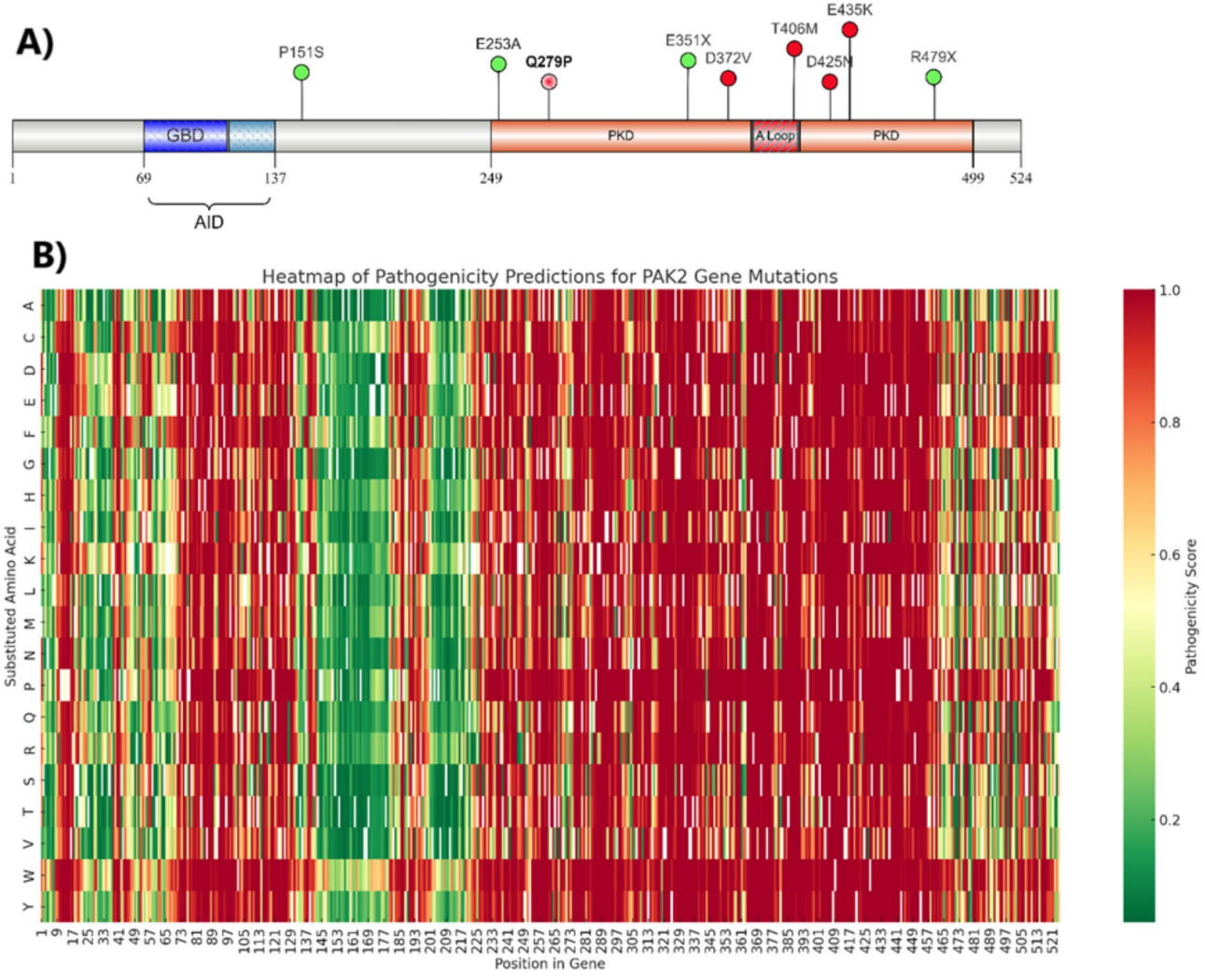

Fig. 1 Schematic representation of *PAK2* functional domains and the variants described in the literature in association with AlphaMissense pathogenicity scores heatmap. **A**) Knobloch syndrome associated variants are represented in red; Neurologic phenotype associated variants are represented in green. GBD: GTPase-Binding Domain; AID: Autoinhibitory Domain; PKD: Protein Kinase Domain; A Loop: Activation Loop. Based on Uniprot and Interpro database. Created thanks to IBS2 2.0 Web Server [30]. **B**) Heatmap generated thanks to ChatGPT 4o with open acces data from AlphaMissense pathogenicity scores database [31]

Table 1 Clinical features of all patients described with a *PAK2* potentially pathogenic variation in the literature

	This study	PAK2 associated Knobloch Syndrome, [25,26,27,28,29]	PAK2 associated Neurologic phenotype [22,23,24]
Genotype	NM_002577.4:c.836 A > C p.(Gln279Pro)	NM_002577.4: c.1303G > A, p.Glu435Lys NM_002577.4:c.1273G > A, p.Asp425Asn NM_002577.4:c.1115 A > T, p.Asp372Val NM_002577.4:c.1217 C > T, p.Thr406Met	NM_002577.4:c.1051G > T, p.Glu351Ter NM_002577:c.758 A > C, p.Glu253Ala NM_002577:c.451 C > T, p.Pro151Ser NM_002577:c.1435 C > T, p.Arg479Ter
De novo	yes	yes (all)	yes (all)
Neurologic defects			
Developmental delay	NA	3/5	1/4
Intellectual deficiency	NA	1/5 (lack of late infancy data)	NA
ASD	NA	1/5	2/4
Other defects	NA	Neural tube defect 2/5	Neural tube defect 2/4
Ophtalmologic defects			No
Retinal detachment	NA	5/5	
Visual impairment	NA	5/5	
Enucleation	NA	2/5	
Pneumologic defects			No
Pleural effusion	Yes (chylothorax)	2/5 (1 chylothorax)	
Interstitial disease	NA	3/5	
Neonatal respiratory distress	Yes	4/5	
Other defects			No
Cardiac defects (ASD, VSD, PDA)	No	4/5	
Purpura fulminans	No	1/5	
Enamel hypoplasia	NA	3/5	
Specific facial features	No	2/5	

AND *PAK2*. We retrieved 5 patients presenting with Knobloch syndrome features and kinase domain missense variants, two siblings from a first clinical description from 1998 [29], one neonate from a 2023 case report [27] and two patients described this year [26, 28]. We also retrieved 4 patients described with non-Kinase domain variants and purely neurological phenotypes such as neural tube defects, neurodevelopmental delay or autism [22–24].

Concerning the Knobloch patients, all the variants occured *de novo* and clustered in the kinase domain of the protein, ranging from aminoacid 249 to 499 (Fig. 1A), in which most of the possible aminoacids changes are predicted to be pathogenic according to the AlphaMissense pathogenicity score (Fig. 1B). The heatmap also shows potential pathogenicity for a lot of variants in the AID but we still have no report of such variants. 3 of the 4 variants already described were functionally tested in vitro and revealed a loss of phosphorylation on Ser141 which is linked to a loss of the kinase function of the protein. All the variants are extremely rare, located at highly conserved position across species and predicted to be pathogenic by various in-silico predictors.

The phenotype review showed that ophthalmic defects are constant. A neurodevelopmental delay seems to occur in some cases and cardiac defects are frequent. We tried to focus on the respiratory phenotype since it was the sole sign we could identify in our case. As we retrieved mention of pleural effusion in two other patients, with one presenting a fetal chylothorax like our fetal presentation, we classified this variant as likely pathogenic with ACMG criteria PS2, PM2_supporting, and PP3.

Despite the potentially poor prognosis associated with these genetic results, the couple decided to go ahead with the pregnancy.

New ultrasound at 33 + 5WG revealed the persistence of a major bilateral pleural effusion. Fetal biometry revealed an intra uterine growth retardation below the 1st percentile. The delivery was induced at 34WG by emergency caesarean section due to fetal heart rate abnormalities.

The newborn was transferred to the neonatal critical care unit and intubated due to refractory hypoxia. Chest X-ray showed major bilateral pulmonary hypoplasia. He had persistent hemodynamic instability with lactic acidosis and pulmonary hypertension despite maximal dose of noradrenaline, milrinone and nitric oxide. In agreement with the parents, a decision to discontinue all active treatments was made and the newborn died at one day of life.

Discussion and conclusions

Although group 1 PAKs have long been known and extensively studied for their multiple roles in numerous essential cellular pathways, their implication in human pathology is still unclear. *PAK1* and *PAK3* are the most

studied and, even if the pathogenic variations reported share molecular similarities with our *PAK2* variants (missenses, clustering in specific domains AID and Kinase), the phenotype associated with these variants is quite different from our *PAK2* kinase domain variations. *PAK1* patients present with neurodevelopmental delay and epilepsy, often associated with brain anomalies and macrocephaly [20] and the transmission is autosomal dominant. The *PAK3* phenotype is the most reported of the three, transmission is X-linked recessive, and it is rather similar to *PAK1*'s with a major difference in head growth since *PAK3* patients tend to show microcephaly [32]. As *PAK1* and *PAK3* are mainly expressed in brain whereas *PAK2* is ubiquitous, it may be understandable that the phenotypes differ and that *PAK2* variants present with non-neurological phenotypes. Another question that remains is the mechanism of these clustered variants in PAKs.

Regarding *PAK1*, some functional studies support a gain of function for two missense variants located in the AID, p.(Tyr131Cys) and p.(Tyr429Cys) [21]. Both destabilize the dimerization of the protein, enhancing the phosphorylation ability of PAK1. For *PAK3*, we have more data that go toward a loss-of-function phenotype. Some missense variants are characterized by a loss of kinase activity [33] while some truncating variants (splice or nonsense) are reported [32]. Finally, some more complex mechanisms combining loss of kinase activity and dominant-negative MAPK deregulating function have also been described [34].

With these data, we can understand how complex it could be to predict the effects of a variation in PAKs domains. Amino acids changes could either impair phosphorylation abilities or impair auto-inhibition leading to loss or gain of functions.

PAK2 is an interesting example of the complexity of these molecular mechanisms associated with PAKs mutations. Data for this gene are even scarcer, but we are starting to accumulate some, as 3 out of the 4 variants already described have been functionally assessed with phosphorylation assays [25, 28], showing a reduced phosphorylation of the Ser141, one of the autophosphorylation sites that allows the protein to exercise its kinase activity on its effectors. This suggests a loss-of-function for these dominant pathogenic variants clustering in the kinase domain. This needs to be balanced as some variants, like p.(Thr402Glu), have rather been shown to activate the catalytic activity of the protein probably due to loss of the autoinhibition. Some variants supposed to cause loss-of-function, like truncating variants, also seem to be associated with purely neurological phenotypes with no other features of Knobloch syndrome [21–24]. This leads to think that the loss of auto-phosphorylation potential of the protein is probably not the only effect of these Kinase domain pathogenic variants and more comprehensive functional studies need to be performed to better understand their pathogenicity.

Two different phenotypes appear to be associated with *PAK2* variants. This literature review confirms the existence of a specific malformative phenotype, Knobloch syndrome 2, including extremely severe ophthalmologic and pulmonary damages in association with *PAK2* kinase domain missense variants. As prenatal pleural effusions are still misunderstood and lack a molecular cause in most of the cases, we wanted to underline the necessity to consider *PAK2* as a potential cause, even in fetuses in whom it may be the sole symptom.

The neurological phenotype seems less clear. Neural tube defects such as encephalocele and meningocele are present in the Knobloch syndrome, and $PAK2^{-/-}$ mice embryos revealed that loss of function of PAK2 was associated with upregulation of the BMP signaling, an essential regulation pathway during neurulation [23]. The authors also showed upregulation of BMP signaling in *PAK2* missenses and splice variants found in fetuses presenting neural tube defects. The missenses were supposed to lead to decrease PAK2 quantity due to enhanced proteasome and autophagy degradation. Interestingly, one of the two missenses (p.Pro151Ser) was located outside of the kinase domain while the other (p.Glu253Ala) was inside it. It would be very interesting to assess the phosphorylation potential of this kinase domain variant. A normal kinase activity could help understand the absence of Knobloch clinical features. It would also suggest that neurological phenotypes, such as neural tube defects, may be more related to a quantitative defect.

Some patients also have a neurodevelopmental phenotype. The mechanisms involved here also seem quite unclear but tend toward a loss of *PAK2* function with truncating mutations associated with autism [22] or language impairment [24]. This is supported by another study showing hypermethylation of *PAK2* in an ASD cohort [35]. Since group 1 PAKs have also been linked to *SHANK3* [36] and their role in the synaptic plasticity is already well known, a role for *PAK2* pathogenic variations in autism disorders could be possible.

In conclusion, pathogenic *PAK2* variations lead to various phenotypes that may sometimes overlap. We still lack data to untangle the molecular and cellular mechanisms explaining it. As loss of PAK2 function seems to be a common finding between these different phenotypes, the explanation could rely in different PAK2 functions impaired. The specific kinase domain impairment seems to lead to Knobloch syndrome while quantitative defects via truncating variants or missenses leading to higher proteolysis could be associated with neurologic defects. More functional studies need to be performed to

better interpret potentially pathogenic variants in *PAK2* in patients presenting compatible phenotypes.

Our case underlines the necessity to consider the pulmonary phenotype, especially in fetuses, with potential severe outcomes at birth, and to investigate *PAK2* variants when facing such prenatal pleural effusion.

As we progress toward a better comprehension of *PAK2*-associated variants, more patients' descriptions are needed for a better refinement of the phenotype since it would facilitate decisions when confronting such a severe phenotype in prenatal presentations where the complete phenotype, especially ophthalmic, is hard to assess.

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Author contributions
LD, HM, CB and CR analyzed and interpreted the genomic patient data. HB performed the obstetrical follow up during the pregnancy. ML was the clinical geneticist of the family. All authors read and approved the final manuscript.

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Data availability
Data concerning the NM_002577.4:c.836 A> C variant are available in ClinVar database (Variation ID: 3362907, Accession: VCV003362907.1). The AlphaMissense dataset used for reproducing PAK2 pathogenicity scores heatmap is available at https://doi.org/10.5281/zenodo.8208688. Extended clinical or sequencing data are available from corresponding authors on reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by our local ethics committee: Comité de Protection des Personnes Bordeaux Outre-Mer III. Written informed consent was received from the patients and the participating families. This study was conducted in accordance with the declaration of Helsinki ethic principles.

Consent for publication
All the participants and parents of minor gave written informed consent for their personal or clinical details along with any identifying images to be published in this study.

Competing interests
The authors declare no competing interests.

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