Expanding the clinical and genetic spectrum of *ALPK3* variants: Phenotypes identified in pediatric cardiomyopathy patients and adults with heterozygous variants

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Introduction Biallelic damaging variants in *ALPK3*, encoding alpha-protein kinase 3, cause pediatric-onset cardiomyopathy with manifestations that are incompletely defined.

Methods and Results We analyzed clinical manifestations of damaging biallelic *ALPK3* variants in 19 pediatric patients, including nine previously published cases. Among these, 11 loss-of-function (LoF) variants, seven compound LoF and deleterious missense variants, and one homozygous deleterious missense variant were identified. Among 18 live-born patients, 8 exhibited neonatal dilated cardiomyopathy (44.4%; 95% CI: 21.5%-69.2%) that subsequently transitioned into ventricular hypertrophy. The majority of patients had extracardiac phenotypes, including contractures, scoliosis, cleft palate, and facial dysmorphisms. We observed no association between variant type or location, disease severity, and/or extracardiac manifestations. Myocardial histopathology showed focal cardiomyocyte hypertrophy, subendocardial fibroelastosis in patients

under 4 years of age, and myofibrillar disarray in adults.

Rare heterozygous *ALPK3* variants were also assessed in adult-onset cardiomyopathy patients. Among 1548 Dutch patients referred for initial genetic analyses, we identified 39 individuals with rare heterozygous *ALPK3* variants (2.5%; 95% CI: 1.8%-3.4%), including 26 missense and 10 LoF variants. Among 149 U.S. patients without pathogenic variants in 83 cardiomyopathy-related genes, we identified six missense and nine LoF *ALPK3* variants (10.1%; 95% CI: 5.7%-16.1%). LoF *ALPK3* variants were increased in comparison to matched controls (Dutch cohort, $P = 1.6 \times 10^{-5}$; U.S. cohort, $P = 2.2 \times 10^{-13}$).

Conclusion Biallelic damaging *ALPK3* variants cause pediatric cardiomyopathy manifested by DCM transitioning to hypertrophy, often with poor contractile function. Additional extracardiac features occur in most patients, including musculoskeletal abnormalities and cleft palate. Heterozygous LoF *ALPK3* variants are enriched in adults with cardiomyopathy and may contribute to their cardiomyopathy. Adults with *ALPK3* LoF variants therefore warrant evaluations for cardiomyopathy. (Am Heart J 2020;225:108-119.)

Pediatric-onset of cardiomyopathy, a disease of the heart muscle causing systolic and/or diastolic dysfunction, is a devastating cause of heart failure in children and the most common indication for heart transplantation in children over 12 months of age.[1] Onset occurs prenatally, at birth, or throughout childhood. Damaging variants in more than 100 genes cause either isolated or syndromic pediatric cardiomyopathy through many different pathological mechanisms.[2-4] *ALPK3* (MIM 617608) is a recently identified pediatric cardiomyopathy gene that encodes alpha-protein kinase 3 (ALPK3), a protein with functions that remain incompletely understood. ALPK3 participates in normal intercalated disc formation and sarcomere organization in both humans and mice.[5-7] *Alpk3*-null mice develop a non-progressive cardiomyopathy characterized by predominantly myocardial hypertrophy and diminished systolic function, as typically occurs in dilated cardiomyopathy (DCM).[6] Cardiomyocytes derived from human-induced pluripotent stem cell (hiPSC-CMs) lacking *ALPK3* display abnormal calcium handling.[7]

We previously reported seven patients from four unrelated consanguineous families with pediatric cardiomyopathy caused by biallelic predicted protein-truncating (loss-of-function, LoF) variants in *ALPK3*.[5,7] Two additional case reports described severe congenital cardiomyopathy including features of both DCM and hypertrophic cardiomyopathy (HCM) from homozygous *ALPK3* LoF variants.[8,9] Extracardiac manifestations have also been observed, including multiple pterygia with skeletal muscle underdevelopment, facial dysmorphisms, and skeletal features.[7-9]

Unlike affected children, the clinical phenotypes of parents and relatives who carry only one damaging *ALPK3* allele are less penetrant. Three of 21 published heterozygous carriers from two families had clinical features of HCM, described as hypertrophy of the interventricular septum,[5,9] whereas other heterozygous carriers had no cardiac disease. It is currently unclear if

these observations indicate that damaging *ALPK3* variants contribute to unexplained cardiomyopathy or modify cardiomyopathy that is caused by a pathogenic or likely pathogenic variant in an established disease gene. To address these issues, we delineated the clinical and genetic spectrum of patients with damaging biallelic *ALPK3* variants and defined the prevalence of heterozygous *ALPK3* variants in two cohorts with adult-onset cardiomyopathy.

Methods
Patient recruitment
Our study was carried out in collaboration with clinicians from seven different countries and institutions. Mutation analysis was performed using next-generation sequencing (NGS), either whole exome sequencing or targeted gene panels. Details on sequencing methods and data analysis are available in the **Data Supplement**. We reviewed clinical data of 19 patients with biallelic variants in *ALPK3* (NM_020778.4), including nine previously reported patients.[5,7-9] HCM was defined as increased ventricular wall thickness (end diastolic wall thickness: z-score ≥ 2) not solely explained by abnormal loading or structural heart conditions such as valve disease, congenital heart disease, or hypertension. DCM was defined as ventricular dilation (LV end-diastolic dimension >2 SD above mean for body surface area) and systolic dysfunction (fractional shortening or LV ejection fraction >2 SD below mean for age) in the absence of abnormal loading conditions.[10,11] Chromosomal analysis was performed in all index patients with pediatric-onset cardiomyopathy—except patient F10P1. The Medical Research Ethical Committees of the University Medical Center Groningen, the Erasmus University Medical Center, Brigham and Women's Hospital, and Boston Children's Hospital approved this study. Informed consent was obtained from all participants or their legal guardians.

Variant interpretation

The pathogenicity of variants was assessed using Alamut Visual software (Interactive Biosoftware, Rouen, France), a gene browser that integrates missense prediction tools (Align GVGD, SIFT, MutationTaster, PolyPhen-2), allele frequencies from different population databases (gnomAD,[12] ESP, GoNL[13]) and disease-specific databases (HGMD, ClinVar, LOVD) and mRNA splicing prediction tools (SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, and Branch Points). A potential splice effect was defined as a difference between reference and mutated scores greater than 10% reported by three or more of five mRNA splicing prediction tools. Deleteriousness of variants was scored using combined annotation dependent depletion (CADD).[14] A scaled CADD score of 10, 20, or 30 indicates the top 10%, 1%, and 0.1% most deleterious substitutions in the human genome, respectively. Variants were interpreted according to the 2015 ACMG guidelines.[15] Variants with a minor allele frequency (MAF) <0.1% in the Genome Aggregation Database (gnomAD) dataset (considering total population and major subpopulations) were considered rare. Nonsense and frameshift variants were considered null variants, with the exception of those residing in the last exon or the last 50 base pairs of the penultimate exon.

Protein multiple sequence alignments

We constructed ALPK3 multiple sequence alignments over a fixed phylogeny of species: human, Hominidae (*Pan troglodytes*), Glires (*Mus musculus*), Laurasiatheria (*Bos taurus*), Marsupialia (*Sarcophilus harrisii*), Aves (*Anas platyrhynchos*), and Teleostei (*Xiphophorus maculatus, Danio rerio*). Sequences were aligned using T-Coffee.

Histology

Paraffin-embedded or frozen cardiac tissue was available for two affected individuals (patient 1 from family 1 (F1P1) and patient 2 from family 2 (F2P2); pedigrees 1 and 2, **Data Supplement**). In addition, we collected muscle biopsy specimens from the lateral portion of the quadriceps femoris muscle from patient 3 from family 2 (F2P3; pedigree 2, **Data Supplement**) and her healthy sister (pedigree 2, **Data Supplement**) and from the spine, taken at scoliosis surgery, from patient 1 from family 3 (F3P1; pedigree 3, **Data Supplement**). Tissues from age-matched donors were used as controls. All samples were histologically examined after hematoxylin and eosin staining using standard techniques. Samples from patient F3P1 were also examined by electron microscopy.

Cohort screening

ALPK3 was evaluated as part of a targeted NGS panel (gene panels B, E or F, **Supplementary Table II**) in 1548 patients (suspected of) having cardiomyopathy who were referred for diagnostic genetic testing at two molecular diagnostic laboratories in the Netherlands between December 2015 and July 2018. *ALPK3* was also evaluated from whole exome sequence data obtained for 149 unrelated U.S. patients of European ancestry who had clinically diagnosed HCM or DCM. In each of these patients, prior NGS analyses of 83 established or putative cardiomyopathy genes (gene panel G, **Supplementary Table II**) had excluded a pathogenic or likely pathogenic variant. Co-segregation analysis was performed for available family members in both of these cohorts.

Haplotype analysis

To investigate whether the recurrent c.4736-1G > A, p. (Val1579Glyfs*30) variant originated from a single mutational event, haplotype analysis was performed using 13 microsatellite markers surrounding *ALPK3*. DNA from six probands (one homozygous carrier (F1P1) and five heterozygous carriers) was analyzed, and DNA samples of three family members of F1P1 who carry the variant were used to verify the phase and reconstruct the haplotype.

Statistical analysis

Sequence data of 64,000 unrelated Non-Finnish Europeans (NFE, assembled by gnomAD) were used as an independent control dataset. Raw data (version 2.1) were downloaded and filtered on PASS quality status. *ALPK3* variants with a MAF >0.1% (2000 alleles) were excluded. The calculation of burden for LoF variants in cardiomyopathy cohorts and gnomAD subjects excluded LoF

Table I. Characteristics of 19 patients with biallelic *ALPK3* variants.

	Proportion	%
Male	9/19	47%
Age of onset of CMP		
Prenatal	4/19	21%
<1 year	10/19	52%
1-18 years	3/19	16%
18+ year	2/19	11%
Mutation type		
LoF/LoF	11/19	58%
LoF/missense	7/19	37%
Missense/missense	1/19	0.5%
Progression DCM to LVH	8/18	44%
Prolonged QTc	13/16	81%
Endpoint		
ICD	4/19	21%
HTx	2/19	11%
Death	4/19	21%
Short stature	9/15	60%
Kyphoscoliosis	6/15	40%
Webbed neck	8/17	47%
Joint contractures	8/19	42%
Cleft palate/VPI	8/18	44%

CMP = cardiomyopathy; DCM = dilated cardiomyopathy; LVH = left ventricular hypertrophy; LoF = loss-of-function; ICD = implantable cardioverter defibrillator; HTx = heart transplantation; VPI = velopharyngeal insufficiency.

variants in the last exon of *ALPK3*. The prevalences of *ALPK3* variants were expressed as proportions (exact 95% binomial confidence intervals [CI]). Differences in prevalence rates between cohorts were estimated as the risk difference with the exact 95% confidence interval of the risk difference and statistically compared using a one-tailed binomial test. Values of $P <.05$ were considered significant.

Results

Identification of *ALPK3* sequence variants

All nine previously reported patients carried biallelic LoF variants in the *ALPK3* gene. Of the 10 new patients described here, two carried biallelic *ALPK3* LoF variants: patient F7P3 (a distant relative of F7P1 and F7P2) carried c.1018C > T, p.(Gln340*) and c.4332delC, p.(Lys1445Argfs*29) and patient F12P1 was homozygous for c.3418C > T, p.(Gln1140*). Seven patients (F7P1, F7P2, F8P1, F9P1, F10P1, F10P2 and F11P1) had compound heterozygous LoF and missense *ALPK3* variants (Tables I and II and **Supplementary Table I**). Patient F13P1 carried a homozygous missense variant, c.5155G > C, p.(Ala1719Pro), which alters an alanine residue within the alpha-kinase domain. The Ala1719Pro substitution was absent in public exome databases and is predicted to be damaging by SIFT and PolyPhen-2.

Identified *ALPK3* variants did not cluster (Figure 1). The alpha-kinase domain has high sequence identity among *ALPK* family members and is required for phosphate modification of other proteins, a fundamental process involved in most signaling and regulatory processes within eukaryotic cells.[16] No additional likely pathogenic or pathogenic variants in genes associated with cardiomyopathy, including *TTN*, nor any pathogenic copy number variants explaining their cardiomyopathy were identified in any of the patients with biallelic *ALPK3* variants (**Data Supplement and Supplementary Table II**). All but two *ALPK3* missense variants had high CADD scores (> 20; Table II), and most of the novel amino acids substituted residues are highly conserved across species (**Data Supplement**). In contrast, the p.(Val812Met) variant in F7P1 and F7P2 had a low CADD score and showed conflicting predictions of pathogenicity. However, the phenotype of both siblings carrying this variant (and a LoF variant on the other allele) showed striking similarities with other patients with biallelic *ALPK3* variants. The p.(Glu199Asp) variant identified in patients F10P1 and F10P2 had a low CADD score and is also a conservative amino acid substitution that may not impact secondary protein structure given the similar properties of these residues (Grantham score: 45). While this missense variant altered a residue within a region of poor sequence alignment, thereby limiting assessment of evolutionary conservation, the variant is absent from gnomAD. Based on the shared phenotype exhibited by F10P1, F10P2, and other carriers of biallelic *ALPK3* variants, we suggest that p.(Glu199Asp) is also damaging. However, functional studies should be carried out to further support our hypothesis.

Clinical features of patients with biallelic *ALPK3* sequence variants

Table I and **Supplementary Tables I and III** provide clinical summary data for the 19 patients (9 male), and additional descriptions are provided in the **Data Supplement**. The age at diagnosis of cardiomyopathy ranged from 20 weeks of gestation to 53 years; two patients were diagnosed after the age of 18 years. Among patients with biallelic LoF variants, median age at diagnosis was 7 days (range: 20 weeks gestation to 9 years). Median age at diagnosis in patients carrying a LoF and a missense variant was 3 months; two of them were diagnosed as adults (range birth to 53 years). Patient F13P1, harboring two missense variants, presented with cardiomyopathy at 35 weeks of gestation.

Prenatal findings were available for 16 patients and included increased nuchal folds and/or fetal hydrops in six patients (37.5%; 95% CI: 15.2%-64.6%). Eight of 18 live-born patients (44.4%; 95% CI: 21.5%-69.2%), including four with a LoF and a missense variant, showed left ventricular or biventricular DCM in the neonatal period that transitioned to ventricular hypertrophy at a later stage (Figure 2A-B and Figure 3). Three patients who died

Table II. Overview of previously published (F1-F6) and novel biallelic variants in *ALPK3*.

| Patient	Nucleotide change	Protein change	Location	gnomAD	Splice prediction	SIFT	PolyPhen-2	CADD
F1P1 (homozygous)	c.4736-1G > A	p.(Val1579Glyfs*30)	intron 9	4/273120	Loss acceptor site	NA	NA	34
F2P1, F2P2, F2P3 (homozygous)	c.3781C > T	p.(Arg1261*)	exon 6	8/235216	No effect	NA	NA	35
F3P1 (homozygous)	c.5294G > A	p.(Trp1765*)	exon 12	Absent	No effect	NA	NA	46
F4P1, F4P2 (homozygous)	c.3792G > A	p.(Trp1264*)	exon 6	Absent	No effect	NA	NA	35
F5P1 (homozygous), F11P1 (comp. het.)	c.2023delC	p.(Gln675Serfs*30)	exon 5	5/238584	No effect	NA	NA	NA
F6P1 (homozygous)	c.1531_1532delAA	p.(Lys511Argfs*12)	exon 5	Absent	No effect	NA	NA	NA
F7P1, F7P2, F7P3 (comp. het.)	c.1018C > T	p.(Gln340*)	exon 4	Absent	No effect	NA	NA	38
F7P1, F7P2 (comp. het.)	c.2434G > A	p.(Val812Met)	exon 6	5/277136	No effect	Tolerated	Probably damaging	2.206
F7P3 (comp. het.)	c.4332delC	p.(Lys1445Argfs*29)	exon 6	Absent	No effect	NA	NA	38
F8P1 (comp. het.)	c.541delG	p.(Ala181Profs*130)	exon 1	5/29436	No effect	NA	NA	NA
F8P1 (comp. het.)	c.3439C > T	p.(Arg1147Trp)	exon 6	15/243424	No effect	Deleterious	Probably damaging	19.77
F9P1 (comp. het.)	c.4997delA	p.(Asn1666Thrfs*14)	exon 10	1/245986	No effect	NA	NA	NA
F9P1 (comp. het.)	c.4091G > C	p.(Gly1364Ala)	exon 6	17/269654	No effect	Deleterious	Probably damaging	26.9
F10P1, F10P2 (comp. het.)	c.5105+ 5G > C	p.(?)	intron 11	Absent	Loss donor site	NA	NA	20.2
F10P1, F10P2 (comp. het.)	c.597G > T	p.(Glu199Asp)	exon 1	Absent	No effect	Tolerated	Benign	11.42
F11P1 (comp. het.)	c.4888G > T	p.(Val1630Phe)	exon 10	Absent	No effect	Deleterious	Probably damaging	29.6
F12P1 (homozygous)	c.3418C > T	p.(Gln1140*)	exon 6	Absent	Loss cryptic donor site	NA	NA	33
F13P1 (homozygous)	c.5155G > C	p.(Ala1719Pro)	exon 12	Absent	No effect	Deleterious	Probably damaging	29.5

*CADD = Combined Annotation Dependent Depletion v1.4; comp. het. = compound heterozygous; gnomAD = Genome Aggregation Database v2.0; NA = not available; PolyPhen-2 = Polymorphism Phenotyping v2; SIFT = Sorting tolerant from intolerant. For variant annotation, human genome assembly GRCh37/hg19 and RefSeq NM_020778.4 was used.

in the neonatal period exhibited DCM or presented with a mixed phenotype of hypertrophy and DCM. Patient F13P1 showed marked changes in cardiac morphology, presenting with mild to moderate biventricular hypertrophy at birth that rapidly progressed to biventricular dilated ventricles without hypertrophy. At age 2 years, the DCM had again transitioned to left ventricular hypertrophy (LVH). Among 16 surviving patients (age >2 years), all (except patient F8P1) had LVH (93.8%; 95% CI: 69.8%-99.8%), and eight patients (age >11 months) also had right ventricular hypertrophy (50%; 95% CI: 24.7%-75.3%). Imaging studies showed progressive LVH in seven of 13 patients (53.8%; 95% CI: 25.1%-80.8%). Echocardiography of patient F4P1 demonstrated features of left ventricular noncompaction (LVNC), and patients F7P1 and F12P1 exhibited LVNC in association with LVH. Patient F9P1 was diagnosed with HCM at 53 years of age based on the finding of midventricular hypertrophy, a morphology that was also observed in three heterozygous carriers in family 3 (pedigree 3 in the **Data Supplement**).

None of the patients with biallelic variants had a structural heart defect (0/18; 95% CI: 0.0%-18.5%).

Electrocardiograms were available for 16 patients and showed ventricular voltages consistent with biventricular hypertrophy or LVH, repolarization abnormalities (inferolateral ST depression), and prolonged QT intervals likely due to LVH (Figure 2D and **Supplementary Table III**). A short PR-interval was noted in two siblings, as is seen in Pompe disease (12.5%; 95% CI: 1.6%-38.3%) (Figure 2C). Rhythm and conduction disorders occurred in seven patients (43.8%; 95% CI:19.8%-70.1%). These included supraventricular tachycardia (n = 2), nonsustained ventricular tachycardia (n = 2), premature ventricular contractions (n = 1), ventricular fibrillation (n = 2), intraventricular conduction delay (n = 1), and second-degree atrioventricular block (n = 1). Four patients received an implantable cardioverter defibrillator (25%; 95% CI: 7.3%-52.4%), and two had a heart transplant at the ages 4 and 28 years, respectively (12.5%; 95% CI:1.6%-38.3%).

Figure 1
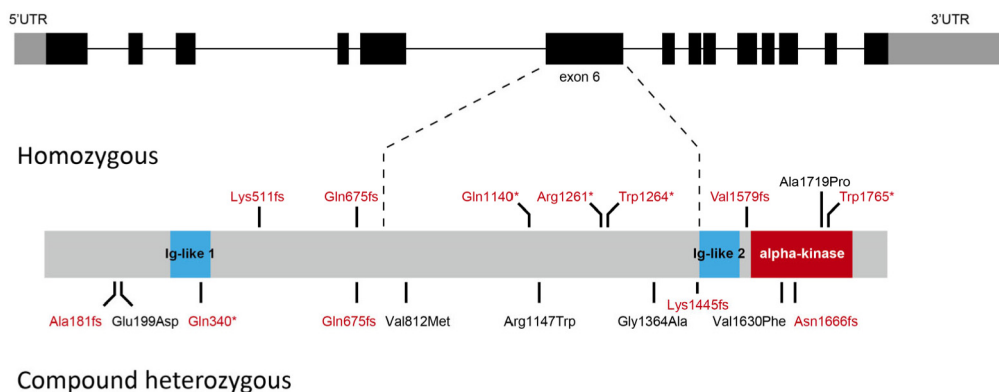
Schematic representation of the structure of *ALPK3* gene (top) and protein and location of disease-associated variants.
The *ALPK3* gene is located on chromosome 15q25 and encodes a member of a superfamily of protein kinases. ALPK3 contains three domains: an alpha-type protein kinase domain and two Ig-like domains. Homozygous variants are displayed on the top of the diagram. Compound heterozygous variants are displayed on the bottom of the diagram. Premature stop codon–introducing variants are indicated in red.

A wide spectrum of extracardiac features (excluding hydrops) was observed in 16 of 18 (88.9%; 95% CI: 65.3%-98.6%) live-born patients with damaging biallelic *ALPK3* variants. At birth all patients were at normal size for their gestational age, but their subsequent growth was delayed. The height of 9/15 patients (60%; 95% CI: 32.3%-83.7%) ranged from 2 to 6 SDs below the normal mean.

Musculoskeletal abnormalities were observed in 11/18 patients (61.1%; 95% CI: 35.7%-82.7%), including severe scoliosis (n = 6) (Figure 4A), webbed neck (n = 8) (Figure 4B), knee and/or shoulder contractures (n = 5), camptodactyly/arthrogryposis (n = 6) (Figure 4C), and spondylolysis (n = 2). Five patients had congenital contractures, while one patient developed contractures and scoliosis later in life. Four of 12 patients (33.3%; 95% CI: 9.9%-65.1%) had delayed motor development with independent walking at ages 18 to 32 months, and three of these children also had a speech delay. Patient F3P1, now aged 14 years, has a learning disorder (nonverbal IQ 74). Hypotonia was present in 4/13 patients (30.8%; 95% CI: 9.1%-61.4%). Cleft palate or velopharyngeal insufficiency occurred in 8/18 patients (44.4%; 95% CI: 21.5%-69.2%). Craniofacial dysmorphic features were present in at least 12/17 patients (70.6%; 95% CI: 44.0%-89.7%), including hypertelorism, ptosis, ankyloglossia, intra-oral pterygia, micrognathia, and low-set ears (Figure 4B). At least 3/12 patients (25.0%; 95% CI: 5.5%-57.2%) had abnormal glucose metabolism. We observed no significant association between the variant type or location and the severity of extracardiac phenotypes.

Clinical features of patients' relatives with heterozygous *ALPK3* variants

Among previously published families, five heterozygous carriers of an *ALPK3* LoF allele showed LVH: three members of family 3 exhibited midventricular hypertrophy at ages 27, 29, and 64, respectively; the father of patient F5P1 was diagnosed with HCM at age 30; and the father of patient F6P1 had asymmetric hypertrophy of the interventricular septum (5/22; 22.7%; 95% CI: 7.8%-45.4%). Cardiac evaluations were normal in 17 other clinically evaluated relatives with heterozygous LoF *ALPK3* variants (17/22; 77.3%; 95% CI: 54.6%-92.2%). Among our newly studied families, none of 20 obligatory heterozygous carriers of a damaging *ALPK3* variant have cardiomyopathy or extracardiac abnormalities (0%; 95% CI: 0.0%-16.8%). As the prevalence of unexplained LVH in the general population is 0.10%,[17] finding LVH in five of 37 (13.5%; 95% CI: 4.5%-28.8%) heterozygous *ALPK3* LoF carriers is unexpected (difference, 13.4 percentage points; 95% CI: 4.4-28.7; $P = 4.2\times10^{-10}$). Although we cannot fully exclude a shared inherited modifier or private mutations in yet unknown HCM genes in these families as an explanation for the LVH, this observation suggests a causal relationship between carrying a heterozygous *ALPK3* LoF variant and LVH.

Histopathologic examination

Post-mortem microscopic examination of myocardial tissue showed (sub)endocardial fibro-elastosis in patients F1P1, F2P1, F5P2, and F5P3. At the DCM stage, no myofiber disarray was observed (patient F1P1). Histopathology of patient F2P1, who had both ventricular

Figure 2
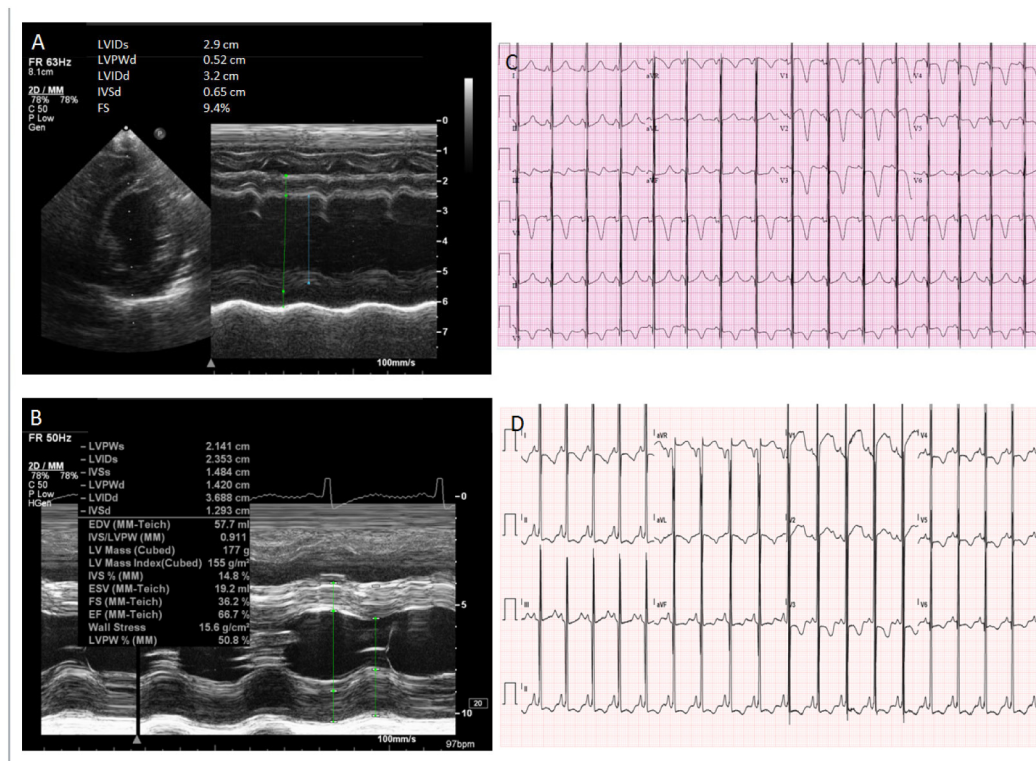
Echocardiographic images and ECGs. Cardiac ultrasounds of patient F10P1: (A) initial presentation with decreased function (shortening fraction 9.4%) and (B) at 11 years of age showing shortening fraction of 36.2%. Initial/final z-scores for IVSd and LVPWd are 2.4/4.7 and 2.64/7.2, respectively. C, ECG of patient F10P1 at age 11 years showing short PR interval (94 ms), short QRS duration (62 ms), marked left ventricular hypertrophy with repolarization abnormality, and prolonged QTc (497 ms), which may be due to QRS abnormality. D, ECG of patient F13P1 at age 3.5 years showing atrial enlargement, marked biventricular hypertrophy, repolarization abnormalities, and prolonged QTc, which is likely secondary to LVH.

dilation and hypertrophy, showed focal cardiomyocyte hypertrophy without myofiber disarray. Patients F7P2 and F7P3 underwent cardiac biopsy at age 4 years and 28 years, respectively, when their DCM progressed to biventricular hypertrophy. Cardiac histopathology of patient F7P3 showed cardiomyocyte hypertrophy with myofiber disarray. A spinal muscle biopsy of patient F3P1 taken at scoliosis surgery showed variation in fiber size, fiber splitting, and numerous central cores (Figure 5A and B). However, subsequent examination of the quadriceps muscle of the same patient did not show any ultrastructural abnormalities (Figure 5C).

Burden of heterozygous LoF *ALPK3* variants identified in patients with adult-onset cardiomyopathy

We assessed the prevalence of *ALPK3* variants in two independent cardiomyopathy cohorts. The Dutch cohort comprised 1548 index patients with predominantly adult-onset cardiomyopathy referred for clinical genetic testing. None had biallelic damaging *ALPK3* variants,

but 24 rare (MAF <0.1%) heterozygous *ALPK3* variants were identified in 39 patients (2.5%; 95% CI: 1.8%-3.4%), including four LoF variants (three frameshift and one splice site variant resulting in exon 10 skipping), 18 missense variants, one stop gain variant in the last exon, and one synonymous variant with a predicted effect on splicing (**Supplementary Table IV**). Ten variants recurred in more than one patient. The heterozygous c.4736-1G > A, p.(Val1579Glyfs*30) variant initially observed in the unaffected parents and sister of patient F1P1 also occurred in five adult cardiomyopathy probands (P025-P029) and four of 273,120 alleles (0.0015%) in gnomAD. A shared haplotype consisting of 5 of 13 polymorphisms located within 2.33 Mb flanking *ALPK3* was identified in eight individuals from five families, suggesting a common founder in the Dutch population (**Supplementary Table V**).

Patients with heterozygous *ALPK3* variants (**Supplementary Table IV**) had the following clinical diagnoses: HCM (13 missense variants, 7 LoF variants, 2 stop gain

Figure 3
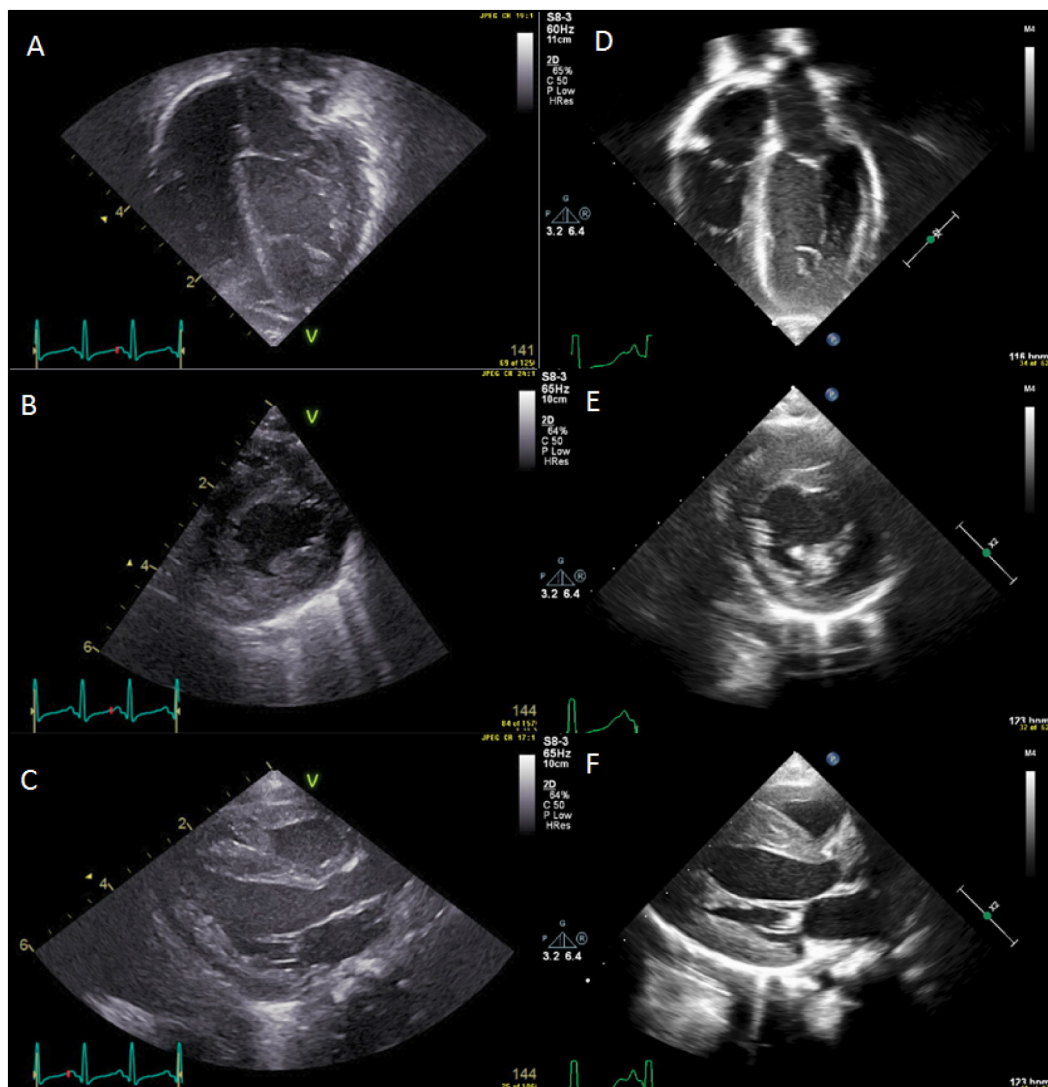
Transthoracic echocardiography images of patient F13P1. A-C, Echocardiographic images at DCM stage with very poor systolic function—age 12 days. (A, systolic 4-chamber view; B, short axis view; C, parasternal long axis view). D-F, Echocardiographic images of patient F13P1 at HCM stage—age 4 years (D, 4-chamber view; E, short axis view; F, parasternal long axis view).

variants in the last exon, and one synonymous variant predicted to affect splicing), DCM (6 missense, 2 LoF), arrhythmogenic cardiomyopathy (ACM, 3 missense, 1 LoF), LVNC (1 missense), mixed/unspecified cardiomy-opathy (2 missense), and one sudden cardiac death with unknown cardiac disease (1 missense). Seven of these patients (17.9%; 95% CI: 7.5%-33.5%) also had a likely pathogenic or pathogenic variant in another cardiomyopathy gene (**Supplementary Table VI**): *MYBPC3* ($n = 4$), *MYH7* ($n = 1$), *TNNI3* ($n = 1$), and *LMNA* ($n = 1$).

The frequency of *ALPK3* LoF variants in the general population approximates the expected frequency, when accounting for protein size (pLI = 0.00; gnomAD[12]), which implies that one null *ALPK3* allele is tolerated. The gnomAD dataset reports 2149 rare (MAF <0.1%) missense or LoF *ALPK3* alleles among ~64,000 NFE (3.4%; 95% CI: 3.2%-3.5%) compared to 38 in 1548 Dutch cardiomy-opathy patients (2.5%; 95% CI: 1.7%-3.4%) (**Supplementary Table IV**; difference, 0.91 percentage points; 95% CI: −2.09%-1.63%; $P = 0.024$). In contrast, we observed significantly more LoF *ALPK3* alleles in Dutch

Figure 4

Extracardiac features in patients with biallelic *ALPK3* variants. A, Anteroposterior X-ray demonstrating S-shaped scoliosis of the thoracic and lumbar spine of patient F3P1. Note: cardiomegaly and implantable cardiac defibrillator in situ. B, Faces of patients F7P1, F11P1, and F13P1. C, Distal arthrogryposis in patient F13P1: bilateral absent flexion creases of dig. V and congenital contractures of dig. I, II and V of the left hand and dig. V of the right hand.

cardiomyopathy subjects (10/1548; 0.65%; 95% CI: 0.3%-1.2%) than in NFE (73/64,000; 0.11%; 95% CI: 0.1%-0.1%; difference, 0.54 percentage points; 95% CI: 0.20-1.07; $P = 1.6 \times 10^{-5}$).

The second cohort comprised 149 unrelated cardiomyopathy patients (HCM, $n = 129$; DCM, $n = 20$) of European ancestry from the United States. Previous genetic analyses in these patients had excluded a pathogenic or likely pathogenic variant in 83 cardiomyopathy genes. Analyses of exome sequencing data identified 15 rare (MAF< 0.1%) *ALPK3* protein–altering variants (15/149; 10.1%; 95% CI: 5.7%-16.1%) (**Supplementary Table IV**): 14 in HCM patients (8 LoF and 6 missense variants) and one in a DCM patient (a

LoF variant). No patients had biallelic variants and none of the variants recurred in this cohort. Similar to what we observed in the Dutch cohort, the proportion of *ALPK3* LoF variants in the U.S. cardiomyopathy cohort (9/149; 6.0%; 95% CI: 2.8%-11.2%) was significantly higher than in gnomAD NFE (73/64,000; 0.11%; 95% CI: 0.09%-0.14%; difference, 5.93 percentage points; 95% CI: 2.69-11.05; $P = 2.2 \times 10^{-13}$).

Discussion

The clinical manifestations of biallelic and heterozygous *ALPK3* variants are quite distinct. Among 19 patients

Figure 5
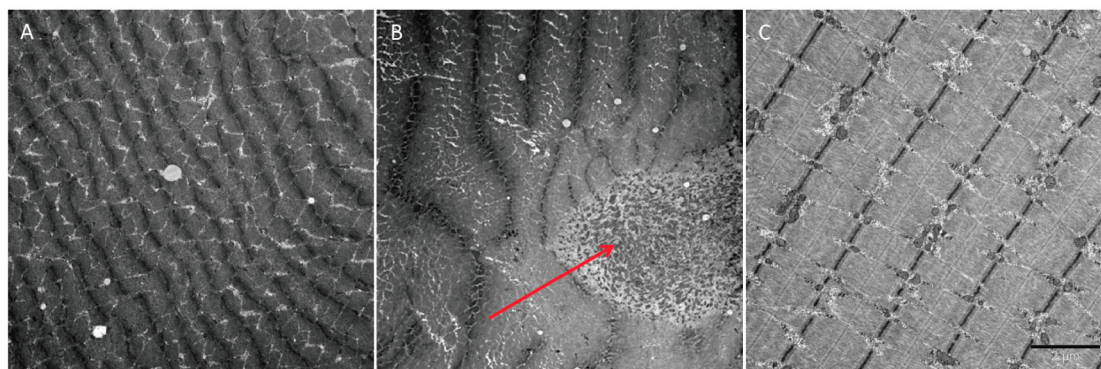
Histopathologic examination of skeletal tissue. A, Electron microscopic (EM) examination of spinal muscle from patient F3P1: relatively unaffected region (4000×). B, Central core (2200×, arrowhead). C, EM of quadriceps muscle sarcomeres from F3P1 showing regular arrangement of contractile protein filaments (8900×).

with biallelic damaging *ALPK3* homozygous or compound heterozygous variants, 17 patients presented with pediatric-onset cardiomyopathy. Strikingly, most cases presented initially with DCM that then transitioned to ventricular hypertrophy with reduced systolic performance—an unusual clinical sequence. *ALPK3* cardiomyopathy often progressed rapidly and six patients died or underwent cardiac transplantation. Most patients had extracardiac manifestations, including craniofacial and musculoskeletal abnormalities, but these were not sufficiently consistent to delineate a recognizable syndrome.

We report, for the first time, biallelic missense variants that cause pediatric-onset cardiomyopathy. The clinical manifestations associated with these variants were similar to those associated with other damaging *ALPK3* variants. These missense variants could result in a conformational change that affects protein folding or flexibility, protein-protein or protein-DNA interaction, or the activity of the alpha-kinase domain. Unfortunately, no 3D structure of ALPK3 is available to predict the consequence of the missense variants. In addition, we demonstrated higher than expected frequencies of heterozygous *ALPK3* LoF variants among adult cardiomyopathy patients in a Dutch and a U.S. cohort. Thirty-seven of these patients were clinically diagnosed with HCM, which is likely related to the hypertrophy phenotype observed in pediatric patients with biallelic *ALPK3* variants. Despite this similarity, there were other notable differences between the clinical features associated with monoallelic and biallelic *ALPK3* cardiomyopathy, including absence or undetected extracardiac phenotypes. Whether these differences reflect graded dose-responses to ALPK3 deficits or distinct mechanisms by which monoallelic or biallelic variants cause disease remains unknown.

Biallelic *ALPK3* variants were associated with a range of morphological and functional abnormalities. Almost half of the live-born pediatric patients presented with DCM that later evolved into ventricular hypertrophy. Three individuals initially displayed a mixed cardiomyopathy with features of both DCM and ventricular hypertrophy that evolved into concentric hypertrophy of both left and right ventricles.[9] This hypertrophic phenotype differs from classic HCM caused by pathogenic variants in genes encoding sarcomere proteins. Notably, hypertrophy was atypical, often biventricular and/or concentric, or apical in distribution. LV dilatation occurred in some pediatric patients, which occurs rarely in HCM and usually decades after diagnosis, with accompanying decrease in systolic performance.[18,19] Like other pediatric cardiomyopathies, *ALPK3* cardiomyopathy can present with features of more than one subtype.[20,21] However, transition from DCM to LVH has not been described before and appears to be unique to biallelic *ALPK3* cardiomyopathy.

The histopathology of biallelic *ALPK3* cardiomyopathy has some features observed in classic HCM,[22] including focal cardiomyocyte hypertrophy, interstitial fibrosis and, at adult age, myofiber disarray. Whether this histopathology precedes the progression to hypertrophy remains unclear. Patients with biallelic variants in *ALPK3* display a variety of rhythm and conduction disturbances reminiscent of those seen in arrhythmogenic cardiomyopathy. We previously showed a reduced plakoglobin signal at intercalated disks of patients with biallelic *ALPK3* variants.[5] A reduced plakoglobin signal has also been documented in ACM[23]; this redistribution of plakoglobin from the junctional pool to the intracellular and nuclear pools likely suppresses the canonical Wnt/beta-catenin signaling, leading to enhanced fibrogenesis and myocyte

apoptosis. ACM and *ALPK3* cardiomyopathy may share the same pathophysiological mechanisms, thus explaining the arrhythmogenic phenotype in patients with biallelic *ALPK3* variants. Alternatively, the rhythm disorders observed in *ALPK3* cardiomyopathy may be secondary to progressive disease.

We observed no association between extracardiac manifestations and allelic heterogeneity: biallelic missense or LoF variants seemed to cause similar phenotypes. The majority of patients with biallelic *ALPK3* variants, including those with one or two missense variants, had musculoskeletal involvement, including contractures and severe progressive scoliosis. Several patients had cleft palate, velopharyngeal insufficiency, and/or facial dysmorphisms. Jaouadi et al also described a patient with a diversified phenotype, including cleft palate, pectus excavatum, bilateral clinodactyly, and facial dysmorphic features like broad forehead, downslanting palpebral fissures, mild ptosis, and low-set posteriorly rotated ears, which fits with the extracardiac features we observed in our cohort.[9] While we cannot exclude that genome-wide inbreeding contributed to the extracardiac features seen in patients with homozygous *ALPK3* variants, their occurrence in multiple unrelated patients with different allelic variants and genetic backgrounds suggests direct effects of *ALPK3* variants.

The expression of *ALPK3* helps to explain these extracardiac phenotypes. The prevalence of skeletal muscle phenotypes in pediatric patients likely reflects *ALPK3* expression in developing skeletal and heart muscle[6,24] and in adult skeletal, smooth, and heart muscles (GTEx (https://commonfund.nih.gov/gtex)). In embryonic mice (E8.5), *Alpk3* expression is detectable around the first branchial arch,[24] which may account for palatal abnormalities. Further support for the syndromic nature of *ALPK3*-related disease arises from GeneNetwork Assisted Diagnostic Optimization (GADO), a method that exploits RNA-seq data from a range of tissues and cell types and uses gene co-regulation to predict gene functions.[25] For *ALPK3*, GADO predicts "muscle contraction" and "myogenesis" as the top phenotypes. Based on a combination of the major shared phenotypic abnormalities in our patients, GADO ranked *ALPK3* in the top 1% of all coding and non-coding human genes (P = .000432) (Supplementary Table VII).

Pathogenicity of heterozygous *ALPK3* variants

Among 37 relatives with a heterozygous LoF variant in *ALPK3* (three ≤18 years of age), five (13.5%) were diagnosed with HCM as adults. In line with this finding, we note that gnomAD reports fewer *ALPK3* LoF variants (transcript NM_020778.4 (ENST00000258888)) than would be expected if these were to occur randomly, despite a pLI = 0.00. While the observed and expected differences are not statistically significant, we suggest that some individuals with *ALPK3* LoF may have an undetected mild or late-onset cardiomyopathy.

To better understand the role of *ALPK3* variants in adult-onset cardiomyopathy, we analyzed two independent patient cohorts. In both cohorts, the frequency of *ALPK3* LoF variants was significantly higher than in the general population. Together these findings provide compelling evidence that *ALPK3* plays important roles in cardiac function and pathologic remodeling. Further evidence for the role of *ALPK3* in cardiac hypertrophy arises from a genome-wide association meta-analysis that identified a novel locus at chromosome 15q25.3, which encompasses *ALPK3* and is strongly associated with two clinically used QRS traits (Cornell and 12-lead sum), reflecting a higher LV myocardial mass. One of the lead SNPs in this GWAS is in strong linkage disequilibrium with two nonsynonymous SNPs in *ALPK3* (P = 9.94e-18).[26] Basic studies are now needed to understand the targets and pathways in which this kinase participates.

Limitations

The majority of pediatric patients with biallelic damaging *ALPK3* variants were characterized in the context of clinical care, and medical examinations, imaging, and other laboratory studies were not consistently obtained across all patients. Phenotypes of heterozygous first-degree relatives from cardiac screening, interviews, and/or medical records were not systematically obtained. The mean coverage of *ALPK3* sequencing data in gnomAD is much lower than in our cohorts, particularly for distal exon 1 sequences and exon 6. Therefore, the number of variants reported in gnomAD may be an underrepresentation.

Conclusions

Our study reinforces the role of *ALPK3* in pediatric cardiomyopathy, and we describe a unique cardiac phenotype with progression of DCM to ventricular hypertrophy as a major feature of *ALPK3*-related disease. We further show that biallelic variants in *ALPK3* can cause a syndromic form of cardiomyopathy with musculoskeletal features as well as craniofacial abnormalities. We also demonstrate an increased burden of heterozygous *ALPK3* LoF variants in two adult-onset cardiomyopathy cohorts. Further study is needed to establish the pathogenicity of heterozygous *ALPK3* variants.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ahj.2020.03.023.

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