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

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Abstract

Background

Biallelic damaging variants in *ALPK3*, encoding alpha-protein kinase 3, cause an enigmatic pediatric cardiomyopathy with manifestations that are incompletely defined. Whether heterozygous *ALPK3* variants contribute to adult-onset cardiomyopathy remains unknown.

Methods

We analyzed clinical manifestations of damaging biallelic *ALPK3* variants in 19 cardiomyopathy patients, including nine previously published cases. Rare heterozygous *ALPK3* variants were also assessed in adult cardiomyopathy patients.

Results

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, 10 exhibited neonatal left ventricular or biventricular dilated cardiomyopathy (DCM) that subsequently transitioned into ventricular hypertrophy. The majority of patients had diverse extracardiac phenotypes, including contractures, scoliosis, cleft palate, and facial dysmorphisms. We observed no association between variant type or location and disease severity and/or extracardiac manifestations. Myocardial histopathology in four patients showed focal cardiomyocyte hypertrophy, subendocardial fibroelastosis in patients under four years of age, and myofibrillar disarray in adults. Among 1,548 Dutch patients referred for initial genetic analyses we identified 39 individuals with rare heterozygous *ALPK3* variants, including 26 missense and 10 LoF variants. Among 149 U.S. cardiomyopathy patients without pathogenic variants in 83 cardiomyopathy-related genes, we identified six missense and nine LoF variants. LoF *ALPK3* variants were increased in comparison to matched controls (Dutch cohort, $p=1.6\text{x}10^{-5}$; U.S. cohort, $p=2.2\text{x}10^{-13}$).

Conclusions

Biallelic damaging *ALPK3* variants cause pediatric cardiomyopathy manifested by DCM transitioning to hypertrophy, often with poor contractile function. Additional extra-cardiac 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 warrant evaluations for cardiomyopathy.

Introduction

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 over 100 genes cause either isolated or syndromic pediatric cardiomyopathy through many different pathological mechanisms [2-4]. *ALPK3* is a recently identified pediatric cardiomyopathy gene that encodes alpha-protein kinase 3, a protein with functions that remain incompletely understood. Alpha-protein kinase 3 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 their 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 inter-ventricular septum [5, 9], whereas other heterozygous carriers had no cardiac disease. Whether or not these observations indicate 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 remains unknown. 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 7 different countries and institutions. Mutation analysis was performed using next-generation sequencing (NGS), either whole exome sequencing or targeted gene panels. 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]. The medical 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 greater than 10% difference between reference and mutated scores 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 from two affected individuals (patient 1 from family 1 (F1P1) and F2P2). In addition, we collected muscle biopsy specimens from the lateral portion of the quadriceps femoris muscle from F2P3 and her healthy sister, and from the spine, taken at scoliosis surgery, from F3P1. 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, D or E, **Supplemental Table 1**) in 1,548 patients (suspected of) having cardiomyopathy who were referred for diagnostic genetic testing in two molecular diagnostic laboratories in the Netherlands between December 2015 and July 2018. *ALPK3* was also evaluated from whole exome sequence data obtained on 149 unrelated U.S. patients of European ancestry, with clinically diagnosed HCM or DCM. In each of these patients, prior NGS analyses of 83 established or putative cardio-

Table 1. Characteristics of 19 patients with biallelic *ALPK3* variants

	n	%
Male	9/19	47%
Age of onset of CMP		
Prenatal	4/19	21%
<1 year	10/19	52%
1-<18 year	3/19	16%
18+ year	2/19	11%
Mutation type		
LoF/LoF	11/19	57.9%
LoF/missense	7/19	36.8%
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; HTx, heart transplantation; ICD, implantable cardioverter defibrillator; LoF, loss-of-function; LVH, left ventricular hypertrophy; VPI, velopharyngeal insufficiency.

myopathy genes (gene panel F, **Supplemental Table 1**) 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 – burden test

Sequence data for 64,000 unrelated non-Finnish Europeans (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 in gnomAD subjects excluded LoF variants in the last exon of *ALPK3*. Frequencies were statistically compared using a one-tailed binomial test. Values of $p<0.05$ were considered significant.

Results

Identification of *ALPK3* sequence variants

All nine previously reported patients carried biallelic LoF variants in the *ALPK3* gene. Of the ten newly studied 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 (**Table 1**, **Table 2** and **Supplemental Table 2**). 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 showed clustering in exon 6 and in sequences encoding the alpha-kinase domain (**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 were identified in any of the patients with homozygous *ALPK3* variants. All but two

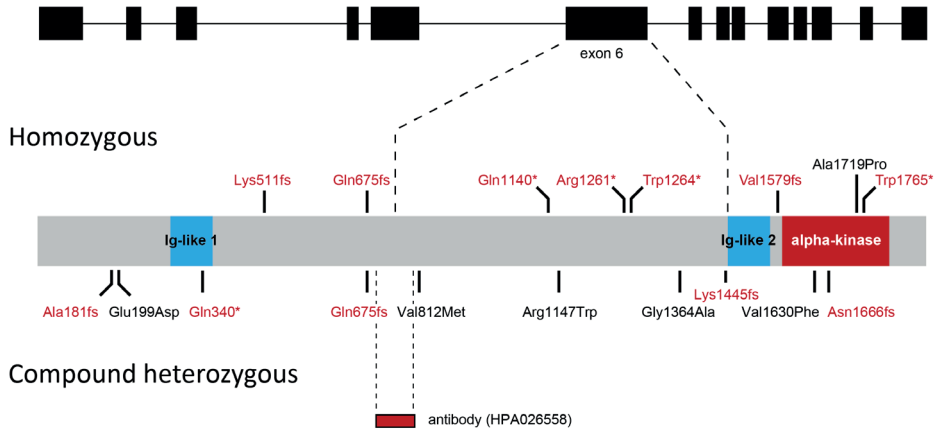

Figure 1. Schematic representation of the structure of ALPK3 gene (top) and protein and location of disease-associated variants. ALPK3 belongs to a superfamily of protein kinases and contains three domains: an alpha-type protein kinase domain and two Ig-like domains. Homozygous variants are displayed on the top of the diagram. Compound heterozygous variants are displayed on the bottom of the diagram. Premature stop codon introducing variants in red.

ALPK3 missense variants had high CADD scores (**Table 2**) and most of the novel amino acids substituted residues that are highly conserved across species. By contrast, the p.(Glu199Asp) variant identified in patients F10P1 and F10P2 is 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 Patients F10P1 and F10P2 and other carriers of damaging biallelic *ALPK3* variants, we suggest that p.(Glu199Asp) is also damaging.

Clinical features of patients with biallelic *ALPK3* variants

Table 1 and **Supplemental Table 2** provide clinical summary data on 19 patients. The age at diagnosis of cardiomyopathy ranged from 20 weeks of gestation to 53 years, although only two patients were diagnosed after the age of 18 years. Among patients with biallelic LoF variants, median age at diagnosis was 7 days. Median age at diagnosis in patients carrying a LoF and a missense variant was 3 months; two of them were diagnosed at adult age. Patient F13P1, harboring two missense variants, presented with cardiomyopathy at 35 weeks of gestation. Prenatal findings were available on 16 patients and included increased nuchal folds and/or fetal hydrops in six patients (37.5%). Eight of 18 live-born patients, 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. Three patients who died 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

Table 2. Overview of previously published and novel biallelic *ALPK3* variants

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

CADD, Combined Annotation Dependent Depletion v1.4; CH, compound heterozygous; gnomAD, Genome Aggregation Database v2.0; HO, homozygous; NA, not applicable; PolyPhen-2, Polymorphism Phenotyping v2; SIFT, Sorting Intolerant From Tolerant.

moderate biventricular hypertrophy at birth that rapidly progressed to biventricular dilated ventricles without hypertrophy. At age two years, the DCM had again transitioned to left ventricular hypertrophy (LVH). Among 16 surviving patients (age >2 years) all, except Patient F8P1, had LVH and eight patients (age >11 months) also had right ventricular hypertrophy. Imaging studies showed progressive LVH in seven of 13 patients. 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.

Electrocardiograms were available on 16 patients and showed prolonged QT intervals, repolarization abnormalities (inferolateral ST depression), and ventricular voltages consistent with LVH. In two siblings a short PQ-interval was noted, as is seen in Pompe disease. Rhythm and conduction disorders occurred in seven patients (44%). 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, and two had a heart transplant at the ages 4 and 28 years, respectively. A wide spectrum of extra-cardiac features (excluding hydrops) was observed in 16 of 18 (89%) live-born patients with damaging biallelic *ALPK3* variants. At birth, all patients had normal size for gestational age, but subsequent growth was delayed. The height of 9/15 patients (60%) ranged from 2 and 6 SDs below the normal mean.

Musculoskeletal abnormalities were observed in 11/18 patients, including severe scoliosis (n=6) (**Figure 2A**), webbed neck (n=8) (**Figure 2B**), knee and/or shoulder contractures (n=5), camptodactyly/arthrogryposis (n=6) (**Figure 2C**), and spondylolysis (n=2). Five patients had congenital contractures while one patient developed contractures and scoliosis later in life. Four of 12 patients (33%) had delayed motor development with independent walking at ages 18-32 months, and three of these children also had a speech delay. Patient F3P1, now aged

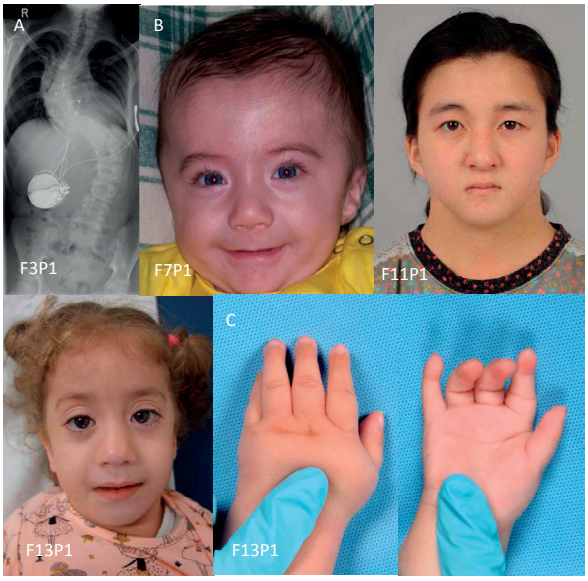

Figure 2. Extracardiac features in biallelic *ALPK3* variants carrying patients. (A) Anteroposterior and lateral X-rays demonstrating S-shaped scoliosis of the thoracic and lumbar spine of patient F3P1. Note: cardiomegaly and implantable cardiac defibrillator in situ. (B) Faces of patients F7P1, F11P1 and F13P1. (C) Distal arthrogryposis in patient F13P1: bilateral absent flexion creases of digit V, congenital contractures of digit I, II and V of left hand and digit V of right hand.

14 years, has a learning disorder (nonverbal IQ 74). Hypotonia was present in 4/13 (31%) patients. Cleft palate or velopharyngeal insufficiency occurred in 8/18 (44%) patients. Craniofacial dysmorphic features were present in at least 12/17 patients (71%), including hypertelorism, ptosis, ankyloglossia, intra-oral pterygia, micrognathia, and low-set ears (**Figure 2B**). At least 3/12 patients (25%) had an abnormal glucose metabolism. We observed no significant association between variant type or location and the severity of extra-cardiac phenotypes.

Clinical features 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. Cardiac evaluations were normal in 17 other clinically evaluated relatives with heterozygous LoF *ALPK3* variants. Among our newly studied families, none of 20 obligatory heterozygous carriers of a damaging *ALPK3* variant have cardiomyopathy or extra-cardiac abnormalities. As the prevalence of unexplained LVH in the general population is 0.10% [17], finding five of 37 (13.5%) of *ALPK3* heterozygous LOF carriers with LVH is highly unexpected (p=4.2x10^{-10}), implying a causal relationship.

Histopathologic examination

Post-mortem microscopic examination of myocardial tissue showed (sub)endocardial fibroelastosis 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 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

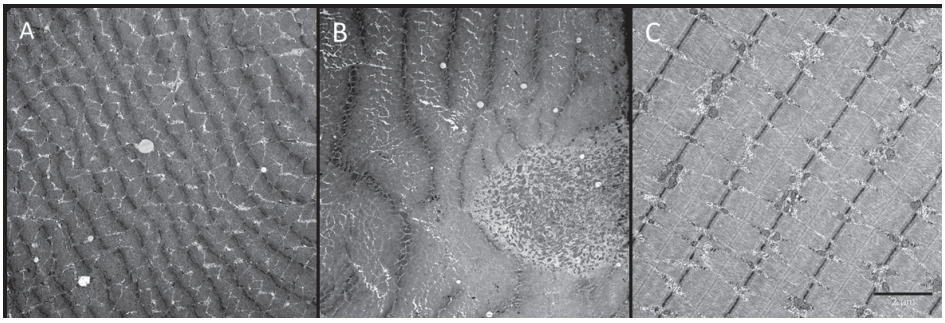

Figure 3. Histopathologic examination of skeletal and heart tissue. Electron microscopic (EM) examination of spinal muscle from F3P1: relatively unaffected region (A. 4000x) and central core (B. 8900x). EM of quadriceps muscle sarcomeres from F3P1 showing regular arrangement of contractile protein filaments (C. 8900x).

F3P1 taken at scoliosis surgery showed variation in fiber size, fiber splitting, and numerous central cores (**Figure 3A-3B**). However, subsequent examination of the quadriceps muscle of the same patient did not show any ultrastructural abnormalities (**Figure 3C**).

Burden of LoF *ALPK3* variants in adult-onset cardiomyopathy

We assessed the prevalence of *ALPK3* variants in two cardiomyopathy cohorts. The Dutch cohort comprised of 1,548 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%), including four LoF variants (three frameshifts 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 predicted effect on splicing (**Supplemental Table 3**). 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 in 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 (**Supplemental Table 4**).

Patients with heterozygous *ALPK3* variants (**Supplemental Table 3**) had the following clinical diagnoses: HCM (13 missense, 7 LoF, 2 stop gain variant in 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 cardiomyopathy (2 missense), and one sudden cardiac death with unknown cardiac disease (1 missense). Seven of these patients (18%) also had a likely pathogenic or pathogenic variant in another cardiomyopathy gene (**Supplemental Table 5**): in *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 2,149 rare (MAF <0.1%) missense or LoF *ALPK3* alleles among ~64,000 non-Finnish Europeans (NFE, 3.4%) compared to 2.5% (38 of 1,548) Dutch cardiomyopathy patients (**Supplemental Table 3**; p=0.024. By contrast, we observed significantly more LoF *ALPK3* alleles in Dutch cardiomyopathy subjects (10/1,548) than in NFE (73/64,000; p=1.6x10^{-5}).

We also studied *ALPK3* LoF variants in 149 unrelated cardiomyopathy patients (HCM, n=129; DCM, n=20) with 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 identified 15 rare (MAF<0.1%) *ALPK3* protein-altering variants (**Supplemental Table 3**): 14 in HCM patients (8 LoF and 6 missense variants) and

one LoF variant in a DCM patient. No patients had biallelic variants and none of the variants recurred in this cohort. The frequency of *ALPK3* LoF variants in the US cardiomyopathy cohort (6.04%) was significantly higher than in the Dutch cardiomyopathy cohort (0.65%; p=6.8x10^{-7}) or in gnomAD NFE (0.11%; p=2.2x10^{-13}).

Discussion

The clinical manifestations of biallelic and heterozygous *ALPK3* variants are quite distinct. Among 19 patients with biallelic damaging *ALPK3* homozygous or compound heterozygous variants, 17 patients presented with pediatric-onset cardiomyopathy. Strikingly, most cases presented initially with DCM that transitioned to ventricular hypertrophy with reduced systolic performance – an unusual clinical sequence. *ALPK3*-related cardiomyopathy often progressed rapidly and six patients died or underwent cardiac transplantation. Most patients had extra-cardiac 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 Dutch and US cohorts. 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 extra-cardiac 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 a concentric hypertrophy of both left and right ventricles [9]. This hypertrophic phenotype differs from classic HCM caused by pathogenic variations 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 decades after diagnosis with accompanying decrease in systolic performance [18, 19]. Like other pediatric cardiomyopathies, A*LPK3* cardiomyopathy can present with features of more than one subtype [20, 21]. However, transition from DCM to LVH has not been described 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 extra-cardiac manifestations and allelic heterogeneity: biallelic missense or LoF variants appeared 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, down-slanting 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 extracardiac features 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 extra-cardiac phenotypes. The prevalence of skeletal muscle phenotypes in pediatric patients likely reflects ALPK3 expression in developing skeletal and heart muscle [6, 24] as well as in skeletal, smooth, and heart muscles in adult humans (GTEx (https://commonfund.nih.gov/gtex)). In embryonic mice (E8.5), Alpk3 is a 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 GeneNet-

work Assisted Diagnostic Optimization (GADO), a method that exploits RNA-seq data from a range of tissues and cell types, and using gene co-regulation to predict gene functions [25]. For *ALPK3*, "muscle contraction" and "myogenesis" were predicted as the top phenotypes. Based on a combination of the major shared phenotypic abnormalities in our patients the *ALPK3* gene is ranked in the top 1% of all coding and non-coding human genes (p=0.000432) (**Supplemental Table 6**).

Pathogenicity of heterozygous *ALPK3* variants

Among 35 relatives of patients with biallelic variants with a heterozygous LoF variant in *ALPK3* (three of whom ≤18 years of age), five (14%) were diagnosed with HCM as adults. No relatives carrying one missense variant have manifested cardiomyopathy. We suggest that these observations imply that a) some carriers have another undetected variant or b) functional levels of ALPK3 contribute to normal cardiac function. In support of these hypotheses, 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 mild or late-onset, undetected cardiomyopathy.

To better understand the role of *ALPK3* variants in adult-onset cardiomyopathy we analyzed a Dutch and US cohort. Among Dutch cardiomyopathy patients, the frequency of rare *ALPK3* LoF alleles was 0.65% or ~5 fold higher than the in the general population (vs. gnomAD, $p=1.6\text{x}10^{-5}$). The US cohort included only cardiomyopathy patients without damaging variants in cardiomyopathy genes. Their frequency of *ALPK3* LoF variants was 6.04%, or ~50 fold higher than the general population frequency (vs. gnomAD, $p=2.2\text{x}10^{-13}$). We suspect that higher burden *ALPK3* LoF variants in the US cohort reflected a high proportion of HCM rather than DCM, prior genetic testing, and differences in sequencing platforms. Regardless of these or other differences, the increased frequency of *ALPK3* LoF variants in cardiomyopathy patients, combined with the emergence of cardiomyopathy in heterozygous relatives of pediatric patients with biallelic *ALPK3* LoF variants provides compelling evidence that this enigmatic kinase plays important roles in cardiac function and pathologic remodeling. Further evidence of the role of *ALPK3* in cardiac hypertrophy arises from a genome-wide association meta-analysis, which identified a novel locus at chromosome 15q25.3, which encompasses *ALPK3*, that 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 of this GWAS is in strong linkage disequilibrium with two nonsynonymous SNPs in *ALPK3* (p=9.94e-18) [26]. Basic studies are needed to understand the targets and pathways in which this kinase participates.

Study 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 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 show a unique cardiac phenotype with progression of DCM to ventricular hypertrophy as a major feature of *ALPK3*-related disease. We demonstrate that biallelic variants in *ALPK3* can cause a syndromic form of cardiomyopathy with musculoskeletal features as well as craniofacial abnormalities. We 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.

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References

1. Wilkinson JD, Landy DC, Colan SD, Towbin JA, Sleeper LA, Orav EJ, Cox GF, Canter CE, Hsu DT, Webber SA, Lipshultz SE. The pediatric cardiomyopathy registry and heart failure: key results from the first 15 years. *Heart Fail Clin.* 2010; 6: 401-13, vii.
2. Harakalova M, Kummeling G, Sammani A, Linschoten M, Baas AF, van der Smagt J, Doevendans PA, van Tintelen JP, Dooijes D, Mokry M, Asselbergs FW. A systematic analysis of genetic dilated cardiomyopathy reveals numerous ubiquitously expressed and muscle-specific genes. *Eur J Heart Fail.* 2015; 17: 484-493.
3. Kindel SJ, Miller EM, Gupta R, Cripe LH, Hinton RB, Spicer RL, Towbin JA, Ware SM. Pediatric cardiomyopathy: importance of genetic and metabolic evaluation. *J Card Fail.* 2012; 18: 396-403.
4. Vasilescu C, Ojala TH, Brilhante V, Ojanen S, Hinterding HM, Palin E, Alastalo TP, Koskenvuo J, Hiippala A, Jokinen E, Jahnukainen T, Lohi J, Pihkala J, Tyni TA, Carroll CJ, Suomalainen A. Genetic Basis of Severe Childhood-Onset Cardiomyopathies. *J Am Coll Cardiol.* 2018; 72: 2324-2338.
5. Almomani R, Verhagen JM, Herkert JC, Brosens E, van Spaendonck-Zwarts KY, Asimaki A, van der Zwaag PA, Frohn-Mulder IM, Bertoli-Avella AM, Boven LG, van Slegtenhorst MA, van der Smagt JJ, van IJcken WF, Timmer B, van Stuijvenberg M, Verdijk RM, Saffitz JE, du Plessis FA, Michels M, Hofstra RM, Sinke RJ, van Tintelen JP, Wessels MW, Jongbloed JD, van de Laar IM. Biallelic Truncating Mutations in ALPK3 Cause Severe Pediatric Cardiomyopathy. *J Am Coll Cardiol.* 2016; 67: 515-525.
6. Van Sligtenhorst I, Ding ZM, Shi ZZ, Read RW, Hansen G, Vogel P. Cardiomyopathy in alpha-kinase 3 (ALPK3)-deficient mice. *Vet Pathol.* 2012; 49: 131-141.
7. Phelan DG, Anderson DJ, Howden SE, Wong RC, Hickey PF, Pope K, Wilson GR, Pebay A, Davis AM, Petrou S, Elefanty AG, Stanley EG, James PA, Macciocca I, Bahlo M, Cheung MM, Amor DJ, Elliott DA, Lockhart PJ. ALPK3-deficient cardiomyocytes generated from patient-derived induced pluripotent stem cells and mutant human embryonic stem cells display abnormal calcium handling and establish that ALPK3 deficiency underlies familial cardiomyopathy. *Eur Heart J.* 2016; 37: 2586-2590.
8. Caglayan AO, Sezer RG, Kaymakcalan H, Ulgen E, Yavuz T, Baranoski JF, Bozaykut A, Harmanci AS, Yalcin Y, Youngblood MW, Yasuno K, Bilguvar K, Gunel M. ALPK3 gene mutation in a patient with congenital cardiomyopathy and dysmorphic features. *Cold Spring Harb Mol Case Stud.* 2017; 3: 10.1101/mcs.a001859. Print 2017 Sep.
9. Jaouadi H, Kraoua L, Chaker L, Atkinson A, Delague V, Levy N, Benkhalifa R, Mrad R, Abdelhak S, Zaffran S. Novel ALPK3 mutation in a Tunisian patient with pediatric cardiomyopathy and facio-thoraco-skeletal features. *J Hum Genet.* 2018; 63: 1077-1082.
10. Konta L, Franklin RC, Kaski JP. Nomenclature and systems of classification for cardiomyopathy in children. *Cardiol Young.* 2015; 25 Suppl 2: 31-42.
11. Maron BJ, Towbin JA, Thiene G, Antzelevitch C, Corrado D, Arnett D, Moss AJ, Seidman CE, Young JB, American Heart Association, Council on Clinical Cardiology, Heart Failure and Transplantation Committee, Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups, Council on Epidemiology and Prevention. Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. *Circulation.* 2006; 113: 1807-1816.
12. Genome Aggregation Database (gnomAD), Version r2.02. Available at: http://gnomad.broadinstitute.org/2018.
13. Boomsma DI, Wijmenga C, Slagboom EP, Swertz MA, Karssen LC, Abdellaoui A, Ye K, Guryev V, Vermaat M, van Dijk F, Francioli LC, Hottenga JJ, Laros JF, Li Q, Li Y, Cao H, Chen R, Du Y, Li N, Cao S, van Setten J, Menelaou A, Pulit SL, Hehir-Kwa JY, Beekman M, Elbers CC, Byelas H, de Craen AJ, Deelen P, Dijkstra M, den Dunnen JT, de Knijff P, Houwing-Duistermaat J, Koval V, Estrada K, Hofman A, Kanterakis A, Enckevort D, Mai H, Kattenberg M, van Leeuwen EM, Neerincx PB, Oostra B, Rivadeneira F, Suchiman EH, Uitterlinden

AG, Willemsen G, Wolffenbuttel BH, Wang J, de Bakker PI, van Ommen GJ, van Duijn CM. The Genome of the Netherlands: design, and project goals. *Eur J Hum Genet.* 2014; 22: 221-227.

14. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet.* 2014; 46: 310-315.
15. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med.* 2015; 17: 405-424.
16. Engh RA, Bossemeyer D. Structural aspects of protein kinase control-role of conformational flexibility. *Pharmacol Ther.* 2002; 93: 99-111.
17. Maron BJ, Gardin JM, Flack JM, Gidding SS, Kurosaki TT, Bild DE. Prevalence of hypertrophic cardiomyopathy in a general population of young adults. Echocardiographic analysis of 4111 subjects in the CARDIA Study. Coronary Artery Risk Development in (Young) Adults. *Circulation.* 1995; 92: 785-789.
18. Biagini E, Coccolo F, Ferlito M, Perugini E, Rocchi G, Bacchi-Reggiani L, Lofiego C, Boriani G, Prandstraller D, Picchio FM, Branzi A, Rapezzi C. Dilated-hypokinetic evolution of hypertrophic cardiomyopathy: prevalence, incidence, risk factors, and prognostic implications in pediatric and adult patients. *J Am Coll Cardiol.* 2005; 46: 1543-1550.
19. ten Cate FJ, Roelandt J. Progression to left ventricular dilatation in patients with hypertrophic obstructive cardiomyopathy. *Am Heart J.* 1979; 97: 762-765.
20. Herkert JC, Abbott KM, Birnie E, Meems-Veldhuis MT, Boven LG, Benjamins M, du Marchie Sarvaas GJ, Barge-Schaapveld DQCM, van Tintelen JP, van der Zwaag PA, Vos YJ, Sinke RJ, van den Berg MP, van Langen IM, Jongbloed JDH. Toward an effective exome-based genetic testing strategy in pediatric dilated cardiomyopathy. *Genet Med.* 2018; 20: 1374-1386.
21. Lee TM, Hsu DT, Kantor P, Towbin JA, Ware SM, Colan SD, Chung WK, Jefferies JL, Rossano JW, Castleberry CD, Addonizio LJ, Lal AK, Lamour JM, Miller EM, Thrush PT, Czachor JD, Razoky H, Hill A, Lipshultz SE. Pediatric Cardiomyopathies. *Circ Res.* 2017; 121: 855-873.
22. Hughes SE. The pathology of hypertrophic cardiomyopathy. *Histopathology.* 2004; 44: 412-427.
23. Asimaki A, Tandri H, Huang H, Halushka MK, Gautam S, Basso C, Thiene G, Tsatsopoulou A, Protonotarios N, McKenna WJ, Calkins H, Saffitz JE. A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy. *N Engl J Med.* 2009; 360: 1075-1084.
24. Hosoda T, Monzen K, Hiroi Y, Oka T, Takimoto E, Yazaki Y, Nagai R, Komuro I. A novel myocyte-specific gene Midori promotes the differentiation of P19CL6 cells into cardiomyocytes. *J Biol Chem.* 2001; 276: 35978-35989.
25. Deelen P, van Dam S, Herkert JC, Karjalainen JM, Brugge H, Abbott KM, van Diemen CC, van der Zwaag PA, Gerkes EH, Folkertsma P, Gillett T, van der Velde J, Kanninga R, van den Akker PC, Jan SZ, Hoorntje ET, te Rijdt WP, Vos YJ, Jongbloed JDH, van Ravenswaaij-Arts CMA, Sinke R, Sikkema-Raddatz B, Kerstjens-Frederikse WS, Swertz MA, Franke L. Improving the diagnostic yield of exome-sequencing, by predicting gene-phenotype associations using large-scale gene expression analysis. 2018.
26. van der Harst P, van Setten J, Verweij N, Vogler G, Franke L, Maurano MT, Wang X, Mateo Leach I, Eijgelsheim M, Sotoodehnia N, Hayward C, Sorice R, Meirelles O, Lyytikainen LP, Polasek O, Tanaka T, Arking DE, Ulivi S, Trompet S, Muller-Nurasyid M, Smith AV, Dorr M, Kerr KF, Magnani JW, Del Greco MF, Zhang W, Nolte IM, Silva CT, Padmanabhan S, Tragante V, Esko T, Abecasis GR, Adriaens ME, Andersen K, Barnett P, Bis JC, Bodmer R, Buckley BM, Campbell H, Cannon MV, Chakravarti A, Chen LY, Delitala A, Devereux RB, Doevendans PA, Dominiczak AF, Ferrucci L, Ford I, Gieger C, Harris TB, Haugen E, Heinig M, Hernandez DG, Hillege HL, Hirschhorn JN, Hofman A, Hubner N, Hwang SJ, Iorio A, Kahonen M, Kellis M, Kolcic I, Kooner IK, Kooner JS, Kors JA, Lakatta EG, Lage K, Launer LJ, Levy D, Lundby A, Macfarlane PW, May D, Meitinger T, Metspalu A, Nappo S, Naitza S, Neph S, Nord AS, Nutile T, Okin PM, Olsen JV, Oostra BA, Penninger JM, Pennacchio LA, Pers TH, Perz S, Peters A, Pinto YM, Pfeufer A, Pilia MG, Pramstaller PP, Prins BP, Raitakari OT, Raychaudhuri S, Rice KM, Rossin EJ, Rotter JI, Schafer S, Schlessinger D, Schmidt CO, Sehmi J, Sillje HHW, Sinagra G, Sinner MF, Slowikowski K, Soliman EZ, Spector TD, Spiering W, Stamatoyan-

nopoulos JA, Stolk RP, Strauch K, Tan ST, Tarasov KV, Trinh B, Uitterlinden AG, van den Boogaard M, van Duijn CM, van Gilst WH, Viikari JS, Visscher PM, Vitart V, Volker U, Waldenberger M, Weichenberger CX, Westra HJ, Wijmenga C, Wolffenbuttel BH, Yang J, Bezzina CR, Munroe PB, Snieder H, Wright AF, Rudan I, Boyer LA, Asselbergs FW, van Veldhuisen DJ, Stricker BH, Psaty BM, Ciullo M, Sanna S, Lehtimaki T, Wilson JF, Bandinelli S, Alonso A, Gasparini P, Jukema JW, Kaab S, Gudnason V, Felix SB, Heckbert SR, de Boer RA, Newton-Cheh C, Hicks AA, Chambers JC, Jamshidi Y, Visel A, Christoffels VM, Isaacs A, Samani NJ, de Bakker PIW. 52 Genetic Loci Influencing Myocardial Mass. *J Am Coll Cardiol.* 2016; 68: 1435-1448.