A homozygous loss of function variant in POPDC3 : from invalidating exercise intolerance to a limb-girdle muscular dystrophy phenotype

A homozygous loss of function variant in *POPDC3* resulting in a clinically diverse spectrum: from invalidating exercise intolerance to a limb-girdle muscular dystrophy phenotype

POPDC3

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ABSTRACT

Objective: To study three siblings and a distantly related individual with a skeletal muscle disorder, harbouring a homozygous splice site variant in *POPDC3*.

Methods: Whole exome sequencing was performed in the index patient and segregation analysis of the identified homozygous variant in *POPDC3* was conducted using Sanger sequencing. Immunohistochemistry, western blot, and mRNA experiments on patients' skeletal muscle tissue as well as on patients' myoblasts were performed to study the pathogenicity of the predicted loss-of-function (LOF) mechanism of the splice variant in *POPDC3*.

Results:

We identified four patients harbouring a homozygous c.486-6T>A splice variant in *POPDC3*. Patients mainly presented with invalidating myalgia and exercise intolerance and limited to no segmentary muscle weakness. CK levels were markedly elevated in all patients. Using mRNA and western blotting studies on muscle biopsies of two patients and primary myoblasts of one patient, a LOF mechanism at the RNA level was shown (r.485_486insauag, p.Ile163*). Muscle biopsies performed in three out of four patients showed non-specific myopathic features with a marked type 2 fibre predominance and the presence of a large number of severely atrophic fibres with pyknotic nuclear clumps.

Conclusions:

We report on a homozygous LOF variant in *POPDC3*, causal in a late juvenile to young adult-onset myopathy (LGMD R26). We show that skeletal muscle symptoms in LGMD R26 may range from an overt limb-girdle muscular dystrophy phenotype to severe exercise intolerance and myalgia, with consistently highly elevated CK levels. We further prove a clear LOF mechanism of *POPDC3* in this rare disorder.

INTRODUCTION

Limb-girdle muscular dystrophies (LGMDs) comprise a phenotypically and genetically heterogeneous group of autosomally inherited myopathies, typically characterized by progressive muscle weakness.[1] Recessive pathogenic variants in two members of the Popeye domain-containing protein (POPDC) family, the blood vessel epicardial substance (*BVES*) gene and *POPDC3* are associated with the rare and recently identified LGMD subtypes, LGMD R25[2-5] and R26[6, 7] respectively. The POPDC proteins are cAMP-binding transmembrane proteins that are abundantly expressed in striated muscle.[8] LGMD R25 is characterized by a striking phenotypical variability, with consistent yet highly varying nature and severity of skeletal muscle involvement and cardiac conduction abnormalities, with all reported patients up to date exhibiting at least subclinical signs of both skeletal muscle and cardiac disease.[2-5] Initially, the p.Ser201Phe variant located within the Popeye domain was reported,[2] while later on a clear loss-of-function (LOF) mechanism was shown through the identification of LOF variants in *BVES*.[5] Similarly, initially only homozygous pathogenic missense variants in *POPDC3* have been reported for LGMD R26, p.Leu155His, p.Leu217Phe, and p.Arg261Gln in 5 patients from 3 ethnically distinct families and the p.Lys154Glu variant in a single index patient.[7] Additionally, a single isolated LGMD patient of consanguineous parents has been reported to harbour a c.486-1G>A splice variant in homozygosity.[9] Patients presented with late juvenile or adult-onset limb girdle muscular weakness with markedly elevated CK levels in the absence of cardiac abnormalities.[6, 7]

Here, we present 3 siblings and a distantly related individual from the same large consanguineous kinship harbouring the novel homozygous pathogenic c.486-6T>A splice site variant in *POPDC3*. Using mRNA and western blotting studies in patients' muscle tissue and patient-derived primary myoblasts, we now confidently show a LOF mechanism of *POPDC3*

causing LMGD R26, a phenotype encompassing highly variable skeletal muscle involvement with consistently highly elevated CKs.

METHODS

Standard protocol approvals, registrations, and patient consents

Ethical approval was granted by the relevant local Ethical Committees of the participating centres. All patients gave their written consent for participation in the study.

Patients and clinical evaluation

We studied four patients presenting with a skeletal muscle disorder, harbouring a rare homozygous variant in *POPDC3*, which was identified by whole exome sequencing (WES). Medical history taking and physical examination were focused on neuromuscular and cardiac symptoms and signs. Muscle strength was evaluated by manual muscle testing (MRC scale). An EMG was performed for patient 2 and 3. Cardiac function was assessed by ECG, Holter monitoring, and echocardiography in all patients.

Muscle MRI

Muscle MRI studies were performed for all patients on a 1.5T MRI platform. Cross-sections at shoulder, abdominal, pelvic, thigh and calf levels were assessed on axial T1-weighted and short tau inversion recovery (STIR) images (not shown) to evaluate patterns of muscle involvement and muscle oedema respectively. Fatty replacement of muscle was graded according to the Mercuri scale.[10]

Analysis of WES data

Genetic analyses

For patient 1, WES was performed using the Twist Human Comprehensive Exome kit with additional custom spike-in (Twist Bioscience). Variants were filtered using VariantDB[11], performing an analysis of a set of genes known to be implicated in myopathies and by extension related neuromuscular disorders. Additionally, exome-wide HPO-based filtering employing Moon software (Diploid, Invitae), was performed. A single candidate variant predicted to influence a splice acceptor site (assessed by following *in silico* splice prediction tools: SpliceSiteFinder-like, MaxEntScan, NNSPLICE and GeneSplicer) in *POPDC3* (reference sequence: NM_022361.5) was identified. Population frequency was determined using the Genome Aggregation Database gnomAD[12], last accessed on August 8th, 2022. The identified variant was validated and after identification of the same variant in a distantly related family member, segregation analysis was performed by Sanger sequencing.

SNP array (Cyto-SNP12v2.1, Illumina) was performed in the distantly related patient 1 and patient 2 from the sibship to confirm a shared haplotype surrounding the variant.

Muscle biopsies

Muscle biopsies of quadriceps muscle were obtained for patients 1, 2 and 4 and analysed following standard histologic and immunohistochemical (IHC) procedures. Standard IHC stainings, including those for dystrophin, α-, β- and γ-sarcoglycans, α- and β-dystroglycan, caveolin 3 and telethonin, were evaluated.

mRNA analysis on patients' muscle

POPDC proteins are abundantly expressed in cardiac and skeletal muscle and show very low expression in lymphocytes.[13] In accordance to this observation we were unable to amplify and sequence the *PODPC3* transcript in cDNA extracted from lymphoblasts from healthy controls.

As a consequence, mRNA studies were subsequently performed in the actual disease tissue, i.e. skeletal muscle and myoblasts.

Total RNA was isolated for patients 2 and 4 and two healthy control individuals from 3-5 mm^3 pieces of quadriceps muscle according to the TRIzol™ protocol (Invitrogen, ThermoFisher Scientific). Homogenisation of the tissue was performed in a GentleMACS™ tube (Miltenyi biotec) using the Dispomix™ homogenisator (Medic tools) (2 rounds of 15 s at 3000 rpm). After DNase treatment to remove residual genomic DNA (Invitrogen, ThermoFisher Scientific), one microgram of total RNA was converted to cDNA using the Superscript® III First Strand Synthesis System (Invitrogen, Thermofisher Scientific) with both oligo dT and random hexamer primers.

Establishing primary myoblast cultures

Additionally, primary myoblast cultures were established for patient 4. Myoblasts were isolated as described by Schmidt *et al*.[14] In brief, after collection of the muscle biopsy tissue, the tissue was manually minced, washed with PBS, and incubated in trypsin (0.05%) in three subsequent fractions of 15 minutes each at 37°C. All fragments, fractions, and supernatants were collected and seeded in cell culture vessels in complete myoblast medium consisting of DMEM with pyruvate, high glucose, and glutamine (Life Technologies; 115944446), supplemented with 10% FCS (Life Technologies; 10270106A), 1% penicillin-streptomycin (Life Technologies; 15140122), and 0.5% chicken embryo extract (MP Biomedicals; 092850145). The medium was changed regularly and cells were passaged upon confluency. Expansion of the cell culture was carried out to obtain sufficient cells for cell sorting.

Cell sorting was performed using CD56-Alexa647 antibodies (Life Technologies; 606-0566-41) and DAPI (Miltenyi Biotec; 130-111-570) for live/dead staining. Preparations for cell sorting consisted of trypsinisation, washing with PBS, and pooling. Cell sorting was carried

out on a MACSQuant Tyto Cell Sorter (Miltenyi Biotec). The $CD56^+$ fraction was seeded in T25 flasks in complete myoblast medium and grown until confluency. Cells were then collected for western blot or seeded in 6-well plates for qPCR experiments (see below).

Human myoblasts isolated from muscle from a 53 years old healthy man (healthy control 1) and a 79 years old female (healthy control 2), and immortalized as previously described, were provided as anonymyzed samples by MYOBANK, a tissue bank affiliated to EUROBIOBANK.[15] Both myoblast lines were cultured separately during regular culture and under experimental conditions. For qPCR, data were pooled for statistical analysis.

Western blotting

Cells for western blot for both patient 4 primary myoblasts and healthy control myoblasts, were first pelleted and lysed, followed by running and blot transfer. Incubation of α-POPDC3 rabbit polyclonal antibody (MyBioSource; MBS859672) as primary antibody was performed overnight at 4°C and Goat-anti-Rabbit-HRP-conjugated (Jackson ImmunoResearch; 111-035-144) as secondary antibody for 1h at RT, followed by incubation with the primary tubulin-alpha mouse monoclonal antibody (Abcam, ab7291) and secondary Goat-anti-mouse-HRP-conjugated (Jackson Immunoresearch; 115-035-146). Imaging was performed with Pierce ECL Plus (ThermoFisher Scientific; 32132) on an Amersham Imaging AI680 (GE Healthcare) imager.

Blocking nonsense-mediated decay with cycloheximide and qPCR on POPDC3 mRNA

For the patient and healthy control myoblasts, subcultures were stimulated at 37°C for 4 h with (i) 100 µg/ml cycloheximide (CHX) to block nonsense-mediated mRNA decay (NMD), (ii) DMSO as negative solvent control or (iii) PBS as untreated control. After incubation, cells were harvested, washed, and shock-frozen at -80°C.

Total RNA from these samples was obtained using the GeneMATRIX Universal RNA Purification kit (Roboklon GmbH; E3598-02). cDNA was synthesised using the High-Capacity cDNA Multiscribe Reverse Transcription kit (Applied Biosystems; 4368814). Quantitative PCR (qPCR) was performed on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) using the Power SYBR Green PCR master mix (ThermoFisher Scientific; 10658255). The signal for *POPDC3* was normalised to those of the housekeeping genes *GAPDH* and *RPLP0*, then fold-changes in mRNA levels were calculated relative topooled control samples.

Data analysis and statistical testing was performed with GraphPad Prism 8.0.1 (GraphPad Software Inc., USA). Additional information according to MIQE guidelines[16] is provided in supplementary file 1.

Data availability statement

Anonymized data will be shared upon reasonable request from any qualified investigator, only for purposes of replicating procedures and results.

RESULTS

Clinical findings

A summarized overview of the clinical symptoms is provided in table 1.

Patient 1, a currently 22-year-old male, was first investigated at 14 years of age, presenting with myalgia, exercise intolerance and markedly elevated CK levels (2440-3938 U/L). An EMG disclosed myopathic abnormalities. A muscle biopsy showed mild myopathic features and a type 2 fibre type predominance. Cardiac screening at the age 20 years was normal.

Prior to this study, there was no known family relationship between patient 1 and patients 2-4 of this study. As patient 1 and 2 shared a common surname and presented a highly similar

clinical phenotype, Sanger sequencing was performed for the candidate variant in *POPDC3* and first confirmed in patient 2 and later on also in patients 3-4 (see genetic findings section below).

Patient 2, a 33-year-old male, manifested complaints of exercise-induced myalgia and fatigue of the lower limb muscles since the age of 25 years. CK levels, repeatedly measured, were elevated in the range of 715-1497 U/L. An EMG performed at that time was normal. During follow-up, the patient developed mild proximal and distal weakness in the legs. A muscle biopsy, performed at the age of 25 years showed scattered necrotic muscle fibres, consistent with a recent episode of rhabdomyolysis (worsened myalgia, myoglobinuria), but also chronic myopathic features as well as multiple severely atrophic muscle fibres with pyknotic nuclear clumps and a marked type 2 fibre type predominance (figure 1). Cardiac screening revealed brief episodes of nocturnal first-degree AV block, without further abnormalities.

Patient 3, a currently 46-year-old brother of patient 2, presented at the age of 45 years with longstanding complaints (approximately 20 years) of myalgia and later on also mild proximal weakness in upper and lower limbs. CK levels were chronically elevated between 2000 and 4000 U/L. Cardiac screening yielded normal results at the age of 45y. Cardiac screening showed nocturnal episodes of borderline first-degree AV block and frequent ventricular premature beats.

Patient 4, sister of patient 2, developed complaints of myalgia at age 38 years. She showed mild hip flexion weakness (5-/5) upon clinical examination, CK was 1025 U/L. Cardiac screening was normal. A muscle biopsy revealed a striking number of very atrophic fibres with pyknotic nuclear clumps apart from non-specific myopathic features and a marked type 2 fibre type predominance (figure 1).

Muscle imaging

T1 weighted muscle MRI images at thigh and calf level are shown in figure 2. Only for patient 4 there was a clear selective pattern of muscle involvement, with severe involvement of medial gastrocnemius muscles and moderate involvement of posterior thigh muscles. STIR imaging revealed an increased fluid signal in adductor muscles, likely linked to the recent episode of rhabdomyolysis. For patient 1 and 2, there is no evident selective increased fatty infiltration of muscles, as both have a rather hypertrophic appearance of muscles and a limited amount of subcutaneous fat.

Genetic findings

By combined WES analysis in patient 1 and Sanger segregation analysis in patients 2-4 we identified a homozygous c.486-6T>A splice variant in *POPDC3* in the four affected individuals described above of two different likely related family branches, unaware of any family relationship (figure 3). SNP array analysis indeed confirmed the presence of a shared 1.2 Mb haplotype surrounding the *POPDC3* variant in the distantly related patient 1 and patient 2 from the sibship (data not shown). WES analysis in patient 1 did not reveal any other candidate variants that could explain the observed phenotype. The parents of patient 1 were shown to both carry this *POPDC3* variant in heterozygous state, indicating that this variant indeed occurs on both alleles in patient 1 (figure 3).

The c.486-6T>A variant has not been reported previously in literature or online databases such as LOVD and is present with a very low frequency of 0.00071% (no homozygotes) in the general gnomAD control population. Different *in silico* splice prediction tools predict a complete or partial loss of the donor splice site of intron 2 of *POPDC3*, while the variant additionally is predicted to activate a cryptic donor splice site 4 base pairs upstream of the wild

type donor splice site, presumably leading to a frameshift and the incorporation of a premature stop codon (figure 3).

mRNA studies

To confirm the predicted splice defect based on the *in silico* prediction algorithms, analysis on cDNA level based on RNA extracted from disease-relevant muscle tissue of patients 2 and 4 was performed. This analysis confirmed the hypothesis of the predominant use of a cryptic donor splice site 4 base pairs upstream of the wild-type intron 2 / exon 3 junction, leading to defective *POPDC3* splicing by the insertion of 4 additional nucleotides (r.485_486insauag) in the *POPDC3* mRNA molecule and the incorporation of a premature stop codon (p.Ile163*) (figure 3), indicating a pathogenic LOF effect of the identified splice variant. qPCR on cDNA of RNA isolated from disease-relevant muscle tissue showed a statistically significant decrease of POPDC3 mRNA in patient 3 (p=0.031) and a marked though statistically not significant decrease (eventueel toch p-waarde als die ietwat in de buurt van significantie is?) in patient 4 compared to pooled healthy controls (figure 4).

Myoblast culture

Primary myoblasts from patient 4 muscle biopsy tissue were isolated and cultured, with no noticeable reduction in cell survival. qPCR performed on primary patient myoblast and two healthy control myoblast lines, treated with CHX, DMSO (vehicle control) or PBS (untreated control) showed a statistically significant lower *POPDC3* mRNA level (p=0.0011) in DMSO-treated primary patient myoblasts compared to DMSO-treated pooled healthy control myoblast lines, in line with the findings from the whole muscle lysate. An increase, though not statistically significant, in *POPDC3* mRNA levels upon inhibition of nonsense-mediated decay in the mutant-POPDC3 myoblasts was observed. Western blotting on cell lysates from

myoblasts isolated from patient 4 showed no detectable levels of POPDC3 protein, contrasting with the presence of POPDC3 in healthy control myoblasts (figure 4).

DISCUSSION

In the present study we diagnosed LGMD R26 in 3 siblings and a distantly related individual harbouring the homozygous pathogenic c.486-6T>A (r.485_486insauag, p.Ile163*) splice site variant in *POPDC3*. More importantly, we firmly establish a clear LOF mechanism at RNA and protein level of POPDC3 in LGMD R26.

As of yet, four homozygous pathogenic missense variants and one splice variant have been reported to be pathogenic in LGMD R26. All missense variants are located in the POPDC domain except for the p.Arg261Gln variant that is located in the intracellular carboxy terminal domain (CTD) domain.[6,7] Additionally, the c.486-1G>A splice variant has recently been reported in a single isolated LGMD patient of consanguineous parents, though phenotypic, genetic, and functional description is limited, raising several questions and inconsistencies. Based on the occurence of mainly missense variants in current literature, a complete LOF mechanism could not yet be established as the main driver of *POPDC3* pathophysiology. In our manuscript, we show strong segregation data supporting the pathogenicity of a homozygous splice site variant. Using mRNA studies using direct sequencing methods and subsequent qPCR on cDNA extracted from patient muscle tissue, we show that the splice site variant results in the introduction of premature stopcoding and consequently a strong decrease in *POPDC3* transcript due to NMD. Biologically, the amount of remaining mRNA which is not immediately eliminated by NMD may differ according to tissue conditions and between individuals. However on the protein level the effect is clearly shown through WB assays on patient-derived myoblasts.

A previous study suggested a LOF mechanism of the reported c.486-1G>A splice donor variant based on analyses on RNA extracted from lymphoblasts and WB on patient's muscle,[9] raising several methodological concerns however. Firstly, POPDC proteins are abundantly expressed in cardiac and skeletal muscle and show very low expression in lymphocytes.[13] We were unable to amplify and sequence *PODPC3* transcript in cDNA from healthy controls lymphocytes, suggesting this cell type is not suitable for studies *POPDC3* mRNA quantification. Secondly, as a frameshift mechanism is proposed, the transcript should be targeted by NMD but this was not experimentally addressed. The authors, however, claim that the WB band observed around 30 kDa (using an antibody targeting the C-terminal domain of PODPC3) represents a truncated form of the protein in the patient's muscle lysate.[9] No control sample or loading control is shown in this experiment.

We show that the skeletal muscle phenotype of LGMD R26 can be highly variable, similarly as for LGMD R25.[5] Earlier reported patients presented with an overt LGMD phenotype,[6, 7] the four patients in this study mainly showed invalidating myalgia and exercise intolerance and little (patient 3 and 4) to no segmentary muscle weakness. Age at onset in this study ranged from 14 years to 38 years and is comparable to earlier reported patients (14-40 years).[6, 7, 9]
CK levels are consistently highly elevated (5-20x upper limit of normal), again paralleling the LGMD R25 phenotype. On top of chronically elevated CKs, this *POPDC3* LOF variant appears to mediate a predisposition to rhabdomyolysis, as shown in patient 2.
Only for patient 4 in the present study a clear selective pattern of muscle involvement is observed on muscle MRI, with early severe involvement of medial gastrocnemius muscles and moderate involvement of posterior thigh muscles corroborating the (rather non-specific) pattern apparently observed in earlier stages in earlier reported LGMD R26 patients.[6] The

implication of the strikingly low amount of subcutaneous fat and hypertrophic appearance of muscles observed in patient 1 and 2 remains unclear. Such a feature was not described in previously reported patients and is otherwise mainly observed in lipodystrophies.[6]

In contrast to consistent cardiac involvement in all reported LGMD R25 patients to date, this was not a feature in the earlier reported LGMD R26 patients.[6, 7] The nocturnal episodes of first degree AV block which were documented for patients 2 and 3 at the age of 33 and 46 years respectively, in the absence of structural cardiac disease, might be an early hint towards eventual *POPDC3*-related involvement of the cardiac conduction system, but long-term cardiac follow-up data are necessary to substantiate this.

Muscle biopsies for patients 1, 2 and 4 did not demonstrate typical dystrophic features (muscle fibre necrosis, endomysial fibrosis) as reported for patients in the first report on LGMD R26.[6] Less specific myopathic features were found including marked type 2 fibre type predominance and the presence of a large number of very atrophic fibres with pyknotic nuclear clumps. The first is rather non-specific and might be accentuated by disuse or might reflect a pathomechanistically relevant shift in fibre types. The significance of the pyknotic nuclear clumps is not entirely understood as they are typically observed in neurogenic causes of muscle fibre atrophy but are also reported in a few myopathies such as myotonic dystrophy type 2.

The integral functional molecular mechanism of pathogenicity of the earlier reported missense variants in *POPDC3* has not been shown yet. It has been proposed that these missense variants may either affect the cAMP affinity or the ability of the (mutant) protein of ligand-induced conformational changes.[6] In case of the LOF variant, all functions of the POPDC3 protein are abolished, yet it is still unknown which downstream disturbance exactly leads to vulnerability and ultimately loss of muscle fibres. Having now established LOF as the clear mechanism for the currently reported splice site variant, the relevance of the original preliminary studies in the

popdc3 knockdown zebrafish model are strengthened and these could be extended in future work. [6]

Similarly as for LGMD R25,[5] this study highlights the diagnostic difficulties that can be faced in case of pauci- or even asymptomatic hyper-CKemia.[17] Clearly, there is no overt dystrophic process at the basis of the markedly high CK levels in our patients. Rather, it might be that there is a membrane instability linked to the interaction of POPDC3 with specific cytoskeletal interactors causing the CK leakage.[6, 8]

We present three siblings and one distantly related individual from a large consanguineous family, harbouring a homozygous LOF variant in *POPDC3*. By identifying an additional pathogenic LOF variant in *POPDC3*, we expand the genetic spectrum of the disorder and provide additional pathomechanistic insights into the disorder. We show that skeletal muscle symptoms may range from an overt limb-girdle muscular dystrophy phenotype to severe exercise intolerance and myalgia. Again, this underlines by extension the role of the POPDC protein family in striated muscle physiology and disease.[8]

COMPETING INTERESTS

The authors declare no competing interests.

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FIGURE LEGENDS

Figure 1. Muscle biopsy findings

Representative muscle biopsy images for patient 2 and 4. (**A**) Hematoxylin and eosin (H&E) staining on the muscle biopsy of patient 4 showed myopathic features with presence of atrophic fibres with pyknotic nuclear clumps (example pointed out by arrow), (**B**) which appear dark on SDH staining. (**C**) ATPase staining (pH 4.6) for patient 4 showing a marked type 2 fibre type predominance. (**D**) Similar features were observed on muscle biopsy of patient 2, with also presence of scattered necrotic muscle fibers.

Figure 2. Muscle MRI findings

Axial T1-weighted images at thigh and calf level are shown for patient 1, 2, 3 and 4 performed at the age of 16, 25, 43, 38 years and respectively.

Figure 3. **Genetic studies in 4 individuals carrying the homozygous c.486-6T>A splice variant in *POPDC3***

A. Pedigree of the studied families harboring the c.486-6T>A *POPDC3* variant. Genotypes are indicated for all family members for whom segregation analysis was performed. Question marks above the two individual pedigrees indicate the supposed (but unreported) consanguinity between the 2 parents in each pedigree, while the upper question mark highlights the supposed (but unreported) relatedness between the two pedigrees based on the identified common haplotype surrounding the c.486-6T>A variant.

B. Visualization of predictions of *in silico* splice prediction tools for the c.486-6T>A *POPDC3* variant. The upper part of the figure shows the results of 4 individual theoretical splice prediction programs for both donor (5') and acceptor (3') splice sites in the wild type situation, while the lower part pictures the predictions in case of the presence of the c.486-6T>A variant.

Based on these predictions the variant activates a cryptic acceptor splice site 4 base pairs upstream of the wild type acceptor splice site. [Screenshot from Alamut Visual Plus 1.4 software, SOPHiA GENETICS].

C. mRNA studies for the c.486-6T>A *POPDC3* variant. cDNA analysis based on RNA extracted from muscle tissue of patient 2 confirming the use of a cryptic acceptor splice site upstream of the wild type intron 2 / exon 3 junction, leading to defective splicing by the insertion of 4 additional nucleotides (r.485_486insauag) in the *POPDC3* mRNA molecule and the incorporation of a premature stop codon (p.Ile163*). [Screenshot from SeqPilot 5.3.3 software, JSI].

Figure 4. mRNA studies and western blotting on patients' muscle and myoblasts

A. qPCR on patient and pooled healthy control whole muscle lysate-derived cDNA shows reduced POPDC3 mRNA levels in whole muscle lysate.

B. Inhibition of nonsense-mediated decay with cycloheximide (CHX) leads to a non-significant increase in POPDC3 mRNA in patient 4 (grey bars) myoblasts compared to DMSO vehicle control. DMSO vehicle control mRNA levels are statistically significantly lower in patient 4 compared to pooled healthy control (black bar).

C. Representative western blot on myoblast cell lysates of patient 4 primary myoblasts compared to healthy control immortalized myoblasts.

475 **TABLES**

476

477 **Table 1**. **Phenotypic information for the patients harbouring the c.486-6T>A splice variant in *POPDC3* in homozygosity**

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**
Sex	Male	Male	Male	Female
Age at onset, y	14	25	36	38y
Presenting symptoms	Myalgia, exercise intolerance	Myalgia, exercise intolerance, rhabdomyolysis	Myalgia, exercise intolerance	Myalgia, mild proximal weakness
Age at last examination, y	21	34	46	38y
Weakness	No marked segmentary weakness	No marked segmentary weakness	Mild weakness of hip flexion (5-/5)	Mild weakness of hip flexion and shoulder abduction (5-/5)
Serum CK (U/L)	2440-3938	1081-1497	1849-2762	1025
EMG (age, y)	NA	No significant abnormalities (25)	No significant abnormalities (43)	NA
Resting ECG	Normal	Normal	Normal	Normal
Echocardiography	Normal	Normal	Normal	Normal
Holter monitoring (age, y)	Normal (20)	Infrequent episodes of nocturnal first-degree AV block (33)	Nocturnal episodes of borderline first-degree AV block, frequent VPB (46)	Normal (38)

Bicycle ergometry testing	Normal	Normal	NA	NA
Biopsy (age, y)	Mild myopathic features, marked type 2 fibre predominance (16)	Findings suggestive of a recent rhabdomyolysis; chronic myopathic features and atrophic muscle fibres with pyknotic nuclear clumps (25)	NA	Myopathic features, marked type 2 fibre type predominance; large number of atrophic fibres with pyknotic nuclear clumps (38)
FVC (% of predicted)	NA	110%	108%	90%

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479 Abbreviations: y, years; CK, creatine kinase; FVC, forced vital capacity; NA, not assessed; VPB, ventricular premature beats