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Bi-allelic *LETM1* variants perturb mitochondrial ion homeostasis leading to a clinical spectrum with predominant nervous system involvement

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Summary

The Leucine zipper-EF-hand containing transmembrane protein 1 (*LETM1*) gene encodes an inner mitochondrial membrane protein with an osmoregulatory function controlling mitochondrial volume and ion homeostasis. The putative association of *LETM1* with a human disease was first suggested in Wolf-Hirschhorn syndrome resulting from *de novo* monoallelic deletion of chromosome 4p16.3, encompassing *LETM1.* Utilizing exome sequencing and international gene-matching efforts, we have identified 18 affected individuals from 11 unrelated families harboring novel and ultra-rare bi-allelic missense and loss-of-function *LETM1* variants and clinical presentations highly suggestive of mitochondrial disease. These manifested as a spectrum of predominantly infantile-onset (14/18, 78%) and variably progressive (50% rapid with premature mortality, 22% moderate, and 28% slow) neurological, metabolic, and dysmorphic symptoms, and multiple organ dysfunction associated with

neurodegeneration. The common features included respiratory chain complex deficiencies (100%), global developmental delay (94%), optic atrophy (83%), sensorineural hearing loss (78%), and cerebellar ataxia (78%) followed by epilepsy (67%), spasticity (53%), and myopathy (50%). Other features included bilateral cataracts (42%), cardiomyopathy (36%), and diabetes (27%).

To better understand the pathogenic mechanism of the identified *LETM1* variants, we performed biochemical and morphological studies on mitochondrial K^+ activities, proteins and shape in patient-derived fibroblasts, muscles and in *S. cerevisiae* as an important model organism for mitochondrial osmotic regulation. Our results demonstrate that bi-allelic *LETM1* variants are associated with defective mitochondrial K^+ efflux, swollen mitochondrial matrix structures, and loss of important mitochondrial oxidative phosphorylation protein components, thus highlighting the implication of perturbed mitochondrial osmoregulation caused by *LETM1* variants in neurological and mitochondrial pathologies.

Keywords: mitochondria, LETM1, mitochondrial diseases, neurodegeneration, Wolf-Hirschhorn syndrome

Introduction

Leucine zipper-EF-hand containing transmembrane protein 1 (*LETM1*), (MIM: 604407) is a ubiquitously expressed and phylogenetically highly conserved nuclear gene encoding the LETM1 protein. LETM1, also named SLC55A1, is part of the new mitochondrial transporter protein SLC55 family that belongs to the SLC solute carrier superfamily,[1] and is the founder of the LETM1 superfamily and listed as one of the EF-hand Ca^{2+}-binding proteins of the MitoCarta library.[2,3] The proteins of the LETM1 superfamily contain leucine zipper and several coiled-coil domains.[2,4] LETM1 is an inner

mitochondrial membrane protein with an osmoregulatory function that controls cation homeostasis, preventing their equilibration with the H^+ electrochemical gradient. While first identified to function as an electroneutral mitochondrial K^+-H^+ exchanger (KHE), LETM1 has also been connected to the regulation of the uptake or extrusion of Ca^{2+}. [2,5-10]

The pathological hallmark of *LETM1* depletion is mitochondrial matrix swelling, fragmentation, and loss of cristae structure, consistently found in all studied organisms,[5] whereas *LETM1* overexpression causes mitochondrial elongation, cristae swelling, and matrix condensation due to imbalance in osmotic homeostasis.[11] Silencing LETM1 homologs in yeast*, Fusarium graminearum*, and *Toxoplasma gondii* results in lethality or loss of virulence. *Drosophila melanogaster* with tissue-specific depleted LETM1 expresses compromised tissue growth and locomotor behavior, as well as impaired evoked synaptic release of neurotransmitters.[12] Homozygous deletion of *LETM1* leads to developmental and embryonic lethality in flies, worms, and mice.[9,11,12]

Consistent with the vital role of mitochondrial osmoregulation, matrix swelling and cation imbalance due to *LETM1* inactivation have wide-reaching and pleiotropic effects on mitochondrial biogenesis and bioenergetics, perturbing glucose and pyruvate utilization, tryptophan and mitochondrial DNA (mtDNA) metabolism, outer mitochondrial membrane integrity and causing necrotic cell death. [9,12,13-17]

The importance of *LETM1* in neuronal function and pathology was first suggested in Wolf-Hirschhorn syndrome (WHS [MIM: 194190]).[4] This genetic syndrome results from *de novo* monoallelic deletion of several genes on the short arm of chromosome 4. Depending on the length of the deletion, WHS might present with a combination of congenital malformations, specific facial dysmorphism, growth and cognitive impairment, microcephaly, hypotonia, and epilepsy.[13] *LETM1* is localized in WHS critical region 2 (WHSCR2), less than 80 kb from WHS critical region 1 (WHSCR1) and is deleted in almost all individuals with the full WHS phenotype. *LETM1* is proposed to be associated with epilepsy and neuromuscular features of WHS.[18,19] Analysis of WHS fibroblasts linked *LETM1* haploinsufficiency with

147 mitochondrial defects. One study reports elevated intracellular Ca^{2+}, decreased Ca^{2+} sensitivity of the
148 mitochondrial permeability transition pore (PTP), increased superoxide and hyperpolarization of the
149 inner membrane;[20] another study reports mtDNA aggregation, pyruvate dehydrogenase (PDH)
150 deficiency and a preferential shift from pyruvate oxidation to ketone body utilization.[14] How the cation
151 transport properties of LETM1 and the broad effects of its dysfunction on other mitochondrial and
152 cellular functions mechanistically contribute to the WHS disease phenotypes is not well understood
153 and is complicated by the multigenic cause for WHS. Other implications of LETM1 impairment in
154 genetic diseases include temporal lobe epilepsy,[21] diabetes,[22] and obesity.[15]

155 Here, we describe 18 affected individuals from 11 unrelated families presenting with clinical features
156 suggestive of a mitochondrial disease largely involving the central nervous system (CNS) in which
157 exome sequencing (ES) identified novel and ultra-rare bi-allelic segregating *LETM1* variants.

158 To functionally characterize the bi-allelic *LETM1* variants, we explored cellular growth and
159 mitochondrial respiratory chain, morphology, osmotic regulation, and KHE activity in patient-derived
160 fibroblasts, muscle samples, and in yeast carrying the variants of interest.

161

162 **Subjects and methods**

163 **Study subjects**

164 Using the GeneMatcher platform[23] and data sharing with collaborators around the world, 11 families
165 with bi-allelic *LETM1* variants were identified. The affected individual from family 8 was recruited from
166 the report by Catania et al.[24] describing a case with a combined pituitary hormone deficiency, ocular
167 involvement, myopathy, ataxia, and mitochondrial impairment carrying variants in several putative
168 disease-causing genes, including rare bi-allelic variants in *OTX2* (MIM: 600037) and *LETM1* as well as
169 rare heterozygous variants in *AFG3L2* (MIM: 604581) and *POLG* (MIM: 174763). Clinical details of the
170 cohort were obtained by the follow-up of the living affected individuals and retrospective analysis of

the available clinical records for deceased cases. Parents and legal guardians of all affected individuals gave their consent for the publication of clinical and genetic information according to the Declaration of Helsinki, and the study was approved by The Research Ethics Committee Institute of Neurology University College London (IoN UCL) (07/Q0512/26) and the local Ethics Committees of each participating center. Consent has been obtained from families 1, 5, and 8 to publish medical photographs and video examinations. Brain magnetic resonance imaging (MRI) scans were obtained from 6 affected individuals and were reviewed by an experienced pediatric neuroradiologist (FA).

ES and data analysis

Proband only or trio ES in 11 families was carried out in DNA extracted from blood-derived leukocytes in 9 different centers following slightly different protocols (Table 1 for methods). ES data analysis and variant filtering and prioritization were performed using in-house implemented pipelines of the local genetic centers (Table 1 for methods). Sanger sequencing was performed to confirm co-segregation in all available family members.

Skin biopsy and primary fibroblast culture, and muscle biopsy

Individuals F1:S1, F1:S2 and (mother-F1:M, father-F1:F), F2:S1, F5:S1, F10:S1, F11:S1, and F11:S2 provided each one skin biopsy, and affected individuals F11:S1, F11:S2, and F5:S1 provided also each one muscle biopsy. Details on fibroblasts cell lines establishment and muscle sample preparations are described in the supplemental material and methods.

Western blotting analysis

Immunoblotting analysis was performed using standard protocols as described previously,[39] detailed descriptions of sample preparation, quantification, and western blotting are in the supplemental material and methods. A list of antibodies used for this study is given in supplemental data.

Cell imaging

Confocal microscopy was performed for fibroblasts from F1, F2, F5, F10, F11, and respective controls following established protocols for life and immune staining described in Durigon et al.[14] and Wilfinger et al.[40] and supplemental material and methods. Transmission electron microscopy is described in the supplemental material and methods.

mtDNA copy number

DNA was extracted from muscle or fibroblasts by proteinase K treatment. The mtDNA content was determined by quantitative real-time PCR using two independent mitochondrial and four independent nuclear DNA sequences as previously described.[41]

Immunohistochemistry

FFPE muscle tissue was cut with a microtome in 4 µm slides. Immunohistochemistry was performed as described previously in Kusikova et al.[39] with some modifications, a detailed description of the method is given in the supplemental material and methods. All antibodies used in this experiment are listed in supplemental data.

Plasmid and LETM1 point variants

Full-length human *LETM1* cDNA fused to C-terminal Hemagglutinin (*HA*)-tag and subcloned into the multi-copy plasmid pVT-103U[42] served as a template to introduce the *LETM1* variants by site-directed mutagenesis. Amino acid replacements and deletions were performed with non-overlapping back-to-back annealing mutagenic primers, using the Q5 site-directed mutagenesis kit (NEB #E0552S) with NEB 5-alpha competent *E. coli* cells (NEB #C2987). All primers were from Microsynth (Balgach, Switzerland) and all the identified variants were confirmed by DNA sanger sequencing. To distinguish the phenotypes of disease-associated *LETM1* variants and non-pathogenic variants, two non-disease-associated *LETM1* (NM_012318.3) missense variants (rare *LETM1* variants but with homozygotes in gnomAD v3.1.1), c.913A>C, p.Ile305Leu and c.1760A>G, p.Lys587Arg, were included in this study. A

list of variants studied in yeast and primers used for site-directed mutagenesis is given in supplemental data.

Yeast transformation

W303 (ATCC 201239) *Saccharomyces cerevisiae* strain *mdm38/yletm1Δ* (lacking the open reading frame *YOL027c,* which encodes the yeast LETM1 homolog)[42] was transformed with the multicopy vector pVT-103U, either empty or containing wildtype *LETM1*[42] or *LETM1* variants using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method[43] and grown on selective media (SD-URA) to ensure the retention of the plasmids. Yeast growth media were described in Zotova et al.[44]

Mitochondrial isolation and KOAc-induced swelling assay

Mitochondria were isolated from yeast cells logarithmically grown in SD-URA by homogenization and differential centrifugation method as described in Nowikovsky et al.[42] and immediately used for KOAc-induced swelling assays. The protocols of Nowikovsky et al.[42] were adapted to smaller volumes. Briefly, isolated yeast mitochondria suspended in breaking buffer (0.6 M sorbitol, 20 mM Tris-HCl pH 7.4) were de-energized with antimycin A (2.5 µM) for 10 min at room temperature (25° C), washed and resuspended in breaking buffer at a concentration of 200 µg/20 µl. As Mg^{2+} is a brake to the KHE,[45] mitochondria were depleted from Mg^{2+} with A23187 (0.5 µM) and EDTA (10 mM) and transferred onto 96 well plates for measurement (200 µg/well). When indicated, quinine (200 µM) served as a control to inhibit KHE-mediated swelling. The 96 well plates were placed in the Thermo Scientific Varioskan LUX Multimode Microplate Reader. The swelling was initiated by injection of KOAc media (55 mM KOAc, 5 mM TES, 0.1 mM EDTA) to a final volume of 200 µl/well and the optical density changes at OD_{540} were immediately recorded at 25° C. Each measurement was performed in 3 independent replicates. Raw swelling data were fitted into a curve showing changes in absorbance versus time to quantify the swelling rate.

Results

Clinical findings

The summary of the core phenotypic features of 18 affected individuals from 11 independent families with bi-allelic *LETM1* variants is provided in Table 2, Figure 1C, and Table S1. Detailed clinical history is provided in the supplemental note (case reports). Video recordings are available for affected individuals from family 1 (Supplemental Videos). The cohort comprises 10 males and 8 females, 9 of whom are currently alive with a median age of 15 years (range 1-39) at the latest available follow-up (Figure 2A). Half of the cases (9/18) succumbed to their rapidly progressing disease at an early age, ranging between 2 months and 8 years old. The ethnic composition of the cohort is diverse including families of Pakistani, Caucasus, Middle Eastern, European, and Mexican origin, with 67% of the cases (12/18) being from consanguineous unions. Only limited clinical data were obtainable from 6 deceased cases belonging to families 3 and 10.

The cohort members had unremarkable prenatal histories with full-term birth in 14/15 cases (93%). Admission to the special care baby unit was necessary in 5/15 cases (33%) due to respiratory, cardiac, and feeding issues during the neonatal period. Most of the cases (14/18, 78%) had an infantile-onset disease manifestation, and 4/18 (22%) presented first symptoms between the ages of 1.5 and 2 years. The common presenting symptoms were global developmental delay, cognitive and motor regression, failure to thrive, central hypotonia, respiratory distress, and feeding difficulties. The disease progressed rapidly in 9/18 (50%), moderately fast in 4/18 (22%), and slowly in 5/18 (28%) cases. Developmental regression was later present in 9/13 (69%) affected individuals with loss of independent ambulation by a mean age of 5.4±3.2 years (range 2-12).

On the most recent follow-up, the affected individuals displayed clinical features suggestive of a mitochondrial disorder. Impaired vision (10/10, 100%) with a mean onset age of 5.2±3.1 years, which was confirmed to be due to optic atrophy in 5/6 (83%), and bilateral sensorineural hearing loss (11/14, 78%) diagnosed at a mean age of 2.6±1.9 years (range from congenital up to 6 years) with hearing aids

fitted in 7/10 (70%) cases were the common neurosensory abnormalities. While cognitive delay and intellectual disability (7/8, 87.5%%) and impaired speech acquisition (6/9, 67%) were among the common neurodevelopmental symptoms, more than half of the cases displayed neuromuscular features including spasticity (8/15, 53%), hypotonia (11/18, 61%), muscular wasting (7/10, 70%), and cerebellar ataxia (7/9, 78%). Other frequent neurological symptoms were nystagmus (7/13, 54%), myopathy (6/12, 50%), hyperkinetic movement disorders (4/12, 33%), and spastic-ataxic gait (3/9, 33%) combined with brisk deep tendon reflexes (4/10, 40%), upgoing plantar response (4/9, 44%), and peripheral neuropathy (3/9, 33%).

Ten of the fifteen affected individuals (67%) developed epileptic seizures by a median age of 5 years (range 0.5-14). The seizure type ranged from infantile spasms and myoclonic jerks to absences, focal, and generalized tonic-clonic seizures. Cases with younger age of seizure onset had frequent episodes spanning from hourly clusters of spasms at peak to seizures once per day. Two affected siblings from family 1 with seizure onset after age 9 and 14 years respectively, had seizures recurring either in clusters 2-3 times every 2-3 months (F1:S2) or once in 2 years (F1:S1). Pharmacoresistance and epileptic encephalopathy were confirmed in one case from family 9. Electroencephalograms, available from 4 cases, showed background slowing (F5:S1), excessive sharp transients (F6:S1), single 3-4 Hz potentials and short trains (F2:S1), and continuous spike-and-slow wave activity, with bursts of faster activity observed during sleep, consistent with epileptic encephalopathy (F9:S1).

Other features consistent with a mitochondrial phenotype included bilateral cataracts (5/11, 45%) cardiomyopathy (5/14, 36%) with pericardial effusion (3/11, 27%), and diabetes (3/11, 27%). Craniofacial abnormalities included occipitofrontal circumference below 3rd percentile in 2/6 cases (33%) and facial dysmorphism (4/10, 40%) with a long thin face, prominent nose, low-set ears, micrognathia, high arched palate, and teeth abnormalities (Figure 1 A).

Whilst not every case had available electrophysiological investigations, biochemical, metabolic studies, and muscle histochemical analysis, the obtainable tests suggested the presence of

mitochondrial dysfunction in the affected individuals. Hence, electromyography and nerve conductions studies available from 5 cases showed neurogenic (3/5) and myopathic changes (2/4). Elevated serum lactate was confirmed in 8/12 (67%) affected individuals. Plasma amino acids were abnormal in 4/9 tested with mildly elevated alanine (501-597 µmol/l, normal range 232-494), glycine, and serine. CSF-alanine was tested and mildly increased in 2 cases. Urine amino acids were tested in 4 cases, and only one case showed abnormal results including increased levels of aspartic, serine, and glycine. Urine organic acids were analyzed in 11 cases and were abnormal in 9 of them with 3-methylglutaconic acid excretion (5/11), moderately elevated beta-hydroxybutyrate and acetoacetate (1/11), and significant elevation of adipic acid (1/11). Muscle biopsy was available from 7 cases and of these, 5 had abnormal findings including scattered necrotic and regenerating COX-deficient fibers with an excess of internal nuclei, lipid depositions within fibers and prominent mitochondrial pattern in vacuolated fibers (F3:S2), COX-deficient multiple ragged-red fibers with increased fiber unisometry (F8:S1), type I fiber predominance with mild glycogen storage (F9:S1) and COX-deficient fibers (F11:S1). Respiratory chain enzyme (RCE) analysis was performed in 11 cases showing isolated or combined mitochondrial respiratory chain deficiencies in all subjects tested (Table 2 and Table S1, Figure 3C).

Brain MRI investigations were available for 6 cases, performed between 6 days and 32 years of age (Figure 1B). In some cases, only a few sections or low-quality images could be reviewed. In 4/6 cases optic nerve and chiasm atrophy were present and in two cases optic nerves were normal. Three cases showed infratentorial abnormalities, with severe pontine hypoplasia and cerebellar atrophy in a case from family 1, and mild vermian hypoplasia in 2 cases from family 3 and family 6. Other minor and non-specific findings were mild supratentorial atrophy and mild ventricular dilatation noted in 2 subjects each.

The affected individual from family 8 was the oldest member of the cohort showing a phenotype consistent with the rest of the individuals that survived into adulthood.

316

317 **Molecular genetic findings**

318 In all index cases, ES at the local genetic centers did not identify causative variants in known disease-
319 associated genes. Filtering for novel and rare protein-altering variants identified bi-allelic variants in
320 *LETM1* (NM_012318.3) in index cases from all families (Table 1). Segregation by Sanger sequencing in
321 families with proband only ES and, where available, trio ES supported LETM1 as a candidate gene
322 (Figure 2A). The proband from family 6 carried a homozygous c.1178G>A, p.Arg393His variant in
323 *LETM1* resulting from maternal uniparental disomy. Known pathogenic variants in mtDNA and mtDNA
324 rearrangements were excluded in all families.

325 The *LETM1* variants (Table 1 for variant characterization and Figure 2B) comprised missense variants
326 causing changes in amino acid charge, size, hydrophobic or "helix breaker" properties, and frameshift
327 variants causing premature or delayed termination. All detected missense variants were located
328 specifically within the conserved LETM domain, while the frameshift variants were localized to the C-
329 terminal part of LETM1 (Figure 2B). Of all the amino acid changes, the only fully conserved amino acid
330 across mammals, vertebrates, invertebrates, plants and yeast is c.1072G>A, p.Asp358Asn, and the
331 semi-conserved ones are c.754-756del, p.Lys252del and c.878T>A, p.Ile293Asn (Figure 2B). The
332 arginine affected by the missense variant c.881G>A, p.Arg294Gln is conserved in all sub-families
333 excluding yeast, and it was found in two independent cases (F4:S1 and F8:S1) of Egyptian and Italian
334 origin, respectively. The proline affected by the variant c.898C>T, p.Pro300Ser is conserved in
335 mammals and zebrafish. Four variants affect the C-terminal stretch of human LETM1 that is absent in
336 the yeast LETM1 homolog (yLetm1p/Mdm38p) as its protein sequence is shorter. The splice variant
337 c.2071-9C>G, p.Val691fs4* (Sashimi plot, Figure S2, Supplemental material for methods) affects two
338 residues conserved across mammals, zebrafish, worm, and plants and introduces a premature stop
339 codon before the second EF loop. The variant c.2094del removes p.Asp699, a negatively charged
340 residue, well-conserved in mammals, fish, worms, and plants that locates close to the second EF loop

and prematurely terminates the protein sequence. The stop-loss variant c.2220G>C, p.*740TyrextTer26 leads to an elongation of 26 amino acids. This variant was present in two independent families of Pakistani origin suggesting a possible founder effect. Five of the ten identified *LETM1* variants were absent across a number of large genetic databases (~1 million alleles), whereas the remaining four variants appear to be ultra-rare (Table 1).

Genotype-phenotype correlation

A remarkable interfamilial phenotypic variability was observed in the present cohort. Four cases from families 1, 2, and 8 have survived into adulthood albeit with a significant disability, while 10 cases from families 3, 4, 9, 10, and 11 had a rapidly progressing disease course leading to early death in 9 of them. Cases from family 5 (age 11 years), family 6 (age 17 months), and family 7 (ages 8 and 15 years) displayed less severe phenotypes. Affected individuals from family 4 and family 8 carrying the recurrent missense *LETM1* c.881G>A, p.Arg294Gln variant exhibited a similar range of symptoms, though F4:S1 displayed more rapid disease progression with significant cardiac involvement and early mortality. Cases of Pakistani origin from family 2 and family 7 with loss-of-function (LoF) *LETM1* c.2220G>C, p.*740TyrextTer26 variant were reported with a similar phenotypic range, which was more severe in family 2, possibly due to older age and longer disease course. No significant intrafamilial phenotypic variability was observed in the cohort.

Effects of the *LETM1* variants on patient-derived fibroblasts and muscle tissue

Loss of mitochondrial volume homeostasis is the most characteristic and universally accepted phenotype of *LETM1* deficiency in human, animal models, plants, and yeast, which leads to mitochondrial fragmentation, matrix swelling, and disorganized cristae as reviewed in Austin et al.[5] Therefore, we first evaluated the mitochondrial morphology in the available fibroblasts. Compared to

365 fibroblasts from healthy donors (C1-C4), fibroblasts from F1:S1 and F1:S2 (compound heterozygotes
366 for c.878T>A;c.2094del, p.Ile293Asn; Asp699Metf*13), F10 (homozygote for c.2071-9C>G,
367 p.Val691fs4*), F2 (homozygote for c.2220G>C, p.*740TyrextTer26), F5 (homozygote for c.1072G>A,
368 p.Asp358Asn), and F11:S2 (homozygote for c.898C>T, p.Pro300Ser) displayed mitochondrial
369 alterations, with significantly increased fragmented shapes seen as donut segments and punctate and
370 enlarged units often separated from the main network (Figure 3A and Figure S1A-C). Elongated
371 mitochondrial shapes were restored by ketone bodies and by nigericin. The use of the membrane
372 potential-dependent mitochondrial dye Mitotracker Red (MTR) revealed an irregular polarization
373 pattern of the mitochondrial network of all patients, with partly depolarized tubules and
374 hyperpolarized patches, as well as a markedly reduced electric potential of mitochondria in F10 (Figure
375 3A and Figure S1A, S1C). Impaired KHE activity in LETM1-deficient cells leads to uncompensated
376 electrophoretic K^+ uptake and consequent mitochondrial swelling.[13] Treatment with the synthetic KHE
377 nigericin to counteract the loss of K^+ homeostasis reverted the decreased membrane potential to
378 control levels in F10 and F5 (Figure S1A, S1C), while addition of ketone bodies had no beneficial effect.
379 Consistent with a lack of KHE activity, mitochondria in F11:S2 cells readily underwent swelling and
380 depolarization (as assessed by *in situ* staining with the potentiometric probe TMRM) upon the addition
381 of low concentrations of valinomycin, a selective K^+ ionophore that allows electrophoretic K^+ uptake.
382 The same concentrations of valinomycin were tolerated by mitochondria of control fibroblasts, and
383 treatment of F11:S2 fibroblasts with the ionophore nigericin restored the normal and elongated shape
384 of mitochondria, a strong indication that the response to valinomycin was due to lack of KHE activity
385 (Figure S1D). Based on the protective effect of ketone bodies as an energy source for LETM1-deficient
386 cells,[14] we tested next whether a tubular network could be better maintained as a result. Ketone
387 bodies completely suppressed MTR fluorescence in fibroblasts from F1:M and F1:F and attenuated its
388 intensity in F5:S1 and F10:S1. Only when increasing the laser intensity, an elongated tubular shape of
389 the mitochondrial network became apparent in the samples of F1:M and F5:S1 (Figure S1A). Thus,
390 elongation of mitochondrial tubules was accompanied by a reduced inner membrane potential, a

391 phenomenon previously described in the context of transient matrix contraction.[46] Replacement of
392 glucose with galactose, known to suppress glycolytic ATP production, in F1:S1 and F11:S2 for up to 5
393 days produced a more dramatic morphological phenotype, in some cases resembling LETM1 siRNA
394 (Figures S1B) and Durigon et al.[14] and it caused cell death after only 48-72 hours in F11:S2.
395 Transmission electron microscopy was performed for F5 and F10 fibroblasts as well as control
396 fibroblasts and confirmed ultrastructural mitochondrial changes associated with *LETM1* variants
397 compared to the elongated tubular shapes of the healthy control mitochondria (Figure 3B). Different
398 morphological stages of mitochondrial alterations were associated with *LETM1* c.2071-9C>G,
399 p.Val691fs4* (F10), including short tubules containing enlarged sections with reduced cristae, swollen
400 matrix devoid of cristae, and perinuclearly distributed spherical ghost shapes resembling a mixture of
401 mitochondrial remnants and vacuoles. Similarly, fibroblasts with the variant *LETM1* c.1072G>A,
402 p.Asp358Asn (F5) showed broad, short, and electron-luce mitochondria, partly devoid of cristae and
403 intermediate shapes between mitochondria and vacuoles.

404 Pathological variants frequently lead to altered expression or stability of the encoded proteins, and so
405 we assessed LETM1 protein levels via immunoblotting. The steady-state levels of LETM1 in fibroblasts
406 from F10 were comparable to controls. Instead, LETM1 was significantly decreased in bi-allelic *LETM1*
407 variant fibroblasts F1:S1 and F1:S2, and F11:S2, and more drastically in F2 (Figure 3C-D).

408 Because LETM1 dysfunction restricts mitochondrial respiratory capacity in yeast and mammals, [14,47]
409 and the clinical and metabolic findings in the affected individuals were consistent with a mitochondrial
410 disorder, we next investigated the abundance of the oxidative phosphorylation (OXPHOS) subunits.
411 Fibroblasts of affected individuals harboring bi-allelic *LETM1* variants, displayed reduced steady-state
412 levels of selected respiratory chain proteins of Complex I and IV, in opposite to increased levels in F10
413 (c.2071-9C>G, p.Val691fs4*) (Figure 3C-D). OXPHOS proteins NUDUFB8 and NDUFA9 were decreased
414 in F1:S1 and F1:S2 fibroblasts and to a higher extent in F11:S2 (Figure 3C-D). Since mitochondrial
415 defects can limit cellular growth, we assessed the proliferation rates of the fibroblast cell lines. While
416 proliferation was comparable for fibroblasts with the single or compound heterozygous variants (F1),

extension variant (F2), or wild type *LETM1* (*LETM1* WT), it was significantly slowed down in *LETM1* c.2071-9C>G, p.Val691fs4* (F10) and absent in *LETM1* c.1072G>A, p.Asp358Asn (F5) fibroblasts (Figure 3E, and Figure S3).

Similar to fibroblasts, LETM1 was significantly reduced in the muscle of F11 (Figure 4A-B). NDUFA4 (Complex I) was reduced in muscles samples from F11 while SDHB (Complex II) displayed a strong tissue-specific upregulation (Figure 4A-B). The immunohistochemistry and western blotting analysis from F5 muscle tissue (Figure 4C-E) revealed even greater reductions for components of Complexes I, III, and IV, increased SDHA, accompanied by decreased enzyme activity of Complex I, and upregulated activity of Complex II and citrate synthase and increased mtDNA copy number (Table S2). Proteins of the ATP synthase remained not significantly changed in all tested cell lines and tissue samples.

Overall, altered LETM1 and OXPHOS protein levels in fibroblasts and muscle samples were observed in most of the cases. Fibroblasts cell culture data indicated that bi-allelic *LETM1* variants result in aberrant mitochondrial morphology, which was more pronounced under galactose challenge (Figure S1B), and often lethal for F11-derived fibroblasts. Consistent with the frequently observed effect of mitochondrial defects on cellular functions and growth, cell proliferation was retarded in F10 and more drastically in F5 fibroblasts. The synthetic KHE nigericin restored mitochondrial morphological aberrations and membrane depolarization, coupling mitochondrial dysfunctions and impaired K^+ homeostasis.

Functional compensation analysis in yeast

Considering that LETM1 controls mitochondrial volume by regulating KHE, we ectopically expressed *LETM1* variants or wild-type in the yeast *S. cerevisiae yletm1*Δ strain to explore the functional impact of *LETM1* variants on mitochondrial KHE activity. All *LETM1* variants listed in the supplemental data (Primers used for site-directed mutagenesis) were included in this analysis. The loss of KHE activity in yeast *letm1* deletion mutants, the complementation by re-expression of the homologous human

LETM1 WT, and the absence of a Ca^{2+} transport system in *S. cerevisiae* mitochondria make the system ideally suited for functional complementation analysis of *LETM1* variants and determination of their pathogenicity with respect to KHE defects.

Light scattering recording of KOAc-induced swelling is a well-established method to measure the mitochondrial electroneutral exchange of K^+ for H^+.[48] Exposure of de-energized mitochondria to hypotonic KOAc buffer elicits the rapid uptake of protonated acetic acid, acidification of mitochondrial matrix, and thereby activation of KHE, which results in mitochondrial K^+ influx, water uptake and thus swelling.[45] Isolated mitochondria from *yLETM1* wild-type cells and *yletm1*Δ cells overexpressing *LETM1* WT or variants or the empty control vector were subjected to KOAc-induced swelling experiments. Recording KHE activity by measuring the decrease in optical density (OD) using light scattering techniques allows discrimination of its main determinants: initial OD, indicating the osmotic state of mitochondria before KOAc addition, and KHE exchange rate per second, indicated by the amplitude from initial to final OD as a function of the time required to achieve it. As shown in Fig 5A, KOAc-induced swelling was sensitive to the KHE inhibitor quinine, confirming the correlation of optical density with KHE activity. Knockout of *yLETM1 (yletm1*Δ) entirely abolished KHE activity, as illustrated by low initial OD and swelling amplitude, which were restored by expression of *LETM1* WT. The non-pathogenic variants (p.Ile305Leu and p.Lys587Arg) performed as well as *LETM1 WT* for the initial OD, and almost as well for the kinetics values. LETM1 with the mutation Val691Argfs* (F10) almost restored the initial OD, so did *LETM1* Lys587Arg (F9) and Arg393His (F6) but their swelling amplitudes were very low. Expression of *LETM1* variants c.754-756del, p.Lys252del (F3), c.878T>A, p.Ile293Asn (F1:M), or c.2220G>C, p.*740TyrextTer26 (F2, F7) marginally compensated K^+ fluxes with extremely slow swelling kinetics; Swelling traces for *yletm1*Δ transformed with *LETM1* c.881G>A, p.Arg294Gln (F4, F8) or *LETM1* c.2094del, p.Asp699Metfs*13 (F1:F) suggested uncontrolled cation leakage (Figure 5A). Overexpression of *LETM1* c.1072G>A, p.Asp358Asn did not rescue KHE. Taken together, these results suggest that mitochondrial reduced K^+ flux dynamics and swollen matrix are indicative of the functional impact of disease-associated *LETM1* variants.

LETM1 protein levels associated with *LETM1* variants were examined using total cell lysates and isolated mitochondria. In comparison to the mitochondrial loading control (Porin, Por1p), LETM1 total protein levels from ectopic *LETM1* WT or variant expression were similar, except those from F1:S1-S2 (*LETM1* c.878T>A; c.2094del, p.Ile293Asn; Asp699Metfs13*), F2, F7 (both *LETM1* c.2220G>C, p.*740Tyrext26) and F11 (*LETM1* c.898C>T, p.Pro300Ser) which showed reduced LETM1 levels in mitochondria (Figure 5B upper panel). The levels of LETM1 from F5 *LETM1* variant (c.1072G>A, p.Asp358Asn) were also low, but not when normalized to Por1p, which was similarly decreased (Figure 5B). LETM1 levels from the variants identified in F3 (c.754-756del, p.Lys252del) and in F4, F8 (both c.881G>A, p.Arg294Gln) were detectable in total lysates and mitochondria prepared from a large-scale intracellular fractionation (Figure 5B lower right panel) but were also reduced. None of the ectopic expression of *LETM1* variants however affected the mitochondrial subcellular localization.

As previously noticed[17,49,50] and shown here (Figure 5B), Cox2p (subunit of CIV) is reduced in *yletm1*Δ strains. Cox2p levels were restored upon ectopic expression of *LETM1* WT or *LETM1* c.878T>A, p.Ile293Asn (F1:M), *LETM1* c.2071-9C>G, p.Val691fs4* (F10) or *LETM1* c.878T>A; c.2094del, p.Ile293Asn; Asp699Metfs13* (F1:S1, F1:S2), but remained absent upon expression of *LETM1* c.754-756del, p.Lys252del (F3), *LETM1* c.881G>A, p.Arg294Gln (F4,8) or *LETM1* c.1072G>A, p.Asp358Asn (F5) (Figure 5B).

*yletm1*Δ shows poor growth on non-fermentable (YPG) substrate.[42] To determine the significance of the *LETM1* variants in rescuing the growth defects of *yletm1*Δ compared to *LETM1* WT, serial dilutions of *yletm1*Δ strains overexpressing an empty plasmid or *LETM1* variants or WT were spotted onto fermentable (YPD) and non-fermentable (YPG) plates and grown at 30 or 37°C (Figure S4). We found a detrimental effect of the mutant phenotype by ectopic expression of *LETM1* c.1072G>A, p.Asp358Asn (F5) variant; this strain was able to grow on selective media but showed worsened growth defect on complete media. Growth was also slowed down at 37°C on YPD by *LETM1* c.898C>T, p.Pro300Ser (F11). On YPG, a marginal rescue was obtained by ectopic expression of *LETM1* c.881G>A,

493 p.Arg294Gln (F4, F8), *LETM1* c.2071-9C>G, p.Val691fs4* (F10) or LETM1 c.878T>A; c.2094del,
494 p.Ile293Asn; Asp699Metfs13* (F1:S1, F1:S2) variants.

495 In summary, ectopic expression in y*letm1*Δ of *LETM1* variants associated with clinical presentations
496 phenocopied *yletm1* loss-of-function, whereas expression of wild-type LETM1 restored the yeast
497 deletion defects in non-fermentable growth and mitochondrial KHE exchange.

498

499 **Discussion**

500 The function of LETM1 is required for the maintenance of mitochondrial cationic and osmotic balance,
501 and swelling of the matrix due to impaired LETM1 has far-reaching consequences. Matrix swelling is
502 supported by the unfolding of inner membranes and loss of cristae invaginations and results in dilution
503 of metabolic substrates. Here, we found that bi-allelic *LETM1* variants identified in the affected
504 individuals with severe clinical features resulted in altered LETM1 levels and the typical aberrant
505 mitochondrial morphology previously described for LETM1-deficient cells. Several OXPHOS subunits
506 were downregulated in fibroblasts or muscle tissue, enzymatic activities were reduced, and mtDNA
507 copy number increased. The fact that nigericin, the synthetic KHE, restored morphological aberrations
508 interconnects these phenotypes to impaired K^+ homeostasis. Decreased membrane potential or
509 increased sensitivity to valinomycin and normalization of this sensitivity by nigericin supports the
510 presence of a defect in K^+/H^+ exchange. Moreover, it is tempting to speculate that OXPHOS decreases
511 proportionally to cristae loss. The finding that loss of KHE activity in *LETM1* defective yeast was
512 restored by ectopic expression of wild-type *LETM1* but not *LETM1* variants strongly support the notion
513 of deregulated mitochondrial K^+ homeostasis caused by the *LETM1* variants. Whether and how Ca^{2+}
514 handling is also perturbed will need to be determined in future studies. Together with the fibroblasts,
515 muscle biopsy and yeast analyses, and with the prior knowledge that the mitochondrial phenotypes
516 in cells match those caused by LETM1 haploinsufficiency, knockdown, or deletion in other eukaryotic

517 species, the present findings amount to compelling evidence that the bi-allelic *LETM1* variants are the
518 cause of the disease in the pedigrees reported in this study.

519

520 **Diseases of mitochondrial morphology and dynamics**

521 Dysfunctional mitochondria result in a large group of clinically heterogeneous MD. Defects in the
522 components, assembly factors, and ancillary proteins of the OXPHOS encoded by the mitochondrial
523 and nuclear DNA account for a large proportion of MD and have been defined as primary MD (PMD).
524 Additionally, defects in non-OXPHOS genes responsible for mitochondrial homeostasis including
525 mitochondrial fission and fusion have also been suggested to cause PMD.[51] Primarily targeting the
526 non-bioenergetic capabilities of the mitochondria, non-OXPHOS gene defects could indirectly affect
527 the OXPHOS system.[52] Hence, the phenotype of non-OXPHOS gene defects could mimic the
528 inactivation of RCE.[12] Indeed, the clinical presentation of MD resulting from OXPHOS gene defects and
529 non-OXPHOS gene defects might significantly overlap ranging from single organ to multiple organ
530 involvement.[52]

531 Whilst there is a plethora of non-OXPHOS genes accounting for PMD,[51,53,54] examples relevant to the
532 context of the present study are the genes regulating mitochondrial shape and interorganellar
533 communication. They regulate mitochondrial dynamics through fusion and fission processes. Defects
534 in these genes have been emerging as a cause of a novel class of inherited neurodegenerative
535 disorders with variable onset ranging from infancy to adulthood.[53,54] Residing in the outer and inner
536 mitochondrial membranes or the cytosol, upon misregulation, they cause altered mitochondrial
537 morphology including matrix swelling, fragmentation, elongation, and abnormal cristae structure,
538 similar to what has been observed in abnormal LETM1 function.[53,54,55,56] Reviews of the disease-
539 causing genes responsible for mitochondrial dynamics are provided in Burté, et al.[53] and
540 Navaratnarajah et al.[54] To date, affected individuals diagnosed with diseases of mitochondrial
541 dynamics present first and foremost with neurological symptoms.[53,54] Being essential for the survival

of all organisms tested so far and having important control over the mitochondrial osmotic balance, morphology, and dynamics, before now, bi-allelic variants in *LETM1* have not been associated with any Mendelian disorder in humans.

Bi-allelic *LETM1* variants present with a phenotypic spectrum of MD largely involving the central nervous system

Here we report on the first association of bi-allelic *LETM1* variants with a spectrum of predominantly infantile-onset neurological, metabolic, dysmorphic, and multiple organ dysfunction syndromes in a cohort of 18 affected individuals from 11 unrelated families. Overall, the disease had a progressive course, though with variable rates of deterioration. Hence, the disease progression varied from rapid, as in families 3, 4, and 9-11 to a slow deterioration as in the oldest cases from families 1, 2, and 8. Similar to the clinical presentation of the defective mitochondrial dynamics genes, bi-allelic *LETM1* variants were associated with an infantile-onset neurodegenerative disorder with a complex phenotype as described for *DNM1L*/*DRP1* (Dynamin 1 like [MIM: 603850]), *OPA1* (OPA1 mitochondrial dynamin-like GTPase [MIM: 605290]), *OPA3* (Outer mitochondrial membrane lipid metabolism regulator OPA3 [MIM: 606580]), *MFF* (Mitochondrial fission factor [MIM: 614785]) and *MSTO1* (Misato Mitochondrial Distribution And Morphology Regulator 1 [MIM: 617619]).[53,54,55,56] The shared phenotype mainly included global developmental delay, regression, and neurosensory impairment combined with neuromuscular symptoms, cerebellar ataxia, seizures, and early mortality. Akin to defects in *OPA3,* 3-methylglutaconic aciduria was a frequent finding in the subjects with bi-allelic *LETM1* variants.[57] Bilateral cataracts and facial dysmorphism observed in the present *LETM1* cohort have also been reported in cases with defective *OPA3* and *MSTO1* genes respectively.[55,56]

All cases with RCE analysis results in the present study showed defects in the OXPHOS system suggesting that *LETM1* defects can affect the mitochondrial ability to generate ATP. This in turn might have mimicked the clinical presentation of OXPHOS MD. Therefore, distinguishing the *LETM1*

phenotype from OXPHOS MD or the aforementioned diseases of mitochondrial dynamics can be challenging without the help of genetic testing, particularly in cases with a rapidly progressive disease course.

The phenotype of defective *LETM1* and WHS

Monoallelic *LETM1* deletion has been suggested to be responsible for epilepsy and neuromuscular features in WHS.[5,19,21,58] Indeed, the current *LETM1* cohort presented with hypotonia and epilepsy. Additionally, though, cases with bi-allelic *LETM1* variants showed a milder spectrum of WHS signs that has not been previously ascribed to the *LETM1* deletion. These included thin habitus, low set ears, microcephaly, micrognathia, and low body weight.[59,60] It has been previously speculated that the most probable cause of growth deficiency, microcephaly, and the characteristic facial features in WHS is due to haploinsufficiency of *WHSC1*, a region located far from *LETM1*.[61] The expression of mild non-neurological symptoms of WHS in our cohort could be due to either putative interaction between *LETM1* and *WHSC1* or other undiscovered mechanisms, including those intrinsically caused by *LETM1* deficiencies.

We have observed some degree of clinical overlap between the presentation of defective *LETM1* and small interstitial deletions in WHS presenting with a milder phenotype. The latter presents with a variable degree of growth and neurodevelopmental delay, microcephaly, thin faces with dysmorphic features, intellectual disability, language impairment, and seizures.[62,63,64] Interestingly, cases with small 4p16.3 deletions encompassing *LETM1* suggested that *LETM1* might not be responsible for seizures in WHS as some cases with *LETM1* deletion did not have seizures by the age of 4 and 9 years, whereas cases with preserved WSHCR-2 including *LETM1* developed seizures.[64] Previous retrospective analysis suggests that several other genes in the terminal 4p region might potentially be involved in seizures in WHS.[5]

Clinical features including lactic acidosis, diabetes, cataract, neuropathy, and proximal myopathy combined with cerebellar ataxia, progressive spastic-ataxic gait, hyperkinetic movement disorders, and pontine/cerebellar atrophy were among the signs of the defective *LETM1* phenotype that are not typical of WHS; instead, they are more typical of archetypal mitochondrial disorders.

Although there have been a handful of reports on microdeletions in WHS describing genotype-phenotype correlations, the association between the specific symptoms of WHS and *LETM1* remains to be fully determined. To understand the full contribution of *LETM1* in WHS cases, further studies would be needed to investigate which phenotypes of WHS can be restored by the re-expression of *LETM1*. Apart from this, the identification of phenotypes that were consistent with both *LETM1* haploinsufficiency in WHS and *LETM1* bi-allelic variants will advance our understanding of the contribution of *LETM1* in WHS.

Genotype-phenotype correlation of bi-allelic *LETM1* variants

This is the first report of bi-allelic *LETM1* variants associated with a human disorder, and the first study to investigate their functional significance using primary fibroblasts, muscle tissue, and *S. cerevisiae*. Similar to *LETM1* knockdown,[13] the bi-allelic *LETM1* variants caused mitochondrial swelling and loss of cristae structures.

The general distribution of the missense and frameshift variants to the highly conserved LETM domain and the C-terminal coiled coils, together with their comparable deleterious effects on mitochondrial morphology and KHE function support the correlation of mitochondrial morphologic defects and imbalanced cation homeostasis. A previous variant analysis of the LETM domain found that Asp359 or the triple combination of Arg382, Gly383, and Met384 is necessary for the organization of cristae structure and growth complementation of *yletm1*Δ strains.[65] The missense variant c.1072G>A, p.Asp358Asn identified here in family 5, which impaired mitochondrial morphology and KHE activity,

615 is adjacent to Asp359. Based on cell-free data showing that the reconstituted LETM domain was
616 sufficient to induce cristae invagination, Nakamura et al.[65] concluded that cristae disorganization due
617 to the single or triple variant occurred independently of ion homeostasis. Our findings are not in
618 contradiction but propose that a regulatory contribution to cristae architecture by the LETM domain
619 may depend on the swelling state of mitochondria in the cellular context.

620 Given the growing consensus that the hallmark of LETM1 deficiency is mitochondrial cation imbalance,
621 we used yeast as a model organism to analyse mitochondrial KHE activity of *LETM1* variants from
622 patients and *LETM1* variants not associated with disease. Based on the results, we propose that light
623 scattering experiments that capture mitochondrial volume status and kinetics of K^+/H^+ exchange are
624 useful to predict the pathogenic potential of *LETM1* variants (Figure S5).

625 Linking clinical features with *in vitro* data, we found that fibroblasts expressing *LETM1* variants
626 c.878T>A; c.2094del, p.Ile293Asn; Asp699Metfs13*, which were identified in the individuals F1:S1 and
627 F1:S2 affected with epilepsy, neurosensory deficiencies, and diabetes, displayed mitochondria with
628 disturbed morphology and membrane potential, reduced LETM1 levels and a severe decrease in
629 respiratory proteins of CI and CIV. Ectopic expression of the variants in yeast marginally rescued
630 mitochondrial KHE activity. Cases harboring the variant c.2071-9C>G, p.Val691fs4* (F10) showed rapid
631 clinical progression and deceased before reaching 1 year of age. Fibroblasts from this case displayed
632 high LETM1 protein levels, indicating that the pathogenic variant and not the lack of protein was
633 associated with the severe phenotypes. Ectopic expression of this variant failed to rescue wild-type
634 KHE activity. The abundance of this non-functional *LETM1* variant suggests that it likely escaped the
635 nonsense-mediated decay as the gained stop codon falls into the last exon.[66] The variant c.898C>T,
636 p.Pro300Ser was identified in family 11 leading to a severe early infantile disease in the homozygous
637 state. Fibroblasts and muscle lysates from those cases showed reduced CI and CIV proteins. Drastic
638 growth defects and lack of KHE activity were induced by this variant in yeast, which could somehow
639 explain the severe clinical conditions caused by this variant. *LETM1* c.2220G>C, p.*740Tyrext26 was

identified in several subjects from F2 and F7 with developmental delay, walking difficulties and, seizures. Fibroblasts from F2:S1 exhibited swollen and fragmented mitochondria and hardly detectable LETM1 protein levels. Ectopic expression in yeast displayed somewhat reduced LETM1 protein levels and poorly improved KHE activity. Since the KHE uses the proton gradient generated by the respiratory chain to drive K^+ flux, and LETM1 is likely involved in the insertion of mitochondrial encoded OXPHOS proteins into the membrane, it is surprising that the reduction of this LETM1 variant did not correlate with decreased OXPHOS components. There are several possible explanations for this. The OXPHOS effects of LETM1 deficiency could be secondary, the OXPHOS components, although not reduced, could not be assembled as efficiently, or genetic compensatory mechanisms could be involved. The affected individual carrying the homozygous variant *LETM1* c.1072G>A, p.Asp358Asn (F5) presented defects in neurosensory functions and Type 3 diabetes. We found that the proliferation of F5-derived fibroblasts was severely impaired. Similarly, yeast growth was also reduced by this variant, and mitochondrial KHE activity could not be restored. Compared to the other variants, c.1072G>A, p.Asp358Asn had the most deleterious effects on mitochondrial morphology, cell proliferation, and KHE activity, predicting this variant to have the most severe consequences. However, the viable state of the affected individual also here raises the possibility of a potential genetic compensatory background. In this respect, increased mtDNA copy number, often considered as an efficient way to overcome OXPHOS deficiencies in diseases and aging,[67] or elevated citrate synthase activity found in muscle specimens may indicate such a compensatory pathway (Figure 4C). Further examination will be required to clarify molecular compensatory mechanisms.

Other *LETM1* variants were analyzed in yeast, as fibroblasts from affected individuals were not available. Yeast data revealed poor complementation of *yletm1*Δ by *LETM1* c.754-756del, p.Lys252del, a variant identified in affected individuals with a neurological, neuromuscular, and craniofacial presentation, rapid progression, and eventually death (F3). Ectopic expression of *LETM1* c.881G>A, p.Arg294Gln, identified in cases with variable disease progression (F4, F8) but similar neuromuscular deficiencies was not able to restore the activity of the mitochondrial KHE, since the swelling traces

revealed continuous but very slow kinetics indicating minimal KHE activity per time unit, thus suggesting leaky mitochondrial membranes. Yeast growth was also impaired by overexpression of this variant. Phenotypic data were rather consistent with severe clinical presentation and early demise in F4.

The affected individual from family 9 was homozygous for the *LETM1* variant c.1139G>C, p.Arg380Pro and presented respiratory insufficiency, epileptic encephalopathy, neuromuscular disorder, and rapid disease progression. The missense variant is located in the middle of the LETM domain, in proximity to the three highly conserved amino acid residues: Arg382, Gly383, Met384 described in Nakamura et al.[65] (Figure 2B), supporting an essential functional role of the LETM stretch between residues 380 and 384.

LETM1 role in cation homeostasis and neurodegenerative phenotype of the cohort

Among the mitochondrial EF-hand-containing proteins, LETM1 has been identified as essential across several cell lines in genome-wide essentiality screens.[68,69] Functionally, LETM1 is required for maintaining mitochondrial homeostasis of K^+, an osmotic and cellularly most abundant cation, and was considered an essential component of the KHE. After LETM1 was identified in a genomic *Drosophila* RNAi screen for mitochondrial CHX, it has been suggested to catalyze the exchange of Ca^{2+} against H^+ in both directions in a ruthenium red-sensitive pattern,[70] which is difficult to reconcile with the CHX, and has been implicated in the pathogenesis of Parkinson's disease through interaction with PINK1.[71] The mitochondrial CHX is part of the mitochondrial Ca^{2+} release system, which compensates for electrophoretic mitochondrial Ca^{2+} uptake mainly through H^+- or Na^+-dependent Ca^{2+} extrusion. While the role of LETM1 as a mitochondrial KHE or CHX has remained controversial, deregulation of the mitochondrial KHE has been shown to affect mitochondrial Ca^{2+} buffering by impacting the Na^+-dependent Ca^{2+} release pathway.[72] Proper maintenance of mitochondrial Ca^{2+} levels is critical to neurons, synaptic function, and neurodevelopment with mishandled mitochondrial Ca^{2+} levels posing

691 a risk of synaptopathies. In turn, synaptopathies may be a harbinger of neurodegenerative disorders.[73]
692 The neurodegenerative phenotype observed in the present LETM1 cohort could partially be explained
693 by impaired mitochondrial Ca^{2+} buffering and ensuing glutamate excitotoxicity, generation of reactive
694 oxygen species, and apoptosis.[74] Consistent with previous studies,[13, 14, 50, 75] exposure to nigericin or
695 ketone bodies improved the mitochondrial morphological phenotypes of fibroblasts from affected
696 individuals, supporting the link between *LETM1* variant and impaired cation homeostasis. While
697 nigericin enables K^+-H^+ exchange and prevents accumulation of matrix K^+, ketone bodies may bypass
698 the deficient Ca^{2+}-dependent catalytic function of the pyruvate dehydrogenase.

699 Unlike yeast LETM1, LETM1 orthologs of more complex organisms possess EF-hands, which may
700 implicate LETM1 in Ca^{2+} sensing or regulation.[76] Focusing on K^+ analysis using yeast, we did not
701 investigate the impact of the reported bi-allelic *LETM1* variants on the mitochondrial Ca^{2+}
702 homeostasis. This would need to be investigated in further studies as it might have future therapeutic
703 implications.[73]

704 Collectively, our results demonstrate that bi-allelic pathogenic *LETM1* variants are associated with
705 defective mitochondrial K^+ efflux, swollen mitochondrial matrix structures, and a reduction in proteins
706 levels and activity of the electron transfer chain. The former highlights the implication of perturbed
707 mitochondrial osmoregulation caused by bi-allelic *LETM1* variants in neurological and mitochondrial
708 pathologies. Data showing that mitochondrial KHE activity is maintained above a functional threshold
709 in non-pathogenic variants suggest that such functional yeast assays could be implemented to
710 routinely determine the pathogenicity of a variant. While the beneficial effect of nigericin
711 strengthened the link to KHE defects, that of ketone bodies, consistent with Durigon et al,[14] supports
712 the promising therapeutic role of ketogenic-based diets.

713

714 **Supplemental data**

Supplemental data include case reports, four figures, four tables, methods, and videos with their descriptions.

Supplemental video legends

Video 1 shows F1:S1 who is non-verbal. She wears glasses and hearing aids. Teeth abnormalities could be seen. Her gait is ataxic and with support only.

Video 2 shows F1:S1 4 years later. Progression in gait impairment could be seen.

Video 3 shows F1:S2 sitting on an armchair. He wears glasses and hearing aids. He is able to understand some questions with a delay and obeys commands. Hearing is impaired. His speech is spastic-dysarthric. He has teeth abnormalities and bilateral clonus of the ankles.

Video 4 shows F1:S2 4 years later. He has got jerky movements in the outstretched arms.

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Declaration of interests

The authors declare no competing interests.

Web Resources

gnomAD, ttps://gnomad.broadinstitute.org/

Ensemble, https://www.ensembl.org/index.html

Uniprot, https://www.uniprot.org/

GeneMatcher, https://genematcher.org/

OMIM, https://www.omim.org/

Iranome, http://www.iranome.ir/

Varnomen, http://varnomen.hgvs.org/

Varsome, https://varsome.com/

Brain RNA-seq, http://www.brainrnaseq.org/

ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/

ClinVar (summary of submissions), https://www.ncbi.nlm.nih.
gov/clinvar/submitters/26957

Database of Genome Variants, http://dgv.tcag.ca/

FlyBase, http://flybase.org/

UKBB, https://www.ukbiobank.ac.uk/

TOPMed, https://bravo.sph.umich.edu/freeze8/hg38/gene/snv/LETM1

Data and code availability

The pathogenic variants identified in this work have been submitted to ClinVar with accession numbers: SCV001981656, SCV001981657, SCV001981658, SCV001981659, SCV001981660, SCV001981661, SCV001981662, SCV001981663, SCV001981664, SCV001981665

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Figure legends

Figure 1. Clinical features and neuroimaging findings of the cases with bi-allelic *LETM1* variants

(A) From left to right facial photos of the affected individuals F1:S1, F1:S2, F5:S1, F8:S1. All cases wear glasses due to bilateral optic atrophy. All cases have prominent noses. F1:S1 and F1:S2 show long thin faces, low-set ears, and teeth abnormalities.

(B) In A (F1:S1), severe cerebellar atrophy (arrows) and pontine hypoplasia (arrowheads) are shown, while in B (F6:S1) only mild vermian hypoplasia is noted. In C arrowheads point at the severe optic nerve and chiasm atrophy in 2 different cases (F5:S1 and F7:S2). Mild ventricular dilatation is present in D (F3:S1).

(C) Clinical features of the affected individuals with bi-allelic *LETM1* variants.

GDD, global developmental delay; ID, intellectual disability; MRI, magnetic resonance imaging; MRC, mitochondrial respiratory chain.

Figure 2. Pedigrees with segregations of *LETM1* variants and LETM1 protein architecture with a partial sequence alignment of the variants.

(A) Family trees of the cases with bi-allelic *LETM1* variants. Square: male, circle: female, black symbols: affected individuals, white symbols: unaffected individuals. M, maternal; P, paternal.

(B) Schematic representation of the human *LETM1* gene organization in introns, shown as a line, and exons, shown as boxes, and of LETM1 protein domains as indicated by the residue numbers and the color code: coiled-coil motifs (light yellow), transmembrane helices (blue), LETM/ribosomal-binding like domain (lavender) and putative EF-hands (green). All identified missense variants in the affected cases (black) and non-pathogenic variants (blue) are mapped according to their positions. The amino acid sequence of human LETM1 was aligned with LETM1 orthologs using Clustal Omega and alignments with LETM1 from other species are shown for all segments that contain missense variants, indicated in bold red letter. Residue conservation is shown below the alignment as fully conserved (*), highly conserved (:) or partially conserved (.). UniProt accession numbers for the *H.s*. (*H. sapiens)*, *M.m*. (*M. musculus)*, S.c. *(S. cerevisiae)*, *D.r*. *(D. rerio)*, *C.e*. *(C. elegans)*, *D.m*. *(D. melanogaster)* and *A.t. (A. thaliana)* LETM1 used in this alignment are O95202, Q9Z2I0, Q08179, Q1LY46, Q9XVM0, P91927 and F4J9G6 respectively.

Figure 3. Effects of *LETM1* variants on mitochondrial morphology and proliferation in fibroblasts

A) *LETM1* variants perturb the mitochondrial network

Confocal images of fibroblasts stained with Mitotracker Red. Shown is a representative overview of the cells (Bars: 5 μm, except F10 10 μm) and details magnified from the box (Bars: 5 μm). C1, C2: healthy donors; F1:S2 c.878T>A, p.Ile293Asn and c.2094del, p.Asp699Metfs*13; F2 c.2220G>C, p.*740TyrextTer26; F5 c.1072G>A, p.Asp358Asn; F10 c.2071-9C>G, p.Val691fs4*. Arrow indicates fragmented mitochondria. For statistics, see Fig S1C.

B) *LETM1* variants cause swollen mitochondria and loss of cristae

The ultrastructure of control (C1) and case (F5 and F10) fibroblasts was investigated by transmission microscopy and images show overviews (left panels, bars: 2 μm) and details (right panels, bar 500 nm). Arrow indicates swollen mitochondria.

C-D) Variants differently affect LETM1 stability and OXPHOS proteins in fibroblasts samples

Total lysates of fibroblasts were analyzed by immunoblotting using the indicated antibodies, GAPDH, or β-actin served as loading control; C2, C3: healthy donors; F1:S1 and F1:S2 c.878T>A and c.2094del, p.Ile293Asn and p.Asp699Metfs*13; F2 c.2220G>C, p.*740TyrextTer26; F10 c.2071-9C>G, p.Val691fs4*; F11 c.898C>T p.Pro300Ser (**C)**. Quantitative graphs from independent experiments representing the protein bands, normalized to the housekeeping proteins, and calculated as a percentage in relation to the controls, data are expressed as mean ± SEM (N=>3 independent experiments). Two-way ANOVA with Dunnet`s multiple comparisons test of F2, F10 against controls (fibroblasts from healthy donors) *p<0.02, **p<0.007, ***p<0.0003, ****p<0.0001, while for F1:S1, F1:S2, F11 as follow; ***P (LETM1 Controls vs F1:S1 or F1:S2 or F11:S2)< 0.0001; **P (Complex I Controls vs F1:S1) = 0.0017, ***P (Complex I Controls vs F1:S2) = 0.0006, and ***P (Complex I Controls vs F11:S2) < 0.0001; *P (Complex IV Controls vs F1:S1) = 0.0229, *P (Complex IV Controls vs F1:S2) = 0.0317 and *P (Complex IV Controls vs F11:S2) = 0.0009; **(D)**.

E) Fibroblast proliferation is prevented by *LETM1* c.1072G>A, p.Asp358Asn

Control (C1 and C2) and case (F5, F10) fibroblasts were grown for 8 days and counted every second day. Cell numbers on day 8 were plotted and the statistical significance was calculated using one-way ANOVA with Dunnett`s multiple comparison test from three independent experiments, n=3, data are means ± SD, ns P > 0.05, **P= 0.0039, ****P< 0.0001.

Figure 4. *LETM1* variants affect the stability of LETM1 and OXPHOS components in muscle samples

A-B) Western blot analysis of LETM1 and components of the OXPHOS complexes I, II, III; and IV in muscle samples from F11 and quantitative graphs

Total lysates of muscle samples from healthy donors (C4, C5) and F11 c.898C>T p.Pro300Ser (S1, S2) were analyzed by immunoblotting using the indicated antibodies, VDAC served as a loading control

(A). Quantitative graphs represent the protein levels relative to controls and normalized to VDAC Data are expressed as mean ± SEM. n=> 3 independent experiments. Two-way ANOVA with Dunnett`s multiple comparisons test. ***P (LETM1 Controls vs F11:S1 and F11 S2) = 0.0004; ***P (CORE2 Controls vs F11:S1) = 0.0001 and ***P (CORE2 Controls vs F11:S2) < 0.0001; ***P (NDUFA9 Controls vs F11:S1 and F11 S2) < 0.0001 *P (SDH Controls vs F11:S1) = 0.0453 and ***P (SDH Controls vs F11:S2) < 0.0001; ***P (COX4 Controls vs F11:S1)= 0.0002 and **P (COX4 Controls vs F11:S2)= 0.0024 **(B)**.

C) Immunohistochemical staining of OXPHOS subunits and VDAC of the muscle of F5 and controls

Muscle samples from healthy donors (C6, C7) and F5 c.1072G>A, p.Asp358Asn were stained for each of the five OXPHOS subunits using the indicated antibodies, VDAC served as a control. Magnification 400 x.

D-E) Western blot analysis of subunits of the OXPHOS complexes, citrate synthase, and GAPDH of the muscle of F5 and controls

Total lysates of muscle samples were analyzed by immunoblotting using the indicated antibodies, VDAC, GAPDH, and CS served as loading controls. C6: healthy donor, F5: c.1072G>A, p.Asp358Asn **(D).** Quantitative graphs representing the protein levels percentage relative to controls (normalized to GAPDH). The statistical significance was calculated using one-way ANOVA with Dunnett`s multiple comparison test, data are means ± SD, N=>3 independent experiments, *P=0.0201, **P=0.0011, ****P< 0.0001.

Figure 5. Functional implication of *LETM1* variants on yeast mitochondria.

A) *LETM1* variants fail to restore KHE activity of yeast *yletm1* Δ

Isolated and de-energized mitochondria were subjected to KOAc and changes of optical density at OD_{540} immediately measured. Upper panel**:** representative traces of KOAc-induced swelling in *yLETM1* WT mitochondria (WT, blue), *yletm1*Δ mitochondria overexpressing the empty plasmid (e, yellow) or

the plasmid carrying LETM1 WT untreated (w, green) or treated (wq, grey) with quinine or the *LETM1* variants: c.754-756del, p.Lys252del (1, red), c.878T>A, p.Ile293Asn (2, bottle green), c.881G>A, p.Arg294Gln (3, aqua), c.898C>T, p.Pro300Ser (4, dark green), c.913A>C, p.Ile305Leu (5, lavender), c.1072G>A, p.Asp358Asn, (6, violet), c.1139G>C, p.Arg380Pro (7, beige), c.1178G>A, p.Arg393His (8, turquois), c.1760A>G, p.Lys587Arg (9, mauve), 2071-9C>G, p.Val691fs4* (10, purple), c.2094del, p.Asp699Metfs*13 (11, dark blue), compound (12, lila) c.2220G>C, p.*740TyrextTer26 (13, olive). Quantified rates of KOAc-induced swelling from 3 independent experiments. Data are means ± SD. One-way ANOVA with Dunnett's multiple comparisons test performed against *yletm*Δ transformed with empty pVT-103U plasmid *p= 0.0426, ** p = 0.0026, ***p= 0.0006, ****p< 0.0001. And for Ile305Leu Lys587Arg relatively to *yletm*Δ transformed with WT, ns >0.05, *p= 0.0169.

B) ectopic expression of *LETM1* variants in *yletm1* Δ

Isolated mitochondria (upper panel) and total protein lysates (lower panel left) from the same strains as in **A)** and subcellular fractions T: total, SN: post-mitochondrial supernatant, M: mitochondria (lower panel right) were immunoblotted using the indicated antibodies, Por1p and Act1p served as mitochondrial and total (and SN) loading control, respectively.

Table 1. Summary of *LETM1* variants identified in the present cohort and 2 non-pathogenic variants

F ID	Center	Method	gDNA Change (chr4 hg 19)	Function	NT change	aa change	gnomAD V3.1.2 and V2.1.1	Other databases	CADD	GERP	SIFT	Poly-Phen	Clinical significance (based on the ACMG criteria)
1	Queen Square Genomics	Proband only ES "Horga. [25]" "Makrythanasis et al.[26]", "Poole et al.[27]"	g.1834673A>T	missense, splice region	c.878T>A	p.Ile293Asn	0	0	28.8	4.61	D	PD	P (PVS1, PS3, PS4, PM2, PM3)
			g.1816277T-	frameshift	c.2094del	p.Asp699Metfs*13	0	1 het allele (UKBB)	-	-	-	-	P (PVS1, PS3, PS4, PP3, PM4)
2	Copenhagen University Hospital	Proband only ES "Barington et al.[28]"	g.1816151C>G	stop_loss	c.2220G>C	p.*740Tyrext	0	0	-	-	-	-	P (PS3, PS4, PM2, PM4)
7	Queen Square Genomics	Proband only ES "Makrythanasis et al. [26]", "Poole et al.[27]"											
3	Wellcome Centre for Mitochondrial Research	Proband only ES "Van Bergen et al.[29]"	g.1836692CTT-	inframe deletion	c.754_756del	p.Lys252del	0	0	-	-	-	-	P (PS3, PS4, PM2, PM4, PP3, PP1)
4	Wellcome Centre for Mitochondrial Research	Proband only ES "Van Bergen et al.[29]"	g.1834670C>T	missense	c.881G>A	p.Arg294Gln	4 het alleles (V2.1.1); 2 het alleles (V3.1.2.)	2 het alleles (UKBB); 3 het alleles (GeneDx); 2 het alleles (TOPMed)	26.3	4.61	D	PD	P (PP1, PS3, PS4, PP3)
8	Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan	Proband only ES "Catania et al.[24]"											
5	Institute of Medical Genetics and Applied Genomics, University of Tuebingen, Germany	Proband only ES "Froukh et al.[30]"	g.1834479C>T	missense	c.1072G>A	p.Asp358Asn	0	0	23.5	4.61	D	B	P (PS3, PS4, PM2, PP3)
6	GeneDX	Trio ES "Rettere et al.[31]", "Bai et al.[32]", "Richards et al.[33]"	g.1827313-C-T*	missense	c.1178G>A	p.Arg393His	13 het alleles (V2.2.1.1); 2 het alleles (V3.1.2)	AF 0.0002 (1K GP); 1 het allele (UKBB); 7 het alleles (GeneDx); 7 het alleles (TOPMed)	26.6	5.06	D	PD	P (PS3, PS4, PP3)
9	Exeter Genomics Laboratory	Trio ES "Williamson et al.[34]"	g.1827352C>G	missense	c.1139G>C	p.Arg380Pro	0	1 het allele (UKBB)	27.4	5.06	D	PD	P (PS3, PS4, PP3)
10	Institute of Human Genetics Technical University of Munich	Proband only ES "Kremer et al.[35]"	g.1814582G>C	splice defect	c.2071-9C>G	p.Val691fsTer4	0	0	-	-	-	-	P (PS3, PS4,PP3, PM2, PM4)
11	Bambino Gesù Children's Hospital, IRCCS	NGS proband only "Calvo et al.[36]" "Legati et al.[37]" "Saoura et al.[38]"	g.1834653G>A	missense	c.898C>T	p.Pro300Ser	0	2 het alleles (GeneDx)	25.8	4.61	D	PD	P (PS3, PS4, PP3)
Non-pathogenic variant 1			g.1834638T>G	missense	c.913A>C	p.Ile305Leu	1 het allele (V2.1.1); 3 het alleles, 1 hom allele (V3.1.2)	4 het alleles (TOPMed)	27.6	4.61	D	PD	B (BS1 BS3)
Non-pathogenic variant 2			g.1818625T>A	missense	c.1760A>G	p.Lys587Arg	2756 het alleles, 39 hom alleles (V2.1.1); 2354 het alleles, 34 hom alleles (V3.1.2)	het alleles 43024.9, hom alleles 1; AF 0.002, 4 hom carriers (UKBB);	25.3	5.04	D	PD	B (BS1, BS3)

	4367 het alleles and 82 hom alleles (TOPMed)

LETM1 isoform is GenBank: NM_012318.3. F,family; ES,exome sequencing; gDNA. genomic DNA; NT, nucleotide; aa, amino acid; ACMG, American College of Medical Genetics; D, deleterious; PD, probably damaging; P, pathogenic; B, benign; AF, allele frequency; het, heterozygous; hom, homozygous; AF – allele frequency.
Other databases: Queen Square Genomics database (23K exomes), ESP, Iranome, 1K GP -1000 Genomes global minor allele frequency, UKBB - UK Biobank, GeneDx database, Middle Eastern database, TOPMed.
*A homozygous LETM1 variant due to maternal uniparental disomy.

Table 2 Clinical Features of Affected Individuals with Bi-allelic *LETM1* Variants

Family ID	F1		F2		F3		F4	F5	F6	F7		F8	F9	F10			F11	
Subject ID	S1	S2	S1	S1	S2	S3	S1	S1	S1	S1	S2	S1	S1	S1	S2	S3	S1	S2
Epidemiology and medical history																		
Gender and current age	F	M	M	F	M	M	M	M	F	M	M	F	F	F	M	F	F	M
Current age/death age	35y	25y	24y	D 1y	D 2.7y	D 1y	D 8y	11y	17m	15y	8y	39y	1y	D 10m	D 2m	D 2m	D 6y	D 4.5m
Age at onset	1y	1.5y	2.5y	4m	6m	4m	4m	7m	birth	1.5y	2y	10m	birth	4m	1m	birth	birth	birth
Type of progression	S	S	S	R	R	R	MD	MD	S	MD	MD	S	R	R	R	R	R	R
GDD/ID	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Regression in development	+	+	+	NA	-	NA	-	-	-	+	+	+	+	NA	NA	NA	+	+
Loss of ambulation (age)	+, 12y	+, 6y	NA	NA	NA	NA	+, 2.5y	-	na	+,5y	+,5y	+,2y	na	NA	NA	NA	NA	NA
Mortality	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+
Main clinical features																		
Age at last examination	35y	25y	24y	>1y	>1y	>1y	NA	11y	2m	15y	8y	37	1	NA	NA	NA	5y	NA
Small weight and height	+	+	+	NA	NA	NA	NA	+	-	+	+	+	+	NA	NA	NA	-	-
Facial dysmorphism	+	+	-	NA	NA	NA	-	-	+	-	-	+	-	NA	NA	NA	NA	NA
Optic atrophy/impaired vision	+	+	+	NA	+	NA	+	+	NA	+	+	+	NA	NA	NA	NA	+	NA
Cataract	-	-	+	NA	NA	NA	+	-	NA	-	-	+	-	NA	NA	NA	+	+
Sensorineural deafness	+	+	+	NA	+	NA	+	+	-	-	-	+	+	+	NA	NA	+	+
Hypotonia	-	-	-	+	+	+	+	+	-	-	-	+	+	-	+	+	+	+
Spasticity/ hypertonia	+	+	+	NA	NA	NA	-	-	-	+	+	-	-	+	+	+	-	-
Cerebellar ataxia	+	+	NA	NA	NA	NA	-	-	na	+	+	+	na	+	NA	NA	+	NA
Myopathy	-	-	-	NA	+	NA	+	+	NA	-	-	+	-	NA	NA	NA	+	+
Hyperkinetic movement disorders	+	+	+	NA	NA	NA	NA	-	-	-	-	-	-	+	NA	NA	-	-
Peripheral neuropathy	+	+	NA	NA	NA	NA	-	-	NA	-	-	-	-	NA	NA	NA	+	NA
Impaired speech/language abilities	+	+	+	NA	NA	NA	-	-	-	+	+	+	na	NA	NA	NA	NA	NA
Impaired/spastic/ataxic gait	+	+	+	na	na	no	-	-	na	+	+	+	na	NA	NA	NA	NA	NA
Seizures	+	+	+	NA	NA	NA	-	-	-	+	+	-	+	+	+	+	+	-
Cardiac involvement	-	-	-	NA	NA	+	+	-	-	-	-	-	-	NA	+	NA	+	+
Diabetes	+	+	-	NA	NA	NA	NA	+	-	-	-	-	-	NA	NA	NA	-	-
Lactic acidosis	-	-	-	NA	+	NA	+	+	NA	NA	NA	NA	-	+	+	+	+	+
Raised urinary 3-MGA	-	-	+	NA	-	NA	-	-	-	NA	NA	+	-	+	+	+	NA	NA
Investigations																		
MRC deficiencies	CI,II,III,IV	CI,IV	CII	NA	CI, II, III,IV	NA	NA	CI, III, IV	NA	NA	NA	CI, III, IV,V	CIV	CI, IV	NA	CI	CI, CIV	CI,IV
Muscle histochemistry	+	+	NA	NA	+	NA	NA	+	NA	NA	NA	+	+	NA	NA	NA	+	-
Brain MRI findings	CA, PA	NA	UR	VM	UR	NA	BA	ONA, CHA	CVH	NA	ONA, CHA	BA, CA	UR	NA	NA	NA	CVH, BSH,	NA

VM, DM

Abbreviations: F(number), family; S, subject; F, female; M, male; y, year; m, months; D, deceased; +, yes; -, no, NA, not available, not available, not suitable, or not performed; S, slow; MD, moderate; R, rapid; na, not applicable; GDD, global developmental delay; ID, intellectual disability; MCR, mitochondrial respiratory complex; C, complex; UR, unremarkable; 3-MGA, 3-methylglutaconic aciduria; CA, cerebellar atrophy; PA, pontine atrophy; VM, ventriculomegaly; BA, brain atrophy, ONA, optic nerve atrophy; CHA, chiasmal atrophy, CVH, cerebellar vermis hypoplasia; BSH, brain stem hypoplasia; DM, delayed myelination