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

Pereira Baptista, JDC

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

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4 Rauan Kaiyrzhanov,[1,38] Sami E. M. Mohammed,[2,38] Reza Maroofian,[1,38] Ralf A. Husain,[3,4] Alessia
5 Catania,[5] Alessandra Torraco,[6] Ahmad Alahmad,[7,8] Marina Dutra-Clarke,[9] Sabine Grønborg,[10]
6 Annapurna Sudarsanam,[11] Julie Vogt,[11] Filippo Arrigoni,[12] Julia Baptista,[13] Shahzad Haider,[14] René G.
7 Feichtinger,[15] Paolo Bernardi,[16] Alessandra Zulian,[16] Mirjana Gusic,[17,18,19,20] Stephanie Efthymiou,[1]
8 Renkui Bai,[21] Farah Bibi,[22] Alejandro Horga,[1,23] Julian A. Martinez-Agosto,[24] Amanda Lam,[25] Andreea
9 Manole,[1] Diego-Perez Rodriguez,[26] Romina Durigon,[26] Angela Pyle,[7] Buthaina Albash,[8] Carlo Dionisi-
10 Vici,[27] David Murphy,[28] Diego Martinelli,[27] Enrico Bugiardini,[1] Katrina Allis,[21] Costanza Lamperti,[5] Lotte
11 Risom,[29] Lucia Laugwitz,[30,31] Michela Di Nottia,[6] Robert McFarland,[7] Laura Vilarinho,[32] Michael Hanna,[1]
12 Holger Prokisch,[17,19] Johannes A. Mayr,[15] Enrico Silvio Bertini,[6] Daniele Ghezzi,[5,33] Elsebet
13 Østergaard,[29,34] Saskia B. Wortmann,[15,17,19,35] Rosalba Carrozzo,[6] Tobias B. Haack,[30,36] Robert W. Taylor,[7]
14 Antonella Spinazzola,[26*] Karin Nowikovsky,[2,37*] Henry Houlden[1*]

15

16 [1]Department of Neuromuscular Diseases, University College London, Queen Square, Institute of
17 Neurology, London, WC1N 3BG, UK;

18 [2]Department of Biomedical Sciences, Institute of Physiology, Pathophysiology and Biophysics,
19 University of Veterinary Medicine Vienna, Vienna, 1210, Austria;

20 [3]Department of Neuropediatrics, Jena University Hospital, Jena, 07747, Germany;

21 [4]Center for Rare Diseases, Jena University Hospital, Jena, 07747, Germany;

22 [5]Unit of Medical Genetics and Neurogenetics, Fondazione IRCCS Istituto Neurologico Carlo Besta,
23 Milan, 20126, Italy;

24 [6]Unit of Muscular and Neurodegenerative Disorders, Laboratory of Molecular Medicine, Bambino
25 Gesù Children's Hospital, IRCCS, Rome, 00146, Italy;

26 [7]Wellcome Centre for Mitochondrial Research, Translational and Clinical Research Institute, Faculty
27 of Medical Sciences, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK;

28 [8]Kuwait Medical Genetics Centre, Al-Sabah Medical Area, 80901, Kuwait;

29 [9]Division of Medical Genetics, Department of Pediatrics, David Geffen School of Medicine, University
30 of California at Los Angeles, Los Angeles, CA, 90095, USA;

31 [10]Center for Rare Diseases, Department of Pediatrics and Department of Genetics, Copenhagen
32 University Hospital Rigshospitalet, Blegdamsvej 9, Copenhagen, 2100, Denmark;

33 [11]West Midlands Regional Genetics Service, Birmingham Women's and Children's Hospital,
34 Birmingham, B15 2TG, UK;

35 [12]Paediatric Radiology and Neuroradiology Department, V. Buzzi Children's Hospital, Milan, 20154
36 Italy;

37 [13]Peninsula Medical School, Faculty of Health, University of Plymouth, PL4 8AA, UK;

38 [14]Paediatrics Wah Medical College NUMS, 44000, Pakistan;

39 [15]University Children's Hospital, Salzburger Landeskliniken (SALK) and Paracelsus Medical University
40 (PMU), Salzburg, 5020, Austria;

41 [16]Department of Biomedical Sciences, University of Padova, Via Ugo Bassi 58/B, Padova, I-35131,
42 Italy;

43 [17]Institute of Neurogenomics, Helmholtz Zentrum München, Neuherberg, 85764, Germany;

44 [18]DZHK (German Centre for Cardiovascular Research), partner site Munich Heart Alliance, Munich,
45 81675, Germany;

46 [19]Institute of Human Genetics, Technical University of Munich, Munich, 81675, Germany;

47 [20]Amalia Children's hospital, Radboudumc, Nijmegen, 6500 HB, The Netherlands;

48 [21]GeneDx Inc, Gaithersburg, MD, 20877, USA;

49 [22]Institute of Biochemistry and Biotechnology, Pir Mehar Ali Shah Arid Agriculture University,
50 Rawalpindi, 44000, Pakistan;

51 [23]Neuromuscular Diseases Unit, Department of Neurology, Hospital Clinico San Carlos and San Carlos
52 Health Research Institute (IdISSC), Madrid, 28040, Spain;
53
54 [24]Department of Human Genetics; Division of Medical Genetics, Department of Pediatrics; David
55 Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA, 90095, USA;

56 [25]Neurometabolic Unit, National Hospital for Neurology and Neurosurgery, London, UK; Department
57 of Chemical Pathology, Great Ormond Street Hospital, WC1N 3BG, London, UK.

58 [26]Department of Clinical Movement Neurosciences, Royal Free Campus, University College of
59 London, Queen Square Institute of Neurology, London, WC1N 3BG, UK;

60 [27]Division of Metabolism, Bambino Gesù Children's Hospital, IRCCS, Rome, 00146, Italy;

61 [28]Department of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology,
62 University College London, WC1N 3BG, United Kingdom;

63 [29]Department of Genetics, Copenhagen University Hospital Rigshospitalet Blegdamsvej,
64 Copenhagen, 2100, Denmark;
65
66 [30]Institute of Medical Genetics and Applied Genomics, University of Tuebingen, 72076, Tübingen,
67 Germany;

68 [31]Department of Neuropediatrics, Developmental Neurology and Social Pediatrics, University of
69 Tübingen, Tübingen, 72076, Germany;

70 [32]Unit of Neonatal Screening, Metabolism & Genetics, Department of Human Genetics, National
71 Institute of Health Dr Ricardo Jorge, Porto, 4000-055, Portugal;

72 [33]Department of Pathophysiology and Transplantation, University of Milan, Milan, 20122, Italy;

73 [34]Institute for Clinical Medicine, University of Copenhagen, Copenhagen, 2200 Denmark;

74 [35]Radboud Center for Mitochondrial Medicine, Department of Pediatrics, Amalia Children's Hospital,
75 Radboudumc, Nijmegen, 6525 EZ, The Netherlands;

76 [36]Centre for Rare Diseases, University of Tuebingen, Tübingen, 72076, Germany;

77 [37]Department of Internal Medicine I, ASCTR and Comprehensive Cancer Center, Medical University
78 of Vienna, Vienna, 1090, Austria;

79

80

81 [38]These authors contributed equally

82 *Correspondence: h.houlden@ucl.ac.uk (H.H.), Karin.nowikovsky@vetmeduni.ac.at (K.N.),
83 a.spinazzola@ucl.ac.uk (A.S.)

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86

87 **Summary**

88 The Leucine zipper-EF-hand containing transmembrane protein 1 (*LETM1*) gene encodes an inner
89 mitochondrial membrane protein with an osmoregulatory function controlling mitochondrial volume
90 and ion homeostasis. The putative association of *LETM1* with a human disease was first suggested in
91 Wolf-Hirschhorn syndrome resulting from *de novo* monoallelic deletion of chromosome 4p16.3,
92 encompassing *LETM1.* Utilizing exome sequencing and international gene-matching efforts, we have
93 identified 18 affected individuals from 11 unrelated families harboring novel and ultra-rare bi-allelic
94 missense and loss-of-function *LETM1* variants and clinical presentations highly suggestive of
95 mitochondrial disease. These manifested as a spectrum of predominantly infantile-onset (14/18, 78%)
96 and variably progressive (50% rapid with premature mortality, 22% moderate, and 28% slow)
97 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

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

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

134 Consistent with the vital role of mitochondrial osmoregulation, matrix swelling and cation imbalance
135 due to *LETM1* inactivation have wide-reaching and pleiotropic effects on mitochondrial biogenesis and
136 bioenergetics, perturbing glucose and pyruvate utilization, tryptophan and mitochondrial DNA
137 (mtDNA) metabolism, outer mitochondrial membrane integrity and causing necrotic cell death. [9,12,13-
138 17]

139 The importance of *LETM1* in neuronal function and pathology was first suggested in Wolf-Hirschhorn
140 syndrome (WHS [MIM: 194190]).[4] This genetic syndrome results from *de novo* monoallelic deletion of
141 several genes on the short arm of chromosome 4. Depending on the length of the deletion, WHS might
142 present with a combination of congenital malformations, specific facial dysmorphism, growth and
143 cognitive impairment, microcephaly, hypotonia, and epilepsy.[13] *LETM1* is localized in WHS critical
144 region 2 (WHSCR2), less than 80 kb from WHS critical region 1 (WHSCR1) and is deleted in almost all
145 individuals with the full WHS phenotype. *LETM1* is proposed to be associated with epilepsy and
146 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

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

178 **ES and data analysis**

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

184 **Skin biopsy and primary fibroblast culture, and muscle biopsy**

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

189 **Western blotting analysis**

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

193 **Cell imaging**

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

198 ***mtDNA copy number***

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

202 ***Immunohistochemistry***

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

207 **Plasmid and LETM1 point variants**

208 Full-length human *LETM1* cDNA fused to C-terminal Hemagglutinin (*HA*)-tag and subcloned into the
209 multi-copy plasmid pVT-103U[42] served as a template to introduce the *LETM1* variants by site-directed
210 mutagenesis. Amino acid replacements and deletions were performed with non-overlapping back-to-
211 back annealing mutagenic primers, using the Q5 site-directed mutagenesis kit (NEB #E0552S) with NEB
212 5-alpha competent *E. coli* cells (NEB #C2987). All primers were from Microsynth (Balgach, Switzerland)
213 and all the identified variants were confirmed by DNA sanger sequencing. To distinguish the
214 phenotypes of disease-associated *LETM1* variants and non-pathogenic variants, two non-disease-
215 associated *LETM1* (NM_012318.3) missense variants (rare *LETM1* variants but with homozygotes in
216 gnomAD v3.1.1), c.913A>C, p.Ile305Leu and c.1760A>G, p.Lys587Arg, were included in this study. A

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

219 **Yeast transformation**

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

225 **Mitochondrial isolation and KOAc-induced swelling assay**

226 Mitochondria were isolated from yeast cells logarithmically grown in SD-URA by homogenization and
227 differential centrifugation method as described in Nowikovsky et al.[42] and immediately used for KOAc-
228 induced swelling assays. The protocols of Nowikovsky et al.[42] were adapted to smaller volumes. Briefly,
229 isolated yeast mitochondria suspended in breaking buffer (0.6 M sorbitol, 20 mM Tris-HCl pH 7.4) were
230 de-energized with antimycin A (2.5 µM) for 10 min at room temperature (25˚C), washed and
231 resuspended in breaking buffer at a concentration of 200 µg/20 µl. As Mg^{2+} is a brake to the KHE,[45]
232 mitochondria were depleted from Mg^{2+} with A23187 (0.5 µM) and EDTA (10 mM) and transferred onto
233 96 well plates for measurement (200 µg/well). When indicated, quinine (200 µM) served as a control
234 to inhibit KHE-mediated swelling. The 96 well plates were placed in the Thermo Scientific Varioskan
235 LUX Multimode Microplate Reader. The swelling was initiated by injection of KOAc media (55 mM
236 KOAc, 5 mM TES, 0.1 mM EDTA) to a final volume of 200 µl/well and the optical density changes at
237 OD_{540} were immediately recorded at 25˚C. Each measurement was performed in 3 independent
238 replicates. Raw swelling data were fitted into a curve showing changes in absorbance versus time to
239 quantify the swelling rate.

240

241 **Results**

242 **Clinical findings**

243 The summary of the core phenotypic features of 18 affected individuals from 11 independent families
244 with bi-allelic *LETM1* variants is provided in Table 2, Figure 1C, and Table S1. Detailed clinical history
245 is provided in the supplemental note (case reports). Video recordings are available for affected
246 individuals from family 1 (Supplemental Videos). The cohort comprises 10 males and 8 females, 9 of
247 whom are currently alive with a median age of 15 years (range 1-39) at the latest available follow-up
248 (Figure 2A). Half of the cases (9/18) succumbed to their rapidly progressing disease at an early age,
249 ranging between 2 months and 8 years old. The ethnic composition of the cohort is diverse including
250 families of Pakistani, Caucasus, Middle Eastern, European, and Mexican origin, with 67% of the cases
251 (12/18) being from consanguineous unions. Only limited clinical data were obtainable from 6
252 deceased cases belonging to families 3 and 10.

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

262 On the most recent follow-up, the affected individuals displayed clinical features suggestive of a
263 mitochondrial disorder. Impaired vision (10/10, 100%) with a mean onset age of 5.2±3.1 years, which
264 was confirmed to be due to optic atrophy in 5/6 (83%), and bilateral sensorineural hearing loss (11/14,
265 78%) diagnosed at a mean age of 2.6±1.9 years (range from congenital up to 6 years) with hearing aids

266 fitted in 7/10 (70%) cases were the common neurosensory abnormalities. While cognitive delay and
267 intellectual disability (7/8, 87.5%%) and impaired speech acquisition (6/9, 67%) were among the
268 common neurodevelopmental symptoms, more than half of the cases displayed neuromuscular
269 features including spasticity (8/15, 53%), hypotonia (11/18, 61%), muscular wasting (7/10, 70%), and
270 cerebellar ataxia (7/9, 78%). Other frequent neurological symptoms were nystagmus (7/13, 54%),
271 myopathy (6/12, 50%), hyperkinetic movement disorders (4/12, 33%), and spastic-ataxic gait (3/9,
272 33%) combined with brisk deep tendon reflexes (4/10, 40%), upgoing plantar response (4/9, 44%), and
273 peripheral neuropathy (3/9, 33%).

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

284 Other features consistent with a mitochondrial phenotype included bilateral cataracts (5/11, 45%)
285 cardiomyopathy (5/14, 36%) with pericardial effusion (3/11, 27%), and diabetes (3/11, 27%).
286 Craniofacial abnormalities included occipitofrontal circumference below 3rd percentile in 2/6 cases
287 (33%) and facial dysmorphism (4/10, 40%) with a long thin face, prominent nose, low-set ears,
288 micrognathia, high arched palate, and teeth abnormalities (Figure 1 A).

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

291 mitochondrial dysfunction in the affected individuals. Hence, electromyography and nerve
292 conductions studies available from 5 cases showed neurogenic (3/5) and myopathic changes (2/4).
293 Elevated serum lactate was confirmed in 8/12 (67%) affected individuals. Plasma amino acids were
294 abnormal in 4/9 tested with mildly elevated alanine (501-597 µmol/l, normal range 232-494), glycine,
295 and serine. CSF-alanine was tested and mildly increased in 2 cases. Urine amino acids were tested in
296 4 cases, and only one case showed abnormal results including increased levels of aspartic, serine, and
297 glycine. Urine organic acids were analyzed in 11 cases and were abnormal in 9 of them with 3-
298 methylglutaconic acid excretion (5/11), moderately elevated beta-hydroxybutyrate and acetoacetate
299 (1/11), and significant elevation of adipic acid (1/11). Muscle biopsy was available from 7 cases and of
300 these, 5 had abnormal findings including scattered necrotic and regenerating COX-deficient fibers with
301 an excess of internal nuclei, lipid depositions within fibers and prominent mitochondrial pattern in
302 vacuolated fibers (F3:S2), COX-deficient multiple ragged-red fibers with increased fiber unisometry
303 (F8:S1), type I fiber predominance with mild glycogen storage (F9:S1) and COX-deficient fibers
304 (F11:S1). Respiratory chain enzyme (RCE) analysis was performed in 11 cases showing isolated or
305 combined mitochondrial respiratory chain deficiencies in all subjects tested (Table 2 and Table S1,
306 Figure 3C).

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

314 The affected individual from family 8 was the oldest member of the cohort showing a phenotype
315 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

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

346

347 **Genotype-phenotype correlation**

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

359

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

361 Loss of mitochondrial volume homeostasis is the most characteristic and universally accepted
362 phenotype of *LETM1* deficiency in human, animal models, plants, and yeast, which leads to
363 mitochondrial fragmentation, matrix swelling, and disorganized cristae as reviewed in Austin et al.[5]
364 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),

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

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

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

435

436 **Functional compensation analysis in yeast**

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

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

445 Light scattering recording of KOAc-induced swelling is a well-established method to measure the
446 mitochondrial electroneutral exchange of K^+ for H^+.[48] Exposure of de-energized mitochondria to
447 hypotonic KOAc buffer elicits the rapid uptake of protonated acetic acid, acidification of mitochondrial
448 matrix, and thereby activation of KHE, which results in mitochondrial K^+ influx, water uptake and thus
449 swelling.[45] Isolated mitochondria from *yLETM1* wild-type cells and *yletm1*Δ cells overexpressing
450 *LETM1* WT or variants or the empty control vector were subjected to KOAc-induced swelling
451 experiments. Recording KHE activity by measuring the decrease in optical density (OD) using light
452 scattering techniques allows discrimination of its main determinants: initial OD, indicating the osmotic
453 state of mitochondria before KOAc addition, and KHE exchange rate per second, indicated by the
454 amplitude from initial to final OD as a function of the time required to achieve it. As shown in Fig 5A,
455 KOAc-induced swelling was sensitive to the KHE inhibitor quinine, confirming the correlation of optical
456 density with KHE activity. Knockout of *yLETM1 (yletm1*Δ) entirely abolished KHE activity, as illustrated
457 by low initial OD and swelling amplitude, which were restored by expression of *LETM1* WT. The non-
458 pathogenic variants (p.Ile305Leu and p.Lys587Arg) performed as well as *LETM1 WT* for the initial OD,
459 and almost as well for the kinetics values. LETM1 with the mutation Val691Argfs* (F10) almost
460 restored the initial OD, so did *LETM1* Lys587Arg (F9) and Arg393His (F6) but their swelling amplitudes
461 were very low. Expression of *LETM1* variants c.754-756del, p.Lys252del (F3), c.878T>A, p.Ile293Asn
462 (F1:M), or c.2220G>C, p.*740TyrextTer26 (F2, F7) marginally compensated K^+ fluxes with extremely
463 slow swelling kinetics; Swelling traces for *yletm1*Δ transformed with *LETM1* c.881G>A, p.Arg294Gln
464 (F4, F8) or *LETM1* c.2094del, p.Asp699Metfs*13 (F1:F) suggested uncontrolled cation leakage (Figure
465 5A). Overexpression of *LETM1* c.1072G>A, p.Asp358Asn did not rescue KHE. Taken together, these
466 results suggest that mitochondrial reduced K^+ flux dynamics and swollen matrix are indicative of the
467 functional impact of disease-associated *LETM1* variants.

468 LETM1 protein levels associated with *LETM1* variants were examined using total cell lysates and
469 isolated mitochondria. In comparison to the mitochondrial loading control (Porin, Por1p), LETM1 total
470 protein levels from ectopic *LETM1* WT or variant expression were similar, except those from F1:S1-S2
471 (*LETM1* c.878T>A; c.2094del, p.Ile293Asn; Asp699Metfs13*), F2, F7 (both *LETM1* c.2220G>C,
472 p.*740Tyrext26) and F11 (*LETM1* c.898C>T, p.Pro300Ser) which showed reduced LETM1 levels in
473 mitochondria (Figure 5B upper panel). The levels of LETM1 from F5 *LETM1* variant (c.1072G>A,
474 p.Asp358Asn) were also low, but not when normalized to Por1p, which was similarly decreased (Figure
475 5B). LETM1 levels from the variants identified in F3 (c.754-756del, p.Lys252del) and in F4, F8 (both
476 c.881G>A, p.Arg294Gln) were detectable in total lysates and mitochondria prepared from a large-scale
477 intracellular fractionation (Figure 5B lower right panel) but were also reduced. None of the ectopic
478 expression of *LETM1* variants however affected the mitochondrial subcellular localization.

479 As previously noticed[17,49,50] and shown here (Figure 5B), Cox2p (subunit of CIV) is reduced in *yletm1*Δ
480 strains. Cox2p levels were restored upon ectopic expression of *LETM1* WT or *LETM1* c.878T>A,
481 p.Ile293Asn (F1:M), *LETM1* c.2071-9C>G, p.Val691fs4* (F10) or *LETM1* c.878T>A; c.2094del,
482 p.Ile293Asn; Asp699Metfs13* (F1:S1, F1:S2), but remained absent upon expression of *LETM1* c.754-
483 756del, p.Lys252del (F3), *LETM1* c.881G>A, p.Arg294Gln (F4,8) or *LETM1* c.1072G>A, p.Asp358Asn (F5)
484 (Figure 5B).

485 *yletm1*Δ shows poor growth on non-fermentable (YPG) substrate.[42] To determine the significance of
486 the *LETM1* variants in rescuing the growth defects of *yletm1*Δ compared to *LETM1* WT, serial dilutions
487 of *yletm1*Δ strains overexpressing an empty plasmid or *LETM1* variants or WT were spotted onto
488 fermentable (YPD) and non-fermentable (YPG) plates and grown at 30 or 37°C (Figure S4). We found
489 a detrimental effect of the mutant phenotype by ectopic expression of *LETM1* c.1072G>A,
490 p.Asp358Asn (F5) variant; this strain was able to grow on selective media but showed worsened
491 growth defect on complete media. Growth was also slowed down at 37°C on YPD by *LETM1* c.898C>T,
492 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

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

545

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

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

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

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

570

571 **The phenotype of defective *LETM1* and WHS**

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

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

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

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

602

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

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

608 The general distribution of the missense and frameshift variants to the highly conserved LETM domain
609 and the C-terminal coiled coils, together with their comparable deleterious effects on mitochondrial
610 morphology and KHE function support the correlation of mitochondrial morphologic defects and
611 imbalanced cation homeostasis. A previous variant analysis of the LETM domain found that Asp359 or
612 the triple combination of Arg382, Gly383, and Met384 is necessary for the organization of cristae
613 structure and growth complementation of *yletm1*Δ strains.[65] The missense variant c.1072G>A,
614 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

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

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

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

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

676

677 **LETM1 role in cation homeostasis and neurodegenerative phenotype of the cohort**

678 Among the mitochondrial EF-hand-containing proteins, LETM1 has been identified as essential across
679 several cell lines in genome-wide essentiality screens.[68,69] Functionally, LETM1 is required for
680 maintaining mitochondrial homeostasis of K^+, an osmotic and cellularly most abundant cation, and
681 was considered an essential component of the KHE. After LETM1 was identified in a genomic
682 *Drosophila* RNAi screen for mitochondrial CHX, it has been suggested to catalyze the exchange of Ca^{2+}
683 against H^+ in both directions in a ruthenium red-sensitive pattern,[70] which is difficult to reconcile with
684 the CHX, and has been implicated in the pathogenesis of Parkinson's disease through interaction with
685 PINK1.[71] The mitochondrial CHX is part of the mitochondrial Ca^{2+} release system, which compensates
686 for electrophoretic mitochondrial Ca^{2+} uptake mainly through H^+- or Na^+-dependent Ca^{2+} extrusion.
687 While the role of LETM1 as a mitochondrial KHE or CHX has remained controversial, deregulation of
688 the mitochondrial KHE has been shown to affect mitochondrial Ca^{2+} buffering by impacting the Na^+-
689 dependent Ca^{2+} release pathway.[72] Proper maintenance of mitochondrial Ca^{2+} levels is critical to
690 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**

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

717 **Supplemental video legends**

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

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

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

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

725

726

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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/

777 GeneMatcher, https://genematcher.org/

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

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

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

781 Varsome, https://varsome.com/

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

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

784 ClinVar (summary of submissions), https://www.ncbi.nlm.nih.

785 gov/clinvar/submitters/26957

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

787 FlyBase, http://flybase.org/

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

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

790

791 **Data and code availability**

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

795

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1017

1018 **Figure legends**

1019 **Figure 1.** Clinical features and neuroimaging findings of the cases with bi-allelic *LETM1* variants

1020 **(A)** From left to right facial photos of the affected individuals F1:S1, F1:S2, F5:S1, F8:S1. All cases wear
1021 glasses due to bilateral optic atrophy. All cases have prominent noses. F1:S1 and F1:S2 show long thin
1022 faces, low-set ears, and teeth abnormalities.

1023 **(B)** In A (F1:S1), severe cerebellar atrophy (arrows) and pontine hypoplasia (arrowheads) are shown,
1024 while in B (F6:S1) only mild vermian hypoplasia is noted. In C arrowheads point at the severe optic
1025 nerve and chiasm atrophy in 2 different cases (F5:S1 and F7:S2). Mild ventricular dilatation is present
1026 in D (F3:S1).

1027 **(C)** Clinical features of the affected individuals with bi-allelic *LETM1* variants.

1028 GDD, global developmental delay; ID, intellectual disability; MRI, magnetic resonance imaging; MRC,
1029 mitochondrial respiratory chain.

1030

1031 **Figure 2.** Pedigrees with segregations of *LETM1* variants and LETM1 protein architecture with a
1032 partial sequence alignment of the variants.

1033 **(A)** Family trees of the cases with bi-allelic *LETM1* variants. Square: male, circle: female, black
1034 symbols: affected individuals, white symbols: unaffected individuals. M, maternal; P, paternal.

1035 **(B)** Schematic representation of the human *LETM1* gene organization in introns, shown as a line, and
1036 exons, shown as boxes, and of LETM1 protein domains as indicated by the residue numbers and the
1037 color code: coiled-coil motifs (light yellow), transmembrane helices (blue), LETM/ribosomal-binding
1038 like domain (lavender) and putative EF-hands (green). All identified missense variants in the affected
1039 cases (black) and non-pathogenic variants (blue) are mapped according to their positions. The amino
1040 acid sequence of human LETM1 was aligned with LETM1 orthologs using Clustal Omega and
1041 alignments with LETM1 from other species are shown for all segments that contain missense variants,
1042 indicated in bold red letter. Residue conservation is shown below the alignment as fully conserved (*),
1043 highly conserved (:) or partially conserved (.). UniProt accession numbers for the *H.s*. (*H. sapiens)*,
1044 *M.m*. (*M. musculus)*, S.c. *(S. cerevisiae)*, *D.r*. *(D. rerio)*, *C.e*. *(C. elegans)*, *D.m*. *(D. melanogaster)* and
1045 *A.t. (A. thaliana)* LETM1 used in this alignment are O95202, Q9Z2I0, Q08179, Q1LY46, Q9XVM0,
1046 P91927 and F4J9G6 respectively.

1047

1048 **Figure 3.** Effects of *LETM1* variants on mitochondrial morphology and proliferation in fibroblasts

1049 **A)** *LETM1* variants perturb the mitochondrial network

1050 Confocal images of fibroblasts stained with Mitotracker Red. Shown is a representative overview of
1051 the cells (Bars: 5 μm, except F10 10 μm) and details magnified from the box (Bars: 5 μm). C1, C2:
1052 healthy donors; F1:S2 c.878T>A, p.Ile293Asn and c.2094del, p.Asp699Metfs*13; F2 c.2220G>C,
1053 p.*740TyrextTer26; F5 c.1072G>A, p.Asp358Asn; F10 c.2071-9C>G, p.Val691fs4*. Arrow indicates
1054 fragmented mitochondria. For statistics, see Fig S1C.

1055 **B)** *LETM1* variants cause swollen mitochondria and loss of cristae

1056 The ultrastructure of control (C1) and case (F5 and F10) fibroblasts was investigated by transmission
1057 microscopy and images show overviews (left panels, bars: 2 μm) and details (right panels, bar 500 nm).

1058 Arrow indicates swollen mitochondria.

1059 **C-D)** Variants differently affect LETM1 stability and OXPHOS proteins in fibroblasts samples

1060 Total lysates of fibroblasts were analyzed by immunoblotting using the indicated antibodies, GAPDH,
1061 or β-actin served as loading control; C2, C3: healthy donors; F1:S1 and F1:S2 c.878T>A and c.2094del,
1062 p.Ile293Asn and p.Asp699Metfs*13; F2 c.2220G>C, p.*740TyrextTer26; F10 c.2071-9C>G,
1063 p.Val691fs4*; F11 c.898C>T p.Pro300Ser (**C**). Quantitative graphs from independent experiments
1064 representing the protein bands, normalized to the housekeeping proteins, and calculated as a
1065 percentage in relation to the controls, data are expressed as mean ± SEM (N=>3 independent
1066 experiments). Two-way ANOVA with Dunnet`s multiple comparisons test of F2, F10 against controls
1067 (fibroblasts from healthy donors) *p<0.02, **p<0.007, ***p<0.0003, ****p<0.0001, while for F1:S1,
1068 F1:S2, F11 as follow; ***P (LETM1 Controls vs F1:S1 or F1:S2 or F11:S2)< 0.0001; **P (Complex I
1069 Controls vs F1:S1) = 0.0017, ***P (Complex I Controls vs F1:S2) = 0.0006, and ***P (Complex I Controls
1070 vs F11:S2) < 0.0001; *P (Complex IV Controls vs F1:S1) = 0.0229, *P (Complex IV Controls vs F1:S2) =
1071 0.0317 and *P (Complex IV Controls vs F11:S2) = 0.0009; **(D)**.

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

1073 Control (C1 and C2) and case (F5, F10) fibroblasts were grown for 8 days and counted every second
1074 day. Cell numbers on day 8 were plotted and the statistical significance was calculated using one-way
1075 ANOVA with Dunnett`s multiple comparison test from three independent experiments, n=3, data are
1076 means ± SD, ns P > 0.05, **P= 0.0039, ****P< 0.0001.

1077

1078 **Figure 4.** *LETM1* variants affect the stability of LETM1 and OXPHOS components in muscle samples

1079 **A-B)** Western blot analysis of LETM1 and components of the OXPHOS complexes I, II, III; and IV in
1080 muscle samples from F11 and quantitative graphs

1081 Total lysates of muscle samples from healthy donors (C4, C5) and F11 c.898C>T p.Pro300Ser (S1, S2)
1082 were analyzed by immunoblotting using the indicated antibodies, VDAC served as a loading control

1083 **(A)**. Quantitative graphs represent the protein levels relative to controls and normalized to VDAC Data
1084 are expressed as mean ± SEM. n=> 3 independent experiments. Two-way ANOVA with Dunnett`s
1085 multiple comparisons test. ***P (LETM1 Controls vs F11:S1 and F11 S2) = 0.0004; ***P (CORE2
1086 Controls vs F11:S1) = 0.0001 and ***P (CORE2 Controls vs F11:S2) < 0.0001; ***P (NDUFA9 Controls
1087 vs F11:S1 and F11 S2) < 0.0001 *P (SDH Controls vs F11:S1) = 0.0453 and ***P (SDH Controls vs F11:S2)
1088 < 0.0001; ***P (COX4 Controls vs F11:S1)= 0.0002 and **P (COX4 Controls vs F11:S2)= 0.0024 **(B)**.

1089 **C)** Immunohistochemical staining of OXPHOS subunits and VDAC of the muscle of F5 and controls

1090 Muscle samples from healthy donors (C6, C7) and F5 c.1072G>A, p.Asp358Asn were stained for each
1091 of the five OXPHOS subunits using the indicated antibodies, VDAC served as a control. Magnification
1092 400 x.

1093 **D-E)** Western blot analysis of subunits of the OXPHOS complexes, citrate synthase, and GAPDH of
1094 the muscle of F5 and controls

1095 Total lysates of muscle samples were analyzed by immunoblotting using the indicated antibodies,
1096 VDAC, GAPDH, and CS served as loading controls. C6: healthy donor, F5: c.1072G>A, p.Asp358Asn **(D).**
1097 Quantitative graphs representing the protein levels percentage relative to controls (normalized to
1098 GAPDH). The statistical significance was calculated using one-way ANOVA with Dunnett`s multiple
1099 comparison test, data are means ± SD, N=>3 independent experiments, *P=0.0201, **P=0.0011,
1100 ****P< 0.0001.

1101

1102 **Figure 5.** Functional implication of *LETM1* variants on yeast mitochondria.

1103 **A)** *LETM1* variants fail to restore KHE activity of yeast *yletm1* Δ

1104 Isolated and de-energized mitochondria were subjected to KOAc and changes of optical density at
1105 OD_{540} immediately measured. Upper panel**:** representative traces of KOAc-induced swelling in *yLETM1*
1106 WT mitochondria (WT, blue), *yletm1*Δ mitochondria overexpressing the empty plasmid (e, yellow) or

1107 the plasmid carrying LETM1 WT untreated (w, green) or treated (wq, grey) with quinine or the *LETM1*
1108 variants: c.754-756del, p.Lys252del (1, red), c.878T>A, p.Ile293Asn (2, bottle green), c.881G>A,
1109 p.Arg294Gln (3, aqua), c.898C>T, p.Pro300Ser (4, dark green), c.913A>C, p.Ile305Leu (5, lavender),
1110 c.1072G>A, p.Asp358Asn, (6, violet), c.1139G>C, p.Arg380Pro (7, beige), c.1178G>A, p.Arg393His (8,
1111 turquois), c.1760A>G, p.Lys587Arg (9, mauve), 2071-9C>G, p.Val691fs4* (10, purple), c.2094del,
1112 p.Asp699Metfs*13 (11, dark blue), compound (12, lila) c.2220G>C, p.*740TyrextTer26 (13, olive).
1113 Quantified rates of KOAc-induced swelling from 3 independent experiments. Data are means ± SD.
1114 One-way ANOVA with Dunnett's multiple comparisons test performed against *yletm*Δ transformed
1115 with empty pVT-103U plasmid *p= 0.0426, ** p = 0.0026, ***p= 0.0006, ****p< 0.0001. And for
1116 Ile305Leu Lys587Arg relatively to *yletm*Δ transformed with WT, ns >0.05, *p= 0.0169.

1117 **B)** ectopic expression of *LETM1* variants in *yletm1* Δ

1118 Isolated mitochondria (upper panel) and total protein lysates (lower panel left) from the same
1119 strains as in **A)** and subcellular fractions T: total, SN: post-mitochondrial supernatant, M:
1120 mitochondria (lower panel right) were immunoblotted using the indicated antibodies, Por1p and
1121 Act1p served as mitochondrial and total (and SN) loading control, respectively.

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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