Lethal Neonatal LTBL Associated with Biallelic *EARS2* Variants: Case Report and Review of the Reported Neuroradiological Features

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Abstract Mitochondrial translation defects are important causes of early onset mitochondrial disease. Although the

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biochemical (combined respiratory chain deficiency) signature and neuroimaging are usually distinctive, they are not diagnostic as the genetic origin of mitochondrial translation defects is heterogeneous. We report a female child, born at term to non-consanguineous parents, who exhibited global hypotonia, failure to thrive, persistent and progressive hyperlactacidaemia with lactic acidosis, liver dysfunction and encephalopathy and died at the age of 5 months. Brain MRI revealed hypogenesis of the *corpus callosum*, T2 signal abnormalities in the medulla oblongata, pons, midbrain, thalami, cerebellar white matter, and a lactate peak on MRS. Muscle histochemistry showed cytochrome *c* oxidase (COX)-deficient and ragged-red fibres, while muscle biochemical studies showed decreased activities of mitochondrial respiratory chain complexes I and IV. Whole exome sequencing (WES) identified biallelic *EARS2* (NM_001083614) variants, a previously reported start-loss (c.1>G, p.Met1?) variant and a novel missense (c.184A>T, p.Ile62Phe) variant. Patient fibroblasts and muscle homogenate displayed markedly decreased *EARS2* protein levels, although decreased steady-state levels of complex I (NDUFB8) and complex IV (MT-CO1 and MT-CO2) subunits were only observed in muscle. Pathogenic variants in *EARS2*, encoding mitochondrial glutamyl-tRNA synthetase (mtGluR), are associated with Leukoencephalopathy involving the Thalamus and Brainstem with high Lactate (LTBL), a mitochondrial disorder characterised by a

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distinctive brain MRI pattern and a biphasic clinical course. We further outline the unique phenotypic spectrum of LTBL and review the neuroradiological features reported in all patients documented in the literature.

Introduction

In recent years, next-generation sequencing (NGS) technologies have allowed identification of many novel disease genes, particularly in those human conditions that are characterised by clinical and genetic heterogeneity. This is true of mitochondrial disorders, which are clinically, biochemically and genetically heterogeneous; respectively, characterised by multisystemic features and mitochondrial respiratory chain complex activity deficiency. Defects of mitochondrial protein translation, which is closely coordinated between both mitochondrial DNA and the nuclear genome, are associated with multiple respiratory chain deficiency (Calvo et al. 2012; Lightowlers et al. 2015).

The mitochondrial amino-acyl tRNA synthetases (mt-aaRSs) are a group of nuclear-encoded enzymes that catalyse the specific attachment and conjugation of each amino acid to their corresponding cognate tRNA; hence, mt-aaRSs are critical for mitochondrial protein synthesis (Diodato et al. 2014). Mutations of mt-aaRSs genes are important causes of autosomal recessive mitochondrial translation disorders associated with multiple mitochondrial respiratory chain complex deficiencies. These disorders often manifest during childhood with typically multi-organ phenotypes. A genotype–phenotype correlation has been ascertained for several mt-aaRS genes with some tissue specificity, probably due to cell-type specific differences in response to mutations (Diodato et al. 2014; Biancheri et al. 2015; Taylor et al. 2014).

Several mt-aaRS defects unveil discrete and specific clinical and neuroradiological phenotypes including *DARS2* (OMIM#611105) (Scheper et al. 2007); *RARS2* (OMIM#611523) (Edvardson et al. 2007); *FARS2* (OMIM#614946) (Elo et al. 2012); *EARS2* (OMIM#614924) (Steenweg et al. 2012) and *AARS2* (OMIM#615889) (Dallabona et al. 2014). Although the biochemical signatures and neuroimaging of combined respiratory chain deficiency can be distinctive, it remains difficult to infer the genetic origin from often heterogeneous phenotypes. Mutations of *EARS2*, encoding the mitochondrial glutamyl-tRNA synthetase (mtGluR), cause Leukoencephalopathy involving the Thalamus and Brainstem with high Lactate (LTBL), also known as Combined Oxidative Phosphorylation Deficiency 12 (COXPD12) (Steenweg et al. 2012). This mitochondrial disorder is characterised by a distinctive brain magnetic resonance

(MR) pattern and a biphasic clinical course; either severe onset with partial clinical and radiological recovery/stagnation or a variable clinical course ranging from mild or intermediate to severe (Steenweg et al. 2012; Biancheri et al. 2015).

We describe the clinical, biochemical and pathological features, alongside the *post-mortem* diagnostic workup of a patient with a severe and fatal form of LTBL. We also review the literature for reported patients with pathogenic *EARS2* variants and highlight the clinical and neuroradiological spectrum of this disorder.

Patient Report and Methods

Patient Report

The proband was a female child, born at term to non-consanguineous parents of Portuguese ancestry. Her birth weight, length and occipitofrontal circumference were normal and she was an otherwise normal baby. At 21 weeks gestation, mild ventriculomegaly was observed by ultrasound and confirmed by foetal brain MR at 22 weeks. A second foetal MR that was performed at 24 weeks was normal.

She was first admitted at day 7 of life due to severe feeding problems, failure to thrive and global hypotonia. Subsequent admissions due to decompensations showed signs of undernutrition and she developed encephalopathy. There was also persistent and progressive lactic acidosis and liver dysfunction. No ketonuria was detected and ammonia was normal. She had no sign of respiratory distress and cardiac evaluation was normal. Newborn screening was also normal. Brain MRI at 2 months revealed symmetrical lesions in brainstem, cerebellum and thalami, suggestive of a mitochondrial disorder (Fig. 1). Delayed myelination was noted (matched with term newborn). MR spectroscopy showed elevated lactate peak (short TE). Analysis of mitochondrial respiratory chain complexes in muscle and liver revealed decreased activities of complex I (19%) and complex IV (11%) only in muscle compared to controls. Histopathology analysis of the muscle revealed cytochrome *c* oxidase (COX)-deficient and ragged-red fibres (Fig. 2a). Liver histology disclosed distention of sinusoids with mix steatosis peri-centrilobular and hemosiderosis. The girl developed multi-organ failure, presenting skin infiltrate and hepatomegaly, and progressive depression of consciousness, went into coma around 4 months old and died at 5 months old. She was the third pregnancy of the couple and second child born. The previous pregnancy was interrupted due to a brain malformation; *corpus callosum* agenesis was described, but no medical records were available.

Fig. 1 Brain MRI at 2 months old revealed bilateral and symmetrical abnormal high signal on T2-weighted images in the posterior part of the medulla oblongata and cerebellar white matter and dentate nucleus (a), posterior part of the pons (b), cerebral peduncles (c), subthalamus

and thalamus (d, e); Delayed myelination in pericentral regions (f); Hypogenesis of the *corpus callosum* was noted (g); Short TE MR spectroscopy demonstrated elevated lactate peak (arrow) (h)

Genetic Studies

Whole mitochondrial genome sequencing of blood, muscle and liver DNA was performed to exclude pathogenic point mutations. Mitochondrial DNA depletion and rearrangements were excluded from blood, muscle and liver using established diagnostic quantitative real-time PCR and long-range PCR assays, respectively. Pathogenic *SURF1* and *POLG1* variants and larger rearrangements were excluded by Sanger sequencing and Multiplex Ligation Probe Amplification (MLPA) according to standard protocols.

Whole Exome Sequencing and Analysis

Exome capture was attained using the Nextera Rapid Exome Capture (37 Mb) kit, sequenced using the Illumina HiSeq 2000 system in 100 base pair reads and aligned to the human reference genome (UCSC hg19). Variants with a minor allele frequency of >0.01 (1%) from in-house and external exome variant databases were excluded. Recessive

(homozygous or compound heterozygous) exonic or splice-site variants of nuclear genes encoding mitochondrial-targeted proteins were prioritised. Polyphen 2, SIFT and Align GVGD were used to predict effect on protein function. Identified recessive variants were validated and segregation studies were performed by Sanger sequencing (Taylor et al. 2014).

Biochemical and Histopathological Studies

Relevant plasma biochemical measurements were liver function tests (AST, ALT, γ GT and LDH), creatine kinase, lactate and amino acids. Measurements of amino acids and organic acids in urine were also performed.

Diagnostic skin, muscle and liver biopsies were performed; samples were frozen and processed for diagnostic histology and histochemistry according to standard procedures. Mitochondrial OXPHOS activities (complexes I–V) were determined in muscle and liver (normalised to citrate synthase activity) as described (Grazina 2012).

Fig. 2 Muscle histochemical and biochemical studies, segregation analysis and p.Ile62 amino acid sequence conservation. **(a)** Sequential COX-SDH histochemical reaction demonstrating a mosaic pattern of COX-deficiency in patient muscle. **(b)** Biochemical activity of mitochondrial respiratory chain complexes I (nmol NADH oxidised·min⁻¹·unit citrate synthase⁻¹), II (nmol DCPIP reduced·min⁻¹·unit

citrate synthase⁻¹), III, IV ($\times 10^{-3}$ K s⁻¹·unit citrate synthase⁻¹) and V (nmol NADH oxidised·min⁻¹·unit citrate synthase⁻¹), as a percentage of residual controls. **(c)** Family pedigree showing confirmation of parental carrier status for the p.Met1? (maternal) and p.Ile62Phe (paternal) *EARS2* variants. **(d)** Multiple sequence alignment of p.Ile62 residue across species

Cell Culture

Cultured fibroblasts from the proband and two controls were grown in minimum essential medium (MEM) (Gibco) with 10% foetal bovine serum (FBS) (Gibco), 1× MEM vitamins (Sigma), 1× non-essential amino acids (Sigma), 50 U/ml penicillin, 50 µg/ml streptomycin (Sigma), 1 mM sodium pyruvate solution (Sigma), 0.05 mg/ml uridine and 2 mM L-glutamine (Sigma).

Western Blotting

Fibroblast cell lysates (50 µg) or muscle lysates (25 µg) were subjected to 12% SDS-PAGE and transferred to PVDF membranes (Immobilon P). Membranes were incubated overnight at 4°C with primary antibodies specific to *EARS2* (HPA043633, Sigma), *SDHA* (ab14715, Abcam), *ATP5B* (ab14730, Abcam), *UQCRC2* (ab14745, Abcam), *MT-CO1* (ab14705, Abcam), *MT-CO2* (ab110258, Abcam), *NDUFB8* (ab110242, Abcam), β -actin (A5316, Sigma) or α -tubulin (ab7291, Abcam), followed by HRP-conjugated

secondary antibodies (DakoCytomation) for 1 h at room temperature and were visualised using ECL-prime (GE Healthcare) and BioRad ChemiDoc MP with Image Lab software according to manufacturer's guidelines.

Results

Diagnostic Mitochondrial Investigations

In agreement with the muscle histopathology, which revealed mitochondrial abnormalities, assessment of mitochondrial respiratory chain complex activities in skeletal muscle demonstrated a combined deficiency involving both complex I and complex IV (Fig. 2b). Assessment of enzyme activities in liver was normal (data not shown). Whole mitochondrial genome sequencing failed to identify likely pathogenic point mutations, while diagnostic quantitative real-time PCR and long-range PCR assays excluded mitochondrial DNA depletion and large-scale mitochondrial DNA rearrangements, respectively. Targeted screening

of *SURF1* and *POLG1* failed to detect likely pathogenic variants.

Whole Exome Sequencing

Prioritisation of rare recessive variants in nuclear genes encoding mitochondrial-targeted proteins identified by whole exome sequencing (WES) revealed two heterozygous variants in *EARS2* (NM_001083614); c.1>G, p.Met1? and c.184A>T, p.Ile62Phe. Segregation analysis confirmed parental carrier status of the identified variants (Fig. 2c). The start-loss p.Met1? variant was described previously in a single *EARS2* patient of Portuguese ancestry (Steenweg et al. 2012). The p.Ile62Phe missense variant was novel and unrepresented in exome variant databases including Exome Aggregation Consortium (ExAC), NHLBI Exome Variant Server (ESP) and the 1000 Genomes Project. This missense change affects a moderately conserved residue within an evolutionary conserved region of the protein (Fig. 2d).

Western Blot Analysis

Patient fibroblasts showed no decrease in steady-state protein levels of respiratory chain complex subunits (Fig. 3a). In contrast, there was a marked loss of NDUFB8 (complex I), MT-CO1 and MT-CO2 (complex IV) protein levels in patient muscle homogenate (Fig. 3b). Steady-state levels of *EARS2* were markedly decreased in both patient fibroblasts and muscle homogenate compared to controls, demonstrating a functional consequence of the *EARS2* mutations.

Discussion

We report the clinical, biochemical and brain image signature of a patient with a severe mitochondrial disease, disclosed to be LTBL. Genetic characterisation identified two heterozygous *EARS2* variants; a maternally inherited start-loss p.Met1? variant and a paternally inherited novel missense p.Ile62Phe change.

The bilateral and symmetrical lesions that predominantly affected grey matter structures and brainstem plus the elevated lactate observed at 2 months of age were suggestive of Leigh syndrome. Muscle studies demonstrated COX-deficient and ragged-red fibres, while complex I and IV respiratory chain activities were significantly reduced. Although liver respiratory chain activity was normal, there was evident liver dysfunction. Liver involvement was also described in six additional *EARS2* patients, which included one or more of neonatal transient icterus, hepatomegaly, fatty liver and steatosis (Steenweg et al. 2012; Talim et al. 2013; Taylor et al. 2014; Pronicka et al. 2016). Post-mortem examination of the liver from the patient reported by Talim et al. (2013) also found COX-deficiency.

Patient fibroblast and muscle samples displayed a marked decrease in *EARS2* protein levels (Fig. 3). This was expected due to the start-loss variant, while the missense may affect *EARS2* stability. Decreased *EARS2* has also been previously demonstrated in fibroblasts with compound heterozygous missense *EARS2* variants (Danhauser et al. 2016). We demonstrated decreased abundance of complex I and IV subunits in the muscle, which

Fig. 3 Western blot analysis of patient fibroblasts and muscle homogenate. Western blots of lysates from patient fibroblasts (a) and skeletal muscle (b) from the proband (P) and two controls (C),

Antibodies against *EARS2*, NDUFB8 (CI), SDHA (CII), UQCRC2 (CIII), MT-CO1 (CIV), MT-CO2 (CIV), and ATP5B (CV) were used, with β -actin and α -tubulin as loading controls

corresponded with impaired complex I and IV activities by direct enzymatic assay (Fig. 2b). In contrast, the abundance of respiratory chain complex subunits was normal in patient fibroblasts (Fig. 3a), which corresponds to the tissue-specific consequences of *EARS2* variants.

To date, 26 patients are reported with LTBL (12 by Steenweg et al. (2012), one by Talim et al. (2013), two by Taylor et al. (2014), one by Biancheri et al. (2015), one by Kohda et al. (2016), one by Kevelam et al. (2016), one by Danhauser et al. (2016), one by Taskin et al. (2016), one by Güngör et al. (2016), two by Şahin et al. (2016) and two by Pronicka et al. (2016)), with our patient being the 26th reported patient. The phenotype of our patient fits the severe group of LTBL patients, mostly due to signs of perinatal presentation of the disease and rapid decline of her clinical status. The relative immature brain associated with a slight delay in myelination limited the evaluation of possible involvement of the cerebral white matter in our patient. Nevertheless, no obvious signal abnormality of the supratentorial white matter was found, namely the spared periventricular rim described in older patients (Steenweg et al. 2012; Biancheri et al. 2015; Kevelam et al. 2016; Güngör et al. 2016; Taskin et al. 2016; Şahin et al. 2016).

Cerebral lesions appeared less expressive than reported previously, perhaps because at 2 months of age it could be too early to observe white matter abnormalities such as leukoencephalopathy. Still, the remaining lesions were typical of LTBL, as the brainstem and cerebellar lesions were present at that early age. Hypogenesis of the *corpus callosum* was also noted, as has been consistently described (Steenweg et al. 2012; Biancheri et al. 2015; Kevelam et al. 2016; Güngör et al. 2016). The collective findings of symmetrical signal changes of the deep white matter (usually sparing the periventricular rim), thalami and brainstem together with increased lactate on MRS is the hallmark of LTBL. In patients described with either mild (Biancheri et al. 2015; Güngör et al. 2016; Taskin et al. 2016; Şahin et al. 2016) or severe disease (Steenweg et al. 2012; Kevelam et al. 2016), the clinical presentation appeared to correlate with the severity of neuroimaging at each stage of this biphasic disease. However, lesions of the main structures involved were not different between patients in each group of severity, except for the significant improvement without new lesions of brain MR/MRS associated with the mild-intermediate forms. In severe situations, a progressive atrophy of the affected structures has been detected (Biancheri et al. 2015). Also, as observed by Steenweg et al. (2012), patients in the severe group had early onset neurodevelopmental compromise and persistent hyperlactacidaemia. Due to the rapid and fatal disclosure, neuroimaging alone was insufficient to predict the outcome of our patient, but she had signs of neurodevelopmental compromise such as hypotonia. She had a fatal course that

was similar to patients reported by Talim et al. (2013) and Danhauser et al. (2016). Unfortunately none of these reports had brain MR reports and images available for comparison, except for *corpus callosum* dysgenesis and agenesis, described for each patient on brain MR and brain sonogram, respectively. The two patients reported by Taylor et al. (2014) (patients 16 and 17) also had a fatal outcome with a severe neurometabolic disorder, with neonatal or very early onset presentation of LTBL.

Of the severe cases, three had compound heterozygous variants and two had a homozygous variant. In total, only five patients have been reported with homozygous pathogenic *EARS2* variants, presenting forms of LTBL with different clinical outcomes. One patient each from Talim et al. (2013) and Taylor et al. (2014) had a homozygous c.193A>G (p.Lys65Glu) missense change affecting a well conserved residue, and had a severe and fatal form of the disease. The patient presented by Biancheri et al. (2015) with a homozygous c.902G>C (p.Gly301Ala) missense change was described as having an intermediate form of LTBL. The patient from Kevelam et al. (2016) had a homozygous c.454_456del (p.Lys152del) in-frame deletion of a highly conserved residue, which presented as a severe form of disease, and was alive at the time of publication (aged 18 years). The fifth patient from Taskin et al. (2016) had a homozygous c.322C>T (p.Arg108Trp) missense change and presented a mild phenotype. Compound heterozygous changes were identified in three additional patients as part of large-scale WES studies but without specific details of phenotypes for comparison (Kohda et al. 2016; Pronicka et al. 2016). Regarding the two heterozygous variants in our patient, the start-loss p.Met1? variant is predicted to abolish the initiation translation site and has only been described previously in a single patient of Portuguese ancestry (Steenweg et al. 2012), suggesting a possible founder effect in this population. The missense p.Ile62Phe variant affects a moderately conserved amino acid within a conserved region of *EARS2*, which we postulate causes the tertiary structure of the protein to become unstable. For the patient here reported, as leukoencephalopathy was not clear on the only MR performed, the diagnosis could therefore be missed without WES analysis.

Hence, from the current 26 patients reported (including this report), almost half (11 patients) presented with a severe form of LTBL with early onset and various *EARS2* pathogenic variants (patients 1, 8, 9, 10 and 11 from Steenweg et al. (2012); Talim et al. (2013); patients 16 and 17 from Taylor et al. (2014); Kevelam et al. (2016); Danhauser et al. (2016), and the current report). Five died soon after the first clinical event (our patient, Talim et al. 2013; Taylor et al. 2014; Danhauser et al. 2016) and six patients had severe neurodevelopmental compromise, although alive at the time of the respective publication,

the oldest being 18 years old (Steenweg et al. 2012; Kevelam et al. 2016). Further studies will be needed to better understand which, if any, specific neuroimaging features would correlate with the severity and course of the disease. We were unable to establish a particular genotype–phenotype correlation among these patients.

A syndromic phenotype has been carefully delineated for LTBL as described by Steenweg et al. (2012), which is consistent with all subsequent patients reported, including our patient. Though the clinical and bio-histochemical characteristics are not specific, when combined with brain MR/MRS pattern there can be enough specificity to implement a targeted *EARS2* gene analysis in similar cases. Supplementary Table S1 summarises and compares patients reported so far with brain MR available.

For our patient, the application of WES was fundamental in reaching a definitive genetic diagnosis, but the functional studies were essential to validate the significance of the novel variant. At the time of presentation, the clinical diagnosis of LTBL had only recently been described in the literature, although we did suspect a nuclear-driven, Mendelian mitochondrial disorder (autosomal recessive inheritance) as the likely cause in our family. The establishment of the molecular diagnosis is always important for accurate genetic counselling for families and should allow the option for prenatal diagnosis or pre-implantation diagnosis for future pregnancies.

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Take-Home Message (Synopsis)

This report reinforces the phenotypic spectrum of pathogenic *EARS2* variants by reporting a severe clinical manifestation and highlights diagnostic methods to recognise biochemical and biological consequences of the genetic error.

Compliance with Ethics Guidelines

Conflict of Interest

Renata Oliveira, Ewen W. Sommerville, Kyle Thompson, Joana Nunes, Angela Pyle, Manuela Grazina, Patrick F. Chinnery, Luísa Diogo, Paula Garcia and Robert W. Taylor declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from the parents of the patient for being included in the study.

Authors' Contributions

Renata Oliveira, Joana Nunes, Manuela Grazina, Luísa Diogo, Paula Garcia and Robert W. Taylor acquired the clinical data and performed genetic, biochemical and histochemical investigations. Ewen W. Sommerville, Angela Pyle and Patrick F. Chinnery generated and analysed WES data. Ewen W. Sommerville and Kyle Thompson undertook the western blot studies. Renata Oliveira, Ewen W. Sommerville and Robert W. Taylor drafted the manuscript; all authors contributed to critical revision of the manuscript.

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