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# Leukoencephalopathy with cysts and hyperglycinemia may result from NFU1 deficiency

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ABSTRACT

Lipoic acid metabolism defects are new metabolic disorders that cause neurological, cardiomuscular or pulmonary impairment. We report on a patient that presented with progressive neurological regression suggestive of an energetic disease, involving leukoencephalopathy with cysts. Elevated levels of glycine in plasma, urine and CSF associated with intermittent increases of lactate were consistent with a defect in lipoic acid metabolism. Support for the diagnosis was provided by pyruvate dehydrogenase deficiency and multiple mitochondrial respiratory chain deficiency in skin fibroblasts, as well as no lipoylated protein by western blot. Two mutations in the *NFU1* gene confirmed the diagnosis. The p.Gly208Cys mutation has previously been reported suggesting a founder effect in Europe.



## 1. Introduction

Lipoic acid is a sulfur-containing cofactor covalently attached to and essential for the function of several key enzymatic complexes such as pyruvate dehydrogenase complex (PDHc), α-ketoglutarate dehydrogenase (α-KGDH), branched chain keto acid dehydrogenase (BCKDH) activity and H protein activity from the glycine cleavage system (GCS) (Cameron et al., 2011; Navarro-Sastre et al., 2011).

Lipoic acid is formed in mitochondria by a series of reactions involving the transfer of an octanoyl-ACP derived from fatty acid biosynthesis onto an apoprotein by LIPT2 and the addition of the sulfur component in a reaction catalyzed by lipoic acid synthase (LIAS), an enzyme possessing two [4Fe–4S] clusters (Hiltunen et al., 2010). The [4Fe–4S] cluster, which is a cofactor of LIAS as well as other mitochondrial proteins (see below), is assembled by a complex metabolic pathway involving proteins such as NFU1 (NFU Iron–Sulfur cluster scaffold homolog (S. cerevisiae), ISCU (Iron–Sulfur cluster scaffold homolog), BOLA3 (bolA family member 3 (*Escherichia coli*)) and IBA57 (IBA57, iron-sulfur cluster assembly homolog (*Saccharomyces. cerevisiae*)) (Ajit Bolar et al., 2013; Cameron et al., 2011).

In this pathway NFU1 probably acts as a scaffold downstream of ISCU during Fe–S cluster biogenesis (Navarro-Sastre et al., 2011), whereas BOLA3 is related to proteins that bind glutaredoxins, which play an unknown role in Fe–S cluster biogenesis. Mutations in either *NFU1* or *BOLA3* disrupt the function of LIAS which adds lipoate moiety to key subunits E2 of PDHc and α-KGDH, and to the related enzymes involved in branched chain amino acid metabolism and glycine degradation. IBA57 is part of the iron–sulfur cluster assembly machinery (Ajit Bolar et al., 2013).

Defects in iron–sulfur cluster biosynthesis pathway ([Fe–S] clusters) lead to abnormal function of the enzyme-bound cofactor lipoate but also of many proteins involved in intermediary metabolism and oxidative phosphorylation, as they participate in electron transfer reactions and in complex I, II and III functions (Rouault and Tong, 2008). This explains the multiple mitochondrial dysfunction syndrome associated with *NFU1*, *BOLA3*, *LIAS*, *ISCU* and *IBA57* mutations (Ajit Bolar et al., 2013; Cameron et al., 2011; Haack et al., 2013; Kollberg et al., 2009; Navarro-Sastre et al., 2011).

Up to now, two reports describe patients with *NFU1* mutations, all presenting with infantile encephalopathy and neurological regression leading to death before the age of 15 months. Most patients had

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pulmonary hypertension, and hyperglycinemia and lactic acidosis were common findings. Interestingly, one mutation, p.Gly208Cys, was frequent in European patients. Some patients apparently presented with leukoencephalopathy but no brain MRI were available (Navarro-Sastre et al., 2011).

Here, we report on a new patient of French origin with two *NFU1* mutations, one shared with the Spanish patients and the other one not reported as yet. The findings at presentation included neurological regression, leukoencephalopathy with cysts and hyperglycinemia. The association of neurological regression mimicking an energetic disease, even with intermittent hyperlactatemia in the basal state, along with hyperglycinemia suggested the diagnosis. Administration of oral lipoic acid was without effect.

## 2. Materials and methods

### 2.1. Patient

The patient, a girl, was the first child of non-consanguineous French parents. She was born after an uneventful pregnancy and spontaneous delivery at term with normal birth parameters. Up to 14 months old, psychomotor development was normal. She could support her head at age one month, sit unaided at age 7 months and had an excellent follow with eyes. The course of the disease was marked by two episodes of metabolic acidosis at 5 and 18 months of age. The first episode occurred in a context of vomiting and dehydration and was associated with mild hyperlactatemia (2.7 mmol/L), liver cytolysis and reversible pulmonary hypertension under symptomatic treatment. The second episode occurred in a context of motor regression without fever, with transient motor deficiency of the right arm, hyperlactatemia (6.9 mmol/L), hypoglycemia (2.6 mmol/L) and acute painful syndrome. Starting from 14 months old, the patient progressively lost standing and sitting abilities, and failure to thrive with poor feeding was noticed. Neurological examination revealed axial hypotonia, severe spastic tetraparesis and extrapyramidal syndrome with numerous opisthotonos episodes daily. A new episode of motor deficiency of the right arm with right facial paralysis occurred at 18 months old, requiring hospitalization in our unit. Progressive aggravation led to artificial feeding. At this time, electroencephalogram (EEG) was pathologic without any physiological rhythms but no burst suppression pattern. At 2 1/2 years, she had abnormal movements and erratic myoclonies. Focal seizures were observed and the EEG showed frontal and temporal spikes and focal discharges suggesting secondary lesional epilepsy.

### 2.2. Metabolic investigation

The first metabolic workup was performed when the patient was referred to our unit at age 17 months. Lactate and pyruvate levels were determined in plasma and CSF by enzymatic methods. Plasma, urinary and CSF amino acids were assayed by nihydrin colorimetry (Jeol Aminotac Analyzers) and urinary organic acids by gas chromatography–mass spectrometry (300MS triple quadrupole, Brüker).

### 2.3. Enzymatic analysis and respiratory chain investigation

Pyruvate dehydrogenase activity was measured by the release of $^{14}CO2$ from 0.2 mM [1-$^{14}$C]pyruvate, using suspensions of leukocytes or DCA-activated fibroblasts disrupted by sonication (Sperl et al., 1993). Polarographic and spectrophotometric assay of mitochondrial respiratory chain complex activities were measured in leukocytes and skin fibroblasts according to standard procedures (Rustin et al., 1994).

### 2.4. Molecular analysis

DNA was extracted from white blood cells, collected from the patient and her parents after informed consent. The *LIAS* (GenBank NG_032111.1), *BOLA3* (GenBank NG_031910) and *NFU1* (GenBank NG_031931.1) genes were sequenced using intronic primers (purchased from Applied Biosystems, Forster City, CA).

### 2.5. Immunoblot analysis in NFU1 and PDHA1 mutant fibroblasts

Total protein extracts were prepared by lysing cultured fibroblasts generated from either control individual or from patients with either *NFU1* mutation, or true PDH E1-α defect. Cell pellets were lysed in Triton X-100 lysis buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM Na orthovanadate, $10^{-4}$ M PMSF, $10^{-6}$ M leupeptin, $10^{-6}$ M pepstatin A and 1% Triton X-100). 40 μg of total protein extracts was separated by SDS-polyacrylamide-gel electrophoresis and transferred to nitrocellulose membranes, blocked in TBS containing 5% nonfat milk for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies. The following antibodies were used: anti-Lipoic Acid (Abcam), anti-Pyruvate Dehydrogenase E1-α subunit (Abcam), anti-DLD (Santa Cruz Biotechnology, Inc.) and anti-α Tubulin (Sigma-Aldrich). Quantitative analyses of immunoblots were performed with the Odyssey infrared image system (LiCor) using DyLight™ 800 conjugated secondary antibodies from LiCor.

### 2.6. In vivo and in vitro supplementation

A trial of lipoic acid (100 mg/kg/day) then ketogenic diet (lipids 60% of calories), was performed during six months and 24 h respectively. This work has been approved by our institutional ethical committee.

Fibroblasts from skin biopsies from a control individual and the patient were cultured in monolayer flasks with HamF10 medium containing 12% fetal calf serum and 100 UI/mL penicillin G and 100 μg/mL streptomycine. The flasks were incubated at 37 °C with 5% carbon dioxide. Culture medium was supplemented with lipoic acid (Sigma) to a final concentration of 10 (+2 mg) and 100 (+20 mg) μM during three weeks. PDH and MRC activities were analyzed on cultured skin fibroblasts before and after three weeks of supplementation.

## 3. Results

### 3.1. Brain MRI

Brain magnetic resonance imaging (MRI) revealed progressive leukoencephalopathy with extensive signal abnormalities in the periventricular cerebral white matter and in the corpus callosum (Fig. 1). The abnormal white matter and corpus callosum were partially cystic or with cavitation. Basal ganglia, cerebellum and brain stem were normal. The MRS spectroscopy with long TE (144) shows no peak of lactate. There was no argument for a stroke-like episode.

### 3.2. Metabolic investigation

Biochemical analysis revealed a mild metabolic acidosis (bicarbonates 15 mmol/L, N > 20 mmol/L) but normal level of lactate and pyruvate in blood (lactate 1.5 mmol/L, N < 2.2 mmol/L; pyruvate 0.07 mmol/L, N < 0.20 mmol/L), in urine (lactate 75 μmol/mmol of creatinine, N < 75 μmol/mmol of creatinine) and CSF (lactate 1.8 mmol/L, N < 1.95 mmol/L; pyruvate 0.11 mmol/L, N < 0.20 mmol/L). These parameters remained normal in the follow-up while lactate was reported as elevated during the two episodes of regression at ages 5 and 18 months (2.7 mmol/L and 6.9 mmol/L respectively; pyruvate not available). Glycine was elevated in blood (1178 μmol/L, N < 264 μmol/L), in urine (3395 μmol/mmol of creatinine, N < 356 μmol/mmol of creatinine) and slightly elevated in CSF (20 μmol/L, N < 16 μmol/L). All other amino acids were normal or low-normal in CSF, plasma and urine. An isolated increase of glycine was observed in all subsequent tests (plasma and urine). Urinary organic acid analysis