Lysine Restriction and Pyridoxal Phosphate Administration in a *NADK2* Patient

Frederic Tort, PhD,[a] Olatz Ugarteburu, MSc,[a] Maria Angeles Torres, MD,[b] Judit García-Villoria, PhD,[a] Marisa Girós, PhD,[a] Angeles Ruiz, MD,[b] Antonia Ribes, PhD[a]

We report the case of a 10-year-old Spanish girl with mutations in *NADK2*. Prenatal central nervous system abnormalities showed ventriculomegaly, colpocephaly, and hypoplasia of the corpus callosum. At birth, axial hypotonia, uncoordinated movements, microcephaly, and generalized cerebellar atrophy were detected. Metabolic investigations revealed high lysine, lactate, and pipecolic acid levels in blood and cerebrospinal fluid. Pyruvate carboxylase and pyruvate dehydrogenase activity in fibroblasts were normal. Beginning at birth she received biotin, thiamine, and carnitine supplementation. A lysine-restricted diet was started when she was 1 month old. Because pipecolic acid was high, pyridoxine was added to treatment. At 3 years old, astatic myoclonic epilepsy appeared, with no response to levetiracetam. We switched pyridoxine to pyridoxal phosphate, with electroclinical improvement. Because the activity of mitochondrial respiratory chain complexes III and IV was slightly low in muscle, other cofactors such as ubidecarenone, idebenone, vitamin E, and creatine were added to the treatment. At 8 years old, plasma acylcarnitine testing was performed, and high levels of 2-trans, 4-cis-decadienoylcarnitine were found. Whole exome sequencing identified a homozygous splice site mutation in *NADK2* (c.956+6T>C; p.Trp319Cysfs*21). This substitution generates exon skipping, leading to a truncated protein. In fact, *NADK2* messenger RNA and the corresponding protein were almost absent. Now, at 10 years of age she presents with ataxia and incoordination. She has oromotor dysphasia but is able to understand fluid language and is a very friendly girl. We hypothesize that the patient's clinical improvement could be due to her lysine-restricted diet together with cofactors and pyridoxal phosphate administration.

Familial hyperlysinemia is a rare autosomal recessive disorder caused by mutations in *AASS,* encoding for the first enzyme in the main lysine degradation pathway, which takes place in the mitochondria.[1,2] The impairment of this catabolic pathway leads to the accumulation of lysine in both plasma and urine. High levels of lysine in body fluids are often concomitant of many inborn errors of metabolism such as urea cycle disorders, pyruvate carboxylase deficiency, and organic acid disorders.[3]

Using next-generation sequencing, a recent study identified disease-causing mutations in *NADK2* in a child presenting with hyperlysinemia, hyperlysinuria, elevated 2-trans, 4-cis-decadienoylcarnitine (C10: 2-carnitine), and 2,4-dienoyl-CoA

abstract

[a]*Secció d'Errors Congènits del Metabolisme–IBC, Servei de Bioquímica i Genètica Molecular, Hospital Clínic, IDIBAPS, CIBERER, Barcelona, Spain; and* [b]*Hospital Universitario Son Espases, Palma de Mallorca, Spain*

Dr Tort designed the study, analyzed exome sequencing, performed and analyzed molecular data, and wrote the initial manuscript; Ms Ugarteburu performed genetic and molecular studies and drafted the initial manuscript; Dr Torres supervised data collection, helped with the follow-up of the patient, and critically reviewed the manuscript; Drs García-Villoria and Girós performed biochemical studies and critically reviewed the manuscript; Drs Ruiz and Ribes designed the study, coordinated and supervised data collection, performed the follow-up of the reported patient, and wrote the initial manuscript; and all authors approved the final manuscript as submitted.

DOI: 10.1542/peds.2015-4534

Accepted for publication Jul 26, 2016

Address correspondence to Antonia Ribes, Secció d'Errors Congènits del Metabolisme–IBC, Servei de Bioquímica i Genètica Molecular, Hospital Clínic, IDIBAPS, CIBERER, C/Mejía Lequerica s/n, Edifici Helios III, Planta Baixa, 08028 Barcelona, Spain. E-mail: aribes@clinic.ub.es

PEDIATRICS (ISSN Numbers: Print, 0031-4005; Online, 1098-4275).

FINANCIAL DISCLOSURE: The authors have indicated they have no financial relationships relevant to this article to disclose.

FUNDING: Supported by the Instituto de Salud Carlos III (FIS PI12/01138) and the Centro de Investigación Biomédica en Red de Enfermedades

To cite: Tort F, Ugarteburu O, Torres MA, et al. Lysine Restriction and Pyridoxal Phosphate Administration in a *NADK2* Patient. *Pediatrics.* 2016; 138(5):e20154534

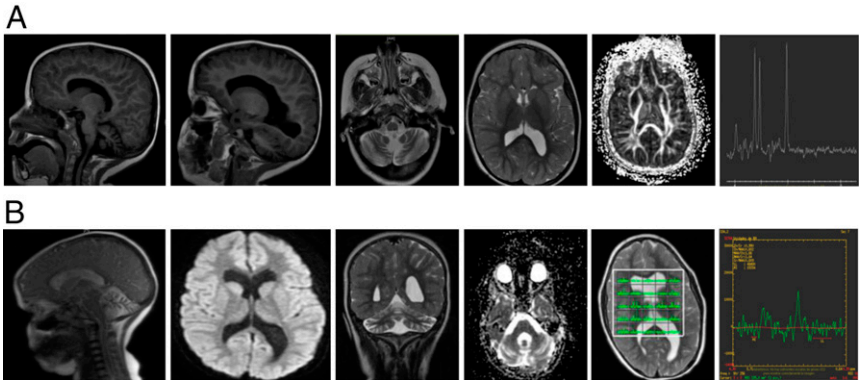

FIGURE 1

Brain MRI studies. At (A) 6 years and (B) 9 years of age no alteration in myelin was found, but a progressive global cerebellar atrophy and a high peak of lactate was observed in WM/BG, especially in CSF (intraventricular), with a relative decrease of the 3 main metabolites (N-acetylaspartate, creatine, and choline). BG, basal ganglia; WM, white matter.

reductase deficiency.[4] *NADK2* encodes for the mitochondrial nicotinamide adenine dinucleotide kinase, which is considered the only biosynthetic source of mitochondrial nicotinamide adenine dinucleotide phosphate (NADP), a cofactor necessary for the activity of enzymes implicated in a large variety of biochemical pathways involved in the mitochondrial function.[5,6]

Here, we report a patient with prenatal central nervous system (CNS) dysgenesia, microcephaly, delayed myelinization, progressive cerebellar atrophy, psychomotor retardation, and astatic myoclonic epilepsy, associated with hyperlysinemia, high levels of lactate and pipecolic acid in body fluids, and deficiency of the mitochondrial respiratory chain complexes III and IV. The use of whole exome sequencing has allowed us to identify a new homozygous mutation in *NADK2,* leading to a premature stop codon and absence of protein. This is the second reported patient with mutations in this gene. Treatment with a low-lysine diet and a cocktail of cofactors, including pyridoxal phosphate (PLP), improved considerably the clinical course of the disease compared with the previously reported patient.[4]

PATIENT PRESENTATION

Written consent was obtained from the parents of the patient for whole exome sequencing analysis and publication of this case report.

We report on a 10-year-old girl born from non consanguineous healthy Spanish parents. Prenatal scan at 34 and 37 weeks' gestational age showed CNS abnormalities with grade I to II ventriculomegaly, colpocephaly, and hypoplasia of the corpus callosum. The patient was born at 40 weeks' gestational age by elective cesarean delivery. She presented with asymmetric intrauterine growth retardation and low birth weight (2400 g, third percentile) and height (45 cm, third percentile). Head circumference was 34 cm (50th percentile). Examination at birth showed central hypotonia along with uncoordinated movements with no seizure activity, lack of sucking reflex, abnormal neonatal reflexes, and generalized hyperreflexia. The anterior fontanelle was bulging, and she showed downward eye deviation to the vertical side. During the first 10 days of life she had persistent signs of hypertensive hydrocephalus that resolved spontaneously but progressed to a persistent microcephaly with generalized cerebellar atrophy (Fig 1). Toxoplasmosis, syphilis, varicella-zoster, parvovirus B19, rubella, cytomegalovirus, and herpes infections were ruled out.

Biochemical investigations revealed high levels of lysine in plasma, urine, and cerebrospinal fluid (CSF). Pipecolic acid and lactate were also high in blood and CSF. Plasma acylcarnitine analysis showed high C10:2-carnitine (Table 1), but no abnormalities were detected in the organic acid profile in urine performed in several occasions. Other metabolic investigations such as coenzyme Q_{10}, biotinidase, sialotransferrins, very long-chain fatty acids, and phytanic and pristanic acids in plasma, as well as creatine, guanidinoacetate, and α-aminoadipic semialdehyde were normal.

Beginning at birth she presented with anorexia, feeding refusal, and frequent vomiting. Because plasma and CSF lactate were slightly high, a disturbance of mitochondrial energy metabolism was suspected. Therefore, biotin (10 mg per day), thiamine (300 mg per day), and carnitine (30 mg/kg per day) were empirically supplemented. At 1 and a half months of age, lysine restriction (45 mg/kg per day) was started, maintaining the total protein intake at 2.7 g/kg per day with hypercaloric supplements. This diet resulted of great benefit for anorexia and vomiting resolution, and plasma lysine decreased drastically, ranging from 40 to 257 μmol/L (control values [CV] 52–196 μmol/L). At 3 months of age high levels of pipecolic acid in plasma and CSF were found (Table 1). Despite normal α-aminoadipic semialdehyde in urine, pyridoxine (50 mg per day) was added empirically to treatment. At 3 years of age she developed epileptic seizures with myoclonic astatic absences. EEG changes showed a generalized spike wave of 2 to 2.5 per second with no clinical or electrical response to

TABLE 1 Biochemical and Clinical Data

	Patient, This Report	Houten et al 2014[4]	Roe et al 1990[7]
Biochemical data			
Metabolites			
Lysine			
Plasma (CV: 52–196 μmol/L)	617–1506	135–831	908–1.052
Urine (CV: 22–171 mmol/mol creatinine)	544–6976	1779	(CV: 81–213)
CSF (CV: 9.0–51.0 μmol/L)	204	226	
Pipecolic acid			
Plasma (CV: 0.1–4.2 μmol/L)	40.9	Normal	NR
CSF (CV: 0.01–0.12 μmol/L)	0.24	Normal	NR
Lactate			
Plasma (CV: 0.5–2.2 mmol/L)	7.57	1.8–7.7	NR
CSF (CV: 1.11–2.44 mmol/L)	3.1	4.1	NR
Acylcarnitines			
C10:2-carnitine (CV: <0.03 μmol/L)	0.20; 2.4	0.2–1.3 (cutoff = 0.07)	Elevated
Organic acids	Normal	Lactate and tricarboxylic acid cycle intermediates	Normal
Mitochondrial respiratory chain activities in muscle (nmol/min/mg protein)			
Complex I (nicotinamide adenine dinucleotide hydride: ubiquinol oxidoreductase) (CV: 33–152)	63	ND	NR
Complex II (CV: 43–117)	68	ND	NR
Complex I + III (nicotinamide adenine dinucleotide hydride: cytochrome C oxidoreductase) (CV: 16–53)	13	ND	NR
Complex II + III (succinate: cytochrome C oxidoreductase) (CV: 20–78)	26	ND	NR
Complex IV (cytochrome C oxidase) (CV: 144–371)	136	ND	NR
Citrate synthase (CV: 132–289)	256	ND	NR
Clinical data			
Age at presentation/age at death	Prenatal/alive at 10 y	8 wk/5 y	Birth/4 mo
Failure to thrive	Yes	Yes	Yes
Progressive leukodystrophy	No	Yes	NR
Microcephaly	Yes	Yes	Yes
Atrophy	Cerebellar atrophy	Cerebral atrophy	NR
Ventriculomegaly	Yes	Yes	Yes
Cephalic abnormalities	Hypoplasia of the corpus callosum	Bilateral basal ganglia T2 abnormalities	NR
Other physical abnormalities	Joint dislocation; Genu valgum; Puffy cheeks	Mild dysmorphic features	Short trunk, arms, and fingers with small feet and a large face
Epilepsy	Yes	Yes	NR
Hypotonia	Yes	NR	Yes
Hypertonia	No	Yes	NR
Movement alterations	Lower limb ataxia	Choreoathetosis clonus spastic quadriplegia	NR
Optical alterations	Optic atrophy; Ophthalmoplegia; Hypermetropia	Nystagmus; Abducens palsies; Cortical blindness	NR
Anorexia or vomiting	Yes	NR	Yes
Pancreatitis	No	Yes	NR

ND, not done; NR, not reported.

levetiracetam (50 mg/kg per day). We switched pyridoxine to PLP (50 mg/kg per day), and an impressive electroclinical improvement was noticed.

Biochemical studies showed normal pyruvate dehydrogenase and pyruvate carboxylase activities in fibroblasts. However, mitochondrial respiratory chain activities in muscle biopsy showed a slight reduction of complexes III and IV (Table 1). Therefore, ubidecarenone (30 mg/kg per day), idebenone (30 mgr per day), and creatine (200 mgr per day) were supplemented. The patient began to walk with assistance at 3 years of age. At present (10 years of age) she presents with static lower limb ataxia and incoordination (Table 1). Walking assistance is still needed. She has oromotor dysphasia but is

able to understand fluid language and is a very friendly girl.

Brain serial MRI scans done in the first 2 years of age showed bilateral ventriculomegaly with frontal and occipital colpocephaly, thin corpus callosum, and an important delay in myelinization, but with normal lactate peak in spectroscopy. At 6 and 9 years of age no alteration in myelin was found, but a progressive global cerebellar atrophy, together with a high peak of lactate and a relative decrease of N-acetylaspartate, creatine, and choline, was evident (Fig 1).

Because no clear diagnosis was found, we first excluded mutations in *AASS* and *ALDH7A1,* reported to be involved in familial hyperlysinemia and pyridoxine-dependent epilepsy, repectively.[1,8] Mutations in mtDNA were also ruled out. Therefore, we performed exome sequencing of the affected patient and her healthy parents (Supplemental Fig 3). A recessive inheritance pattern was hypothesized. Because a mitochondrial dysfunction was suspected, we filtered for variants in genes annotated in the MitoCarta, an inventory of proteins with strong support of mitochondrial localization.[9] However, none of them were predicted to have a significant effect on the encoded proteins. During this project a parallel study identified for the first time mutations in *NADK2* in a patient with clinical and biochemical characteristics that resembled those of our patient. A retrospective analysis of the genetic data revealed that *NADK2* was not annotated in the MitoCarta, although it is known to be localized in the mitochondria.[5,6] We therefore reconsidered our initial filtering approach, and a more accurate analysis with less stringent filters identified a homozygous mutation in *NADK2,* annotated as a low-impact variant affecting the donor splice site sequence of exon 9 (c.956+6T>C). The mutation was confirmed by Sanger sequencing (Supplemental Fig 4A). Although *in silico* analysis predicted a normal splicing for the mutated pre–messenger RNA (mRNA),[10,11] molecular studies demonstrated that the identified mutation produced an aberrant splicing that generates the skipping of exon 9, leading to a truncated protein with a premature termination codon (p.Trp319Cysfs*21), probably degraded by the nonsense-mediated mRNA decay mechanism (Fig 2, Supplemental Fig 4B). Accordingly, mRNA and protein expression analysis of the patient's fibroblasts demonstrated a strong reduction of *NADK2* transcripts compared with controls and a complete absence of protein expression (Fig 2 C and D).

DISCUSSION

The patient presented here was homozygous for a mutation (c.956+6T>C; p.Trp319Cysfs*21) in *NADK2*, encoding for the mitochondrial NAD kinase.[5,6] During this project a parallel study also identified disease-causing mutations in *NADK2* in 1 patient presenting with a clinical and biochemical phenotype resembling that of the patient described here.[4] To our knowledge this is the second reported individual with mutations in *NADK2*.

Nicotinamide adenine dinucleotide kinase 2 (NADK2) is considered to be the only biosynthetic source of NADP into the mitochondria that is a cofactor of several enzymes needed for a large variety of biochemical reactions.[5,6] Thus, a defect in NADP biosynthesis is expected to cause general mitochondrial impairment. The metabolic profile of the patient described here is very similar to that seen in the previously reported *NADK2* patient and to another patient who could have the same diagnosis, but unfortunately no material was available to perform additional studies (Table 1).[4,7] All of them showed elevated amounts of lysine in body fluids and a significant accumulation of C10:2-carnitine in blood. The fact that aminoadipic semialdehyde synthase, as well as other enzymes of the fatty acid β-oxidation, such as 2,4-dienoyl-CoA reductase, need NADP for their function explains the increase of lysine and C10:2-carnitine in these patients. Interestingly, and contrary to the previously reported case, our patient also showed elevated amounts of pipecolic acid in plasma and CSF (Table 1). Pipecolic acid is an intermediate of lysine catabolism pathway that is produced into the peroxisome. This alternative route is predominant in the brain.[3] Deficiency of NADK2 might inactivate the main mitochondrial lysine catabolic pathway, overloading the peroxisomal pathway, which might lead to the subsequent accumulation of pipecolic acid in plasma and CSF (Supplemental Fig 5).

We highlight the better clinical evolution and prolonged survival of our patient compared with the severe clinical course of those previously reported[4,7] (Table 1). One of these patients[4] and the patient reported here were both treated with lysine restriction, but PLP, thiamine, vitamin E, ubidecarenone, idebenone, and creatine were administered only to our patient. We hypothesize that the impressive electroclinical responsiveness observed in our patient could be due mainly to the administration of PLP. The rationale for this treatment was the fact that pipecolic acid was high, and consequently piperidine-6-carboxylate (P6C) could also be

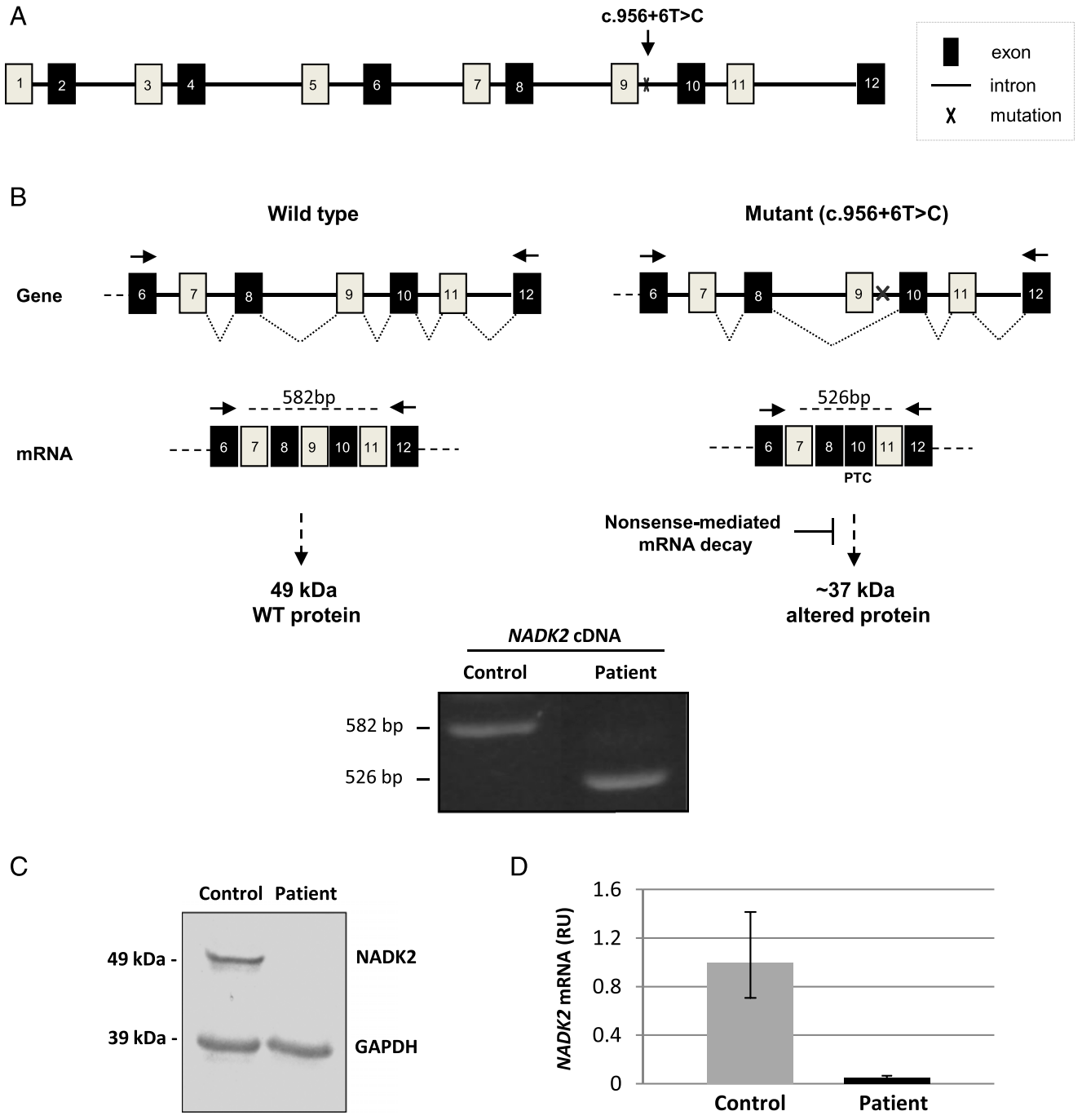

FIGURE 2

Molecular studies. A, *NADK2* genomic organization. The c.956+6C>T mutation is indicated. B, Reverse transcription polymerase chain reaction (RT-PCR) analysis demonstrated the exon skipping caused by the c.956+6C>T mutation. Arrows indicate oligos used for polymerase chain reaction (PCR). C, Immunoblotting showed absence of NADK2 protein in patient's fibroblasts. D, Quantitative RT-PCR demonstrated reduced *NADK2* mRNA levels in patient's fibroblasts.

transiently high and condense with PLP via the Knoevenagel reaction, as happens in patients with pyridoxine-dependent epilepsy (*ALDH7A1* mutations).[8,12] In this way, α-aminoadipic semialdehyde, which is in equilibrium with P6C, could be kept within the normal range, and the pool of PLP could be reduced in our patient (Supplemental Fig 5).[8,12]

The fact that PLP is a cofactor necessary for the activity of several enzymes of the CNS provides a potential explanation for the clinical

improvement of our patient upon PLP administration. Unfortunately, the available CSF sample was too small to perform additional studies to fully demonstrate this hypothesis.

In summary, we highlight the importance of an accurate biochemical characterization and the identification of specific biomarkers to direct the analysis and interpretation of next-generation sequencing data. In this case, high lysine and C10: 2-carnitine levels allowed us to identify the second patient with NADK2 deficiency reported so far. In addition, we suggest that the clinical improvement may be due mainly to a lysine-restricted diet and PLP administration.

ACKNOWLEDGMENTS

We thank Leslie Matalonga, Xènia Ferrer-Cortès, and Àngela Arias for technical support and helpful comments. We also thank Dr Ann B. Moser (Kennedy Krieger Institute, Baltimore, MD) for pipecolic acid determination, Paz Briones for respiratory chain activities, Célia Pérez-Cerdá for α-aminoadipic semialdehyde determination, and the CNAG team for their excellent technical and bioinformatics support. We are also grateful to the families involved in this study.

ABBREVIATIONS

C10: 2-carnitine: 2-trans, 4-cis-decadienoylcarnitine
c-DNA: complementary DNA
CNS: central nervous system
CSF: cerebrospinal fluid
CV: control value
mRNA: messenger RNA
NADK2: nicotinamide adenine dinucleotide kinase 2
NADP: nicotinamide adenine dinucleotide phosphate
P6C: piperidine-6-carboxylate
PCR: polymerase chain reaction
PLP: pyridoxal phosphate
RT-PCR: reverse transcription polymerase chain reaction

Raras, an initiative of the Instituto de Salud Carlos III (Ministerio de Ciencia e Innovación, Spain). This study was supported by the 2013 CNAG Call: 300 exomes to elucidate rare diseases and Agència de Gestió d'Ajuts Universitaris i de Recerca (2014: SGR 393).

POTENTIAL CONFLICT OF INTEREST: The authors have indicated they have no potential conflicts of interest to disclose.

REFERENCES

1. Sacksteder KA, Biery BJ, Morrell JC, et al. Identification of the alpha-aminoadipic semialdehyde synthase gene, which is defective in familial hyperlysinemia. *Am J Hum Genet*. 2000;66(6):1736–1743
2. Markovitz PJ, Chuang DT, Cox RP. Familial hyperlysinemias. Purification and characterization of the bifunctional aminoadipic semialdehyde synthase with lysine-ketoglutarate reductase and saccharopine dehydrogenase activities. *J Biol Chem*. 1984;259(19):11643–11646
3. Houten SM, Te Brinke H, Denis S, et al. Genetic basis of hyperlysinemia. *Orphanet J Rare Dis*. 2013;8:57
4. Houten SM, Denis S, Te Brinke H, et al. Mitochondrial NADP(H) deficiency due to a mutation in *NADK2* causes dienoyl-CoA reductase deficiency with hyperlysinemia. *Hum Mol Genet*. 2014;23(18):5009–5016
5. Ohashi K, Kawai S, Murata K. Identification and characterization of a human mitochondrial NAD kinase. *Nat Commun*. 2012;3:1248
6. Zhang R. MNADK, a novel liver-enriched mitochondrion-localized NAD kinase. *Biol Open*. 2013;2(4):432–438
7. Roe CR, Millington DS, Norwood DL, et al. 2,4-Dienoyl-coenzyme A reductase deficiency: a possible new disorder of fatty acid oxidation. *J Clin Invest*. 1990;85(5):1703–1707
8. Mills PB, Struys E, Jakobs C, et al. Mutations in antiquitin in individuals with pyridoxine-dependent seizures. *Nat Med*. 2006;12(3):307–309
9. Pagliarini DJ, Calvo SE, Chang B, et al. A mitochondrial protein compendium elucidates complex I disease biology. *Cell*. 2008;134(1):112–123
10. Carmel I, Tal S, Vig I, Ast G. Comparative analysis detects dependencies among the 5′ splice-site positions. *RNA*. 2004;10(5):828–840
11. Shapiro MB, Senapathy P. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res*. 1987;15(17):7155–7174
12. Plecko B, Paul K, Paschke E, et al. Biochemical and molecular characterization of 18 patients with pyridoxine-dependent epilepsy and mutations of the antiquitin (*ALDH7A1*) gene. *Hum Mutat*. 2007;28(1):19–26

Lysine Restriction and Pyridoxal Phosphate Administration in a *NADK2* Patient

Frederic Tort, Olatz Ugarteburu, Maria Angeles Torres, Judit García-Villoria, Marisa Girós, Angeles Ruiz and Antonia Ribes

Pediatrics 2016;138;
DOI: 10.1542/peds.2015-4534 originally published online October 18, 2016;

PEDIATRICS®
OFFICIAL JOURNAL OF THE AMERICAN ACADEMY OF PEDIATRICS

Lysine Restriction and Pyridoxal Phosphate Administration in a *NADK2* Patient

Frederic Tort, Olatz Ugarteburu, Maria Angeles Torres, Judit García-Villoria, Marisa Girós, Angeles Ruiz and Antonia Ribes

Pediatrics 2016;138;
DOI: 10.1542/peds.2015-4534 originally published online October 18, 2016;