

## Regular Article

# Triple therapy with pyridoxine, arginine supplementation and dietary lysine restriction in pyridoxine-dependent epilepsy: Neurodevelopmental outcome



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## ARTICLE INFO

## Article history:

Received 27 March 2015

Received in revised form 22 May 2015

Accepted 22 May 2015

Available online 23 May 2015

## Keywords:

Alpha-aminoadipic semialdehyde

Antiquitin

Arginine supplementation

Epileptic encephalopathy

Pyridoxine dependent epilepsy

## ABSTRACT

Pyridoxine-dependent epilepsy (PDE) is an epileptic encephalopathy characterized by response to pharmacologic doses of pyridoxine. PDE is caused by deficiency of  $\alpha$ -aminoadipic semialdehyde dehydrogenase resulting in impaired lysine degradation and subsequent accumulation of  $\alpha$ -aminoadipic semialdehyde. Despite adequate seizure control with pyridoxine monotherapy, 75% of individuals with PDE have significant developmental delay and intellectual disability. We describe a new combined therapeutic approach to reduce putative toxic metabolites from impaired lysine metabolism. This approach utilizes pyridoxine, a lysine-restricted diet to limit the substrate that leads to neurotoxic metabolite accumulation and L-arginine to compete for brain lysine influx and liver mitochondrial import. We report the developmental and biochemical outcome of six subjects who were treated with this triple therapy. Triple therapy reduced CSF, plasma, and urine biomarkers associated with neurotoxicity in PDE. The addition of arginine supplementation to children already treated with dietary lysine restriction and pyridoxine further reduced toxic metabolites, and in some subjects appeared to improve neurodevelopmental outcome. Dietary lysine restriction was associated with improved seizure control in one subject, and the addition of arginine supplementation increased the objective motor outcome scale in two twin siblings, illustrating the contribution of each component of this treatment combination. Optimal results were noted in the individual treated with triple therapy early in the course of the disease. Residual disease symptoms could be related to early injury suggested by initial MR imaging prior to initiation of treatment or from severe epilepsy prior to diagnosis. This observational study reports the use of triple therapy, which combines three effective components in this rare condition, and suggests that early diagnosis and treatment with this new triple therapy may ameliorate the cognitive impairment in PDE.

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## 1. Introduction

Pyridoxine-dependent epilepsy (PDE) is an early infantile epileptic encephalopathy characterized by a positive response to pharmacologic

doses of pyridoxine. PDE is an autosomal recessive condition caused by mutations in *ALDH7A1*. PDE results from the deficiency of  $\alpha$ -aminoadipic semialdehyde dehydrogenase with subsequent accumulation of  $\alpha$ -aminoadipic semialdehyde ( $\alpha$ -AASA) and  $\Delta^1$ -piperidine-6-

**Abbreviations:** 5HIAA, 5-hydroxyindolacetic acid;  $\alpha$ -AASA,  $\alpha$ -aminoadipic semialdehyde; ABAS II, Adaptive Behavioral Assessment System-II; AIMS, Alberta Infant Motor Score; CSF, cerebral spinal fluid; DOL, days of life; EEG, electroencephalography; FMOc, fluorenylmethoxycarbonyl; GA1, glutaric aciduria type I; HVA, homovanilic acid; P6C,  $\Delta^1$ -piperidine-6-carboxylate; PDE, pyridoxine dependent epilepsy; PLP, pyridoxal 5'-phosphate.

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carboxylate (P6C), which are linear and cyclic molecules, respectively, that are in equilibrium. The accumulated P6C inactivates the active vitamers of B<sub>6</sub> (pyridoxal 5'-phosphate; PLP) by forming a Knoevenagel condensation product [1]. PLP is a cofactor for over 140 enzymatic reactions including those involved in the synthesis and degradation of amino acids and neurotransmitters [2,3], and decreased availability of PLP for neurotransmitter synthesis is believed to be important to the pathogenesis of PDE, and is the basis of treatment with pyridoxine. Low cerebrospinal fluid (CSF) concentrations of PLP have been noted in vitamin B<sub>6</sub> responsive seizure disorders, although are not always identified in affected individuals with PDE [4,5].

The classic presentation of PDE is the neonatal onset of treatment-refractory seizures that have a dramatic response to pyridoxine supplementation, although onset of seizures may occur in childhood, multiple seizure types may be observed, and the clinical response to pyridoxine may be delayed [6,7]. Treatment with pharmacologic doses of pyridoxine provides sufficient pyridoxine to overcome the apparent sequestration of PLP due to condensation with P6C, although rarely patients have responded to physiological doses of pyridoxine [8]. The majority of patients are reported to achieve seizure control with pyridoxine alone, although additional antiepileptic drugs may be required in some patients for optimal seizure management [9]. The identification that folinic acid-responsive seizures are also a result of deficiency of  $\alpha$ -aminoacidic semialdehyde dehydrogenase has led to concomitant treatment with folinic acid in some individuals [10]. The mechanism of response to folinic acid is not understood, and the benefit of folinic acid in the treatment of PDE has not been established.

Despite adequate seizure control, 75% of individuals with PDE have significant developmental delay and intellectual disability on pyridoxine monotherapy [9,11]. Even with early diagnosis and optimal seizure control, significant cognitive impairment has been noted in children and adults suggesting that pyridoxine supplementation alone is not sufficient to treat all neurologic aspects of the disease [11,12]. The degree of intellectual disability does not correlate with the age of seizure onset, seizure type, age at diagnosis, or biochemical findings at presentation [9,11].

Identification of alpha-aminoacidic semialdehyde dehydrogenase deficiency as the cause of PDE, an enzyme within the cerebral lysine degradation pathway, suggested that patients may benefit from dietary limitation of lysine [1,13]. The impaired lysine metabolism in PDE results in significant accumulation of  $\alpha$ -AASA and P6C, which are likely neurotoxic and may contribute to the pathogenesis of PDE. In a previously reported case series, implementation of a lysine-restricted diet resulted in decreased plasma and urinary  $\alpha$ -AASA levels and an improved developmental outcome [14]. As a result, a lysine-restricted diet has been recommended as adjunct therapy in PDE, and dietary guidelines have been developed by an international working group [15].

Glutaric aciduria type I (GA I) is another disorder of lysine catabolism, and results in elevated neurotoxic metabolites glutaric acid and 3-hydroxyglutaric acid. A lysine-restricted diet is a crucial component of treatment of GA I [16], and has a demonstrated neuroprotective effect [17,18], although dietary therapy alone is not sufficient to prevent all neurologic sequelae [19]. In a mouse model of GA I, dietary lysine restriction reduced the glutaric acid concentrations in the brain, although the 3-hydroxyglutaric acid concentration remained unchanged [20]. Lysine is a dibasic amino acid that is transported at epithelia of the intestine, the kidney and the blood-brain barrier by the cationic transporters that also transport the dibasic amino acids arginine and ornithine [21–23]. As a result, the use of arginine to compete with lysine for transport has been suggested. Both arginine supplementation and a low ratio of dietary lysine to dietary arginine have been recommended in the treatment of individuals with GA I [24]. Experimental evidence for the efficacy of this treatment was demonstrated in the mouse model of GA I, in which, arginine supplementation alone mimicked the results of the lysine-restricted diet in the reduction of neurotoxic brain

metabolites [20]. Arginine supplementation combined with a lysine-restricted diet was able to further reduce the brain glutaric acid concentration, compared to monotherapy with either lysine restriction or arginine, as well as the brain 3-hydroxyglutaric acid concentration [20]. This supports the hypothesis that supplemental arginine can decrease brain lysine metabolism through competitive inhibition, and suggests an additive benefit of both a lysine-restricted diet and arginine fortification in cerebral lysine disorders. Recently arginine supplementation was used as an alternative to the lysine-restricted diet in a child with PDE with a decrease in cerebral lysine and urine and CSF  $\alpha$ -AASA levels as well as neurologic improvement [25].

We present the clinical outcome of six subjects with PDE who were treated with a novel treatment combination of pyridoxine supplementation, a lysine restricted diet, and arginine supplementation. We refer to this treatment as “triple therapy” for PDE.

## 2. Methods

The study is a retrospective review of available medical records from three centers in North America. For subjects 1–4, treatment for PDE was performed as part of clinical care. For subjects 5 and 6 the British Columbia Children's Hospital Review Board approved the study as an “innovative treatment protocol.” Consent for participation and publication of study results was obtained from each subject's parent or legal guardian. Medical records were reviewed for six subjects diagnosed with PDE through biochemical testing and mutations in *ALDH7A1*. Subjects were initially treated with pyridoxine supplementation and both a lysine restricted diet and arginine supplementation were added to the treatment regimen either sequentially (in five subjects) or concurrently (1 subject). Summary clinical and genetic information and information regarding treatment and outcome are listed in Table 1. Information regarding the effect of a lysine-restricted diet alone in subjects 1, 5 and 6 were reported previously [14,26]. Data extracted from medical records included plasma or CSF pipercolic acid, AASA, P6C, lysine, arginine, 5-hydroxyindolacetic acid (5HIAA) and homovanilic acid (HVA). Brain MR imaging findings and clinical evaluation of cognitive and motor development were also recorded.

### 2.1. Subjects

Subject 1 was the first child of non-consanguineous parents of European ancestry born after an unremarkable pregnancy. The subject presented at nine days of life (DOL) with hypoglycemia, acidosis, and episodes of stiffening with brief jerking and twitching movements of her extremities. Electroencephalography (EEG) was indicative of epileptic encephalopathy with mild burst suppression. She received 100 mg of IV pyridoxine at 11 DOL with no immediate changes noted on EEG. Pyridoxine supplementation was continued at 30 mg/kg/day and improvement in the subject's clinical status and EEG was noted 24 h after initial treatment. The diagnosis of PDE was established through elevated plasma levels of pipercolic acid,  $\alpha$ -AASA and P6C. The subject had complete resolution of clinical seizures at 11 DOL with a normal EEG at 20 DOL. Brain MR imaging performed at 12 DOL noted numerous foci of restricted diffusion scattered throughout the bilateral anterior and posterior periventricular white matter (Fig. 1A, 1B). A lysine-restricted diet was initiated at 1 month of life through the addition of metabolic formula thereby limiting lysine from natural sources. Arginine supplementation was added to her treatment regimen at 3 months of age at 150 mg/kg/day and increased to 200 mg/kg/day at two years of age (Table 1).

Subject 2 was the second child of a consanguineous union of parents originating from El Salvador. Her pregnancy history was unremarkable, and she presented to care with seizure like activity at 13 DOL and an EEG noted multifocal onset short electrographic seizures with the clinical correlate of apnea. Brain MR imaging, at that time, showed mild diffuse cerebral swelling without evidence of ischemia. Pyridoxine

supplementation was initiated at 16 DOL with resolution of clinical seizures, although clinical improvement was attributed to a concurrent increase in the subject's topiramate treatment. Pyridoxine supplementation was discontinued at 20 DOL and traditional pharmacologic therapy was continued. The subject remained seizure free until 90 DOL at which time she presented with a seizure in the setting of an upper respiratory infection. She continued to have episodes of breakthrough seizures and episodes of status epilepticus resulting in changes to her pharmacologic therapy, which included trials of seven different antiepileptic medications. The diagnosis of PDE was established at 264 DOL with elevated plasma  $\alpha$ -AASA and P6C, and triple therapy was initiated immediately after diagnosis. Dietary therapy was initiated through the use of a metabolic formula thereby limiting lysine from natural sources. Arginine supplementation was added to her treatment regimen at 150 mg/kg/day (Table 1).

Subjects 3 and 4 are identical male twins born after an uneventful pregnancy, and presented at one week of age with seizures and metabolic acidosis. Initial EEG showed a burst suppression pattern that persisted despite phenobarbital and topiramate loading. Brain MR imaging revealed T2 hyperintensities within the periventricular white matter in both twins. Clinical seizures resolved with a pyridoxine trial, however EEGs continued to show burst-suppression activity, which led to discontinuation of pyridoxine after a two-day trial. Clinical seizures recurred for both twins one week after discontinuation of therapy, and a second trial of pyridoxine was administered at one month of age with resolution of clinical and electrographic seizures. The diagnosis of PDE was established by analysis of monoamine metabolites in CSF using high-performance liquid chromatography (HPLC) with electrochemical detection. Two compounds associated with PDE were identified in CSF [13]. In addition, there was elevation of urine  $\alpha$ -AASA and plasma pipercolic acid, a related metabolite. The twins were treated with pyridoxine and folinic acid supplementation, and folinic acid was discontinued at 5 months of age. A lysine-restricted diet was initiated

at three months of age, and arginine supplementation was initiated at 12 months of age at 150 mg/kg/day (Table 1).

Subject 5 was the first child of non-consanguineous parents of European ancestry and prenatal history was notable for repetitive fetal movements likely representing fetal hiccups. Subject 5 presented with intractable seizures, hypoglycemia, lactic acidosis, bilateral temporal lobe hemorrhages and thalamic changes on cranial MR imaging during the neonatal period. She was treated with pyridoxine and folinic acid resulting in resolution of clinical seizures, although she continued to have an abnormal EEG showing mild background suppression and multifocal spikes 1.5 months of age. The diagnosis of PDE was established at five weeks of age based on elevated urinary  $\alpha$ -AASA. Diet was restricted in lysine and supplemented with a lysine-free amino-acid mixture formula according to the PDE consortium recommendations [15]. Arginine supplementation (150 mg/kg/day) was added to her therapy at 6.3 years of age (Table 1).

Subject 6 was the first child of non-consanguineous parents of European ancestry born after a pregnancy where maternal idiopathic thrombocytopenic purpura and splenomegaly were noted. Subject 6 presented to care at 3 DOL with seizures and was treated at 2 months of age with pyridoxine and phenytoin after focal epilepsy was identified. The diagnosis of PDE was established as a result of elevated urine  $\alpha$ -AASA. He continued to have clinical seizures despite very high doses of pyridoxine (40 mg/kg/day), folinic acid, clobazam and phenobarbital. At 3 years of age, he developed electrophysiological evidence of sensorimotor neuropathy. Dietary lysine restriction was initiated at 3.5 years of age, with subsequent cessation of clinical seizures allowing a decrease of pyridoxine dosage to 20 mg/kg/day and discontinuation of antiepileptic drugs, although clobazam was restarted at six years of age. Lysine was restricted in the diet and supplemented with an amino-acid mixture formula according to the PDE consortium recommendations [15]. Arginine supplementation (150 mg/kg/day) was added to his therapy at 8 years of age (Table 1).

**Table 1**  
Demographic, treatment and outcome data in those individuals treated with triple therapy.

	Subject 1	Subject 2	Subject 3 <sup>a</sup>	Subject 4 <sup>a</sup>	Subject 5	Subject 6
<b>Demographic information</b>						
Genotype	c.448_458del11ntfsX45; p.E399Q	p.G274E; p.S317L	p.P403L; unknown <sup>b</sup>	p.P403L; unknown <sup>b</sup>	p.V278V; p.G398A	p.V250V; p.E399Q
Age at presentation	9 days	13 days	7 days	7 days	3 days	3 days
Clinical symptoms	Intractable seizures, hypoglycemia	Intractable seizures	Seizures, metabolic acidosis	Seizures, metabolic acidosis	Intractable seizures, hypoglycemia, lactic acidosis	Seizures
Age at diagnosis	11 days	264 days	1 month	1 month	5 weeks	2 months
<b>Treatments</b>						
B6 Tx (age)	30 mg/kg/day (11 d) 25 mg/kg/day (200 d)	25 mg/kg/day (284 d)	20 mg/kg/day (1 m)	20 mg/kg/day (1 m)	15 mg/kg/day (5 w) 30 mg/kg/day (3 y)	40 mg/kg/day (2 m) 20 mg/kg/day (3.5 y)
Dietary Tx (age)	Lysine restriction (28 d)	Lysine restriction (288 d)	Lysine restriction (3 m)	Lysine restriction (3 m)	Lysine restriction (11 m)	Lysine restriction (3.5 y)
Arginine Tx (age)	150 mg/kg/day (99 d) 200 mg/kg/day (2.3 y)	150 mg/kg/day (288 d)	150 mg/kg/day (12 m)	150 mg/kg/day (12 m)	150 mg/kg/day (6.3 y)	150 mg/kg/day (8 y)
Other Tx(s)	NA	Folinic acid, AE	Folinic acid	Folinic acid	Folinic acid, AE	Folinic acid, AE
<b>Outcome after treatments</b>						
After B6 Tx	Resolution of seizures and improved EEG	Significant improvement in developmental milestones.	Seizure control	Seizure control	Resolution of seizures until 5 years	Breakthrough seizures continued
After Dietary Tx	Continued normal developmental milestones	Continued developmental delay <sup>c</sup>	Developmental improvement	Developmental improvement	Developmental and EEG improvements	Resolution of seizures, developmental improvement
After Arginine Tx	Continued normal developmental milestones		Further developmental improvement	Further developmental improvement	Resolution of seizures, increased focus and improved memory <sup>c</sup>	Initial improvement in speech and language development

AE, anti-epileptics; d, days; m, months; NA, not applicable; Tx, treatment; w, weeks; y, years.

<sup>a</sup> Subjects 3 and 4 are identical twins with a similar clinical course and treatment.

<sup>b</sup> Analysis for copy number alterations in *ALDH7A1* was negative.

<sup>c</sup> All therapies were started within a 4 day period.

### 3. Clinical outcomes and results

#### 3.1. Clinical outcomes

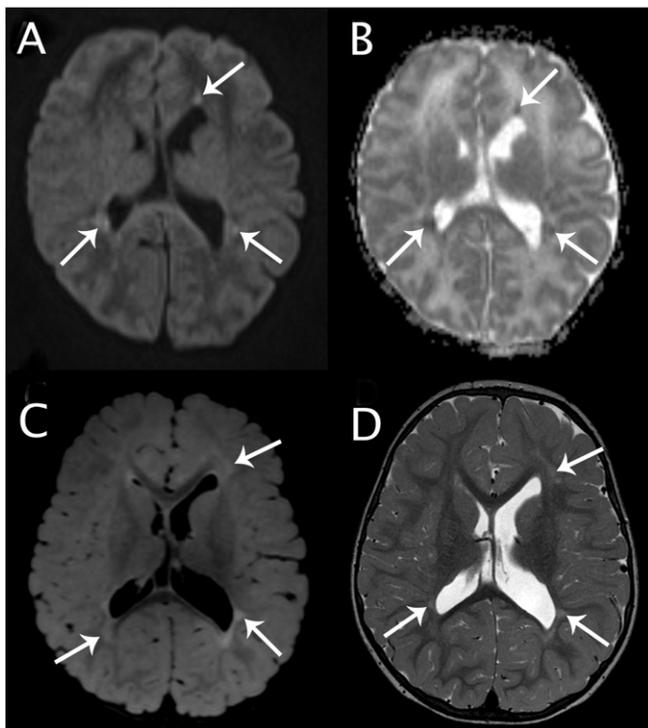
Subject 1's development was assessed through regular developmental evaluations. Her development has continued to be age appropriate, and she has not had seizures since the initiation of treatment at 11 days of age. The plasma metabolites including pipercolic acid,  $\alpha$ -AASA and P6C decreased with the initiation of a lysine-restricted diet and appeared to decrease further with the initiation of arginine supplementation (Supplemental Fig. 1). Serial measurements of CSF amino acids, PLP and  $\alpha$ -AASA were performed; a decrease in CSF lysine and an increase in CSF PLP following the initiation of treatment were noted, though there was an increase in  $\alpha$ -AASA (Supplemental Table I). Repeat MR imaging, performed at 25 months of life, demonstrated white matter volume loss and gliosis corresponding to the areas of previously injured brain (Fig. 1C, 1D) as well as a mild thinning of the posterior corpus callosum (Supplemental Fig. 2). At 26 months of life she exhibited a stiff gait with unilateral increased tone and physical therapy was initiated, although her other developmental milestones continued to be age appropriate.

Subject 2 exhibited global developmental delay at diagnosis at 9 months of age; she could not sit unsupported and had minimal interaction with her environment. Her symptoms of hypotonia and developmental delay were, in part, attributable to her anti-epileptic therapy, which was weaned over time. Anti-epileptics were discontinued by 26 months of age. Triple therapy was initiated shortly after diagnosis. Within four months she was able to sit on her own, started to communicate with her parents and attempted to stand. Despite immediate developmental improvement, the subject's development remained significantly delayed. Folinic acid (5 mg/day) was added to her treatment at 1.4 years of age without notable improvement in her development. At 2 years 9 months of age the Bayley Scales of Infant and Toddler Development – 3rd edition identified diffuse cognitive and motor deficits with a cognitive composite score of 60 and motor composite

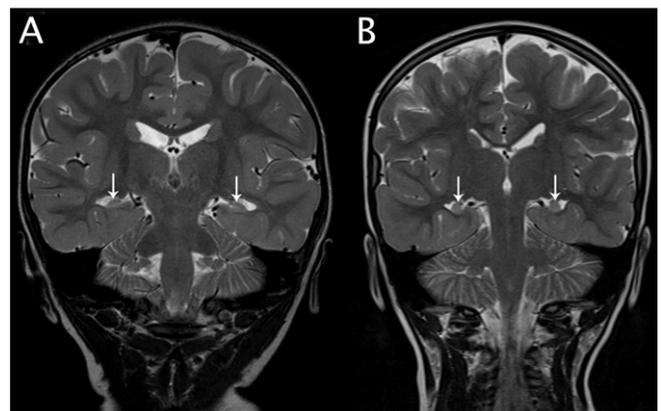
score of 50 (scores have a mean of  $100 \pm 15$ ). The Adaptive Behavioral Assessment System-II (ABAS II) noted limited ability to perform daily activities with a general adaptive composite score of 45 (mean of  $100 \pm 15$ ). She has severe speech and language delay producing only a few spoken words and signs. The subject has remained seizure free since the initiation of triple therapy with the exception of one breakthrough seizure two months after initial diagnosis. Plasma metabolites including pipercolic acid,  $\alpha$ -AASA and P6C decreased with initiation of triple therapy (Supplemental Fig. 3). CSF metabolites were measured prior to initiation of treatment and again 1.6 years after initiation of treatment, which showed that the CSF lysine level was halved in comparison with the pre-treatment value and at the lower limit of the reference range though the CSF  $\alpha$ -AASA was unchanged (Supplemental Table II). Follow-up MR imaging of the brain revealed atrophy and increased T2 signal of the bilateral hippocampi, consistent with hippocampal sclerosis, with no additional abnormality noted (Fig. 2A).

Subjects 3 and 4 had good head control and were able to roll from supine to prone position on pyridoxine monotherapy, but subject 4 was noted to have focal neurological deficits with hand fisting and upper and lower limb spasticity at three months of age. The limb spasticity and hyperreflexia 3+ in upper and lower limbs in both twins, and left hand fisting in subject 4 continued throughout the first year. They showed progression of their developmental milestones over the course of treatment; they were commando crawling but were not yet pulling to stand at 1 year of age. The Alberta Infant Motor Score (AIMS) [27,28] was used to assess their gross motor development, where they followed the 20th centile. There were signs of mild to moderate speech delay, as they had not started babbling at one year of age. In addition, the twins appeared to show transient regression of language development and unresponsiveness to external stimuli during periods of febrile illness.

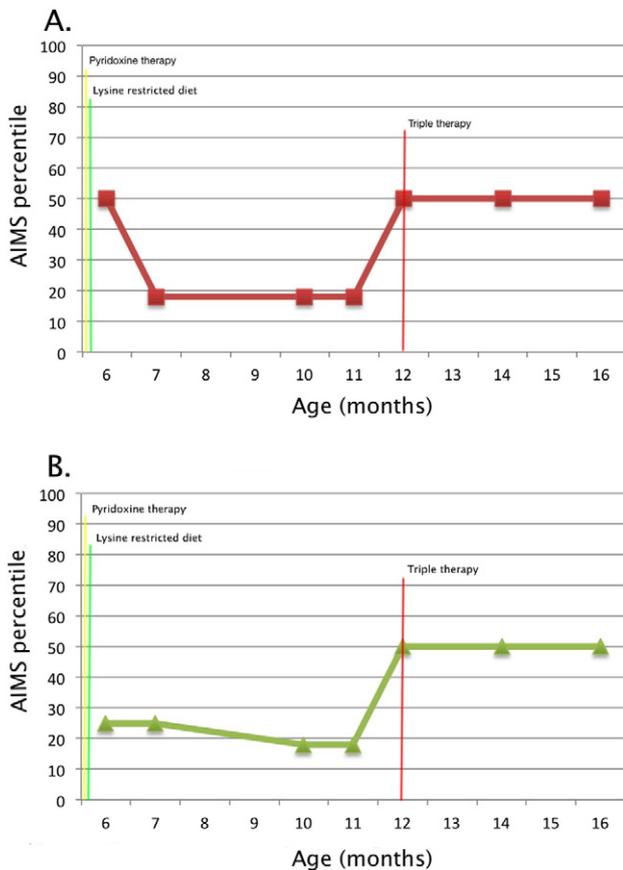
Adjunct therapy was started sequentially at 3 months and 12 months of age and both plasma and urinary  $\alpha$ -AASA decreased with initiation of a lysine-restricted diet and appeared to decrease further with the initiation of triple therapy (Supplemental Figs. 4, 5). Both subjects had notable improvement in their muscle tone with reduced spasticity for subject 4. In addition, the twins began to stand and to walk with support, and their AIMS percentiles improved and continued to follow the 50th percentile (Fig. 3). At 18 months of age the twins were assessed by a speech therapist, and found to have a 6-month delay in receptive language and a 12-month delay in expressive language. For subject 3, a follow-up MR imaging of the brain at 18 months revealed hyperintensities on T2 weighted imaging in the centrum semiovale and in the frontal and parietal lobes, which were more prominent on the left. For subject 4, T2 hyperintensities were



**Fig. 1.** Brain MR imaging over time in a subject with PDE. Legend: brain MR imaging performed at 12 DOL in subject 1 reveals numerous foci of restricted diffusion scattered throughout the bilateral anterior and posterior periventricular white matter [A (DWI) and B (ADC)]. Repeat MR imaging of the same patient at 25 months of life demonstrates consequent white matter volume loss and gliosis [C (FLAIR) and D (T2)].



**Fig. 2.** Brain MR imaging noting mesial temporal sclerosis in subjects with PDE. Legend: brain MR imaging (T2) performed at 2 years 6 months of life, in subject 2, illustrates atrophy and increased T2 signal of the bilateral hippocampi consistent with sclerosis (A). Brain MR imaging (T2), in subject 5, performed at 2 years of life demonstrates similar findings of bilateral mesial temporal sclerosis (B).



**Fig. 3.** Improved developmental outcome following triple therapy. Legend: subjects 3 (A) and 4 (B) had improved development once triple therapy was initiated. Development was measured by AIMS evaluations and expressed as a percentile (y-axis) over time (x-axis).

also seen in the centrum semiovale and periventricular parietal white matter, and were more severe on the right. In addition there was focal dilatation of the temporal horn of the right lateral ventricle suggestive of focal atrophy. By 22 months of age both subjects understood 20 words with only a mild delay in receptive language, however, their expressive language development remained severely delayed. At 2 years of age the subjects' neurological exam revealed normal axial and peripheral tone, and persistently brisk reflexes remained in upper and lower extremities. Transient periods of regression and unresponsiveness to external stimuli were no longer observed during periods of febrile illness while treated with triple therapy.

Subject 5 was clinically seizure free on pyridoxine monotherapy, but with continuously abnormal EEG showing mild background suppression and multifocal spikes at age 1.5 months. She had neurodevelopmental testing (Bayley-III) at ages 4 and 8 months, which showed truncal hypotonia, moderate delay in fine and gross motor skills with age appropriate communication and language skills. Dietary therapy was started at 11 months of age and neurodevelopmental testing (Gesell Developmental Test) at ages 12, 19, 26, and 31 months showed improvements in age-appropriate skills, with the exception of mild fine motor delay.

At age 3 years frequent spike and wave activity as well as paroxysmal theta activity in bilateral anterior quadrants, albeit without clinical correlates, recurred and the pyridoxine dosage was increased from 15 to 30 mg/kg/day. At the age of 5 years she suffered severe constipation, and was temporarily unable to comply with the lysine-free formula. During the period she was unable to comply with lysine restriction, she had several generalized tonic-clonic seizures requiring the addition of ethosuximide to her therapy. Her constipation was treated aggressively and at the age of 6.3 years triple therapy was started with resolution of clinical seizures. MR imaging of the brain, performed 2.5 months after

starting arginine supplementation, was unchanged compared with previous MR imaging at age 2 years and showed bilateral mesial temporal sclerosis (Fig. 2B). Her EEG recording noted a slow and dysrhythmic background showing no change when compared to her previous EEG done at age 4 years. Neurodevelopmental testing prior to triple therapy showed that her fine motor skills were underdeveloped and her overall visual-spatial reasoning and integration were below average, affecting her school performance. She currently utilizes both vision training and therapeutic horseback riding. Following initiation of triple therapy, a decrease in her behavioral problems and an increase in focus and memory were reported; due to logistical challenges it was not possible to perform formal testing. Plasma pipercolic acid and urine  $\alpha$ -AASA decreased with the initiation of dietary therapy and continued to decline with the addition of supplemental arginine (Supplemental Fig. 6) and CSF pipercolic acid and  $\alpha$ -AASA were lower on triple therapy than with dietary restriction alone (Supplemental Table III).

Subject 6 had poor gross motor and very poor fine motor skills (Peabody Motor Scales II) as well as a significant delay in language expression (1st percentile) and comprehension (5th percentile) (Pre-School Language Scale – 4th edition) at 3 years of age on pyridoxine and clobazam. Two weeks after the initiation of dietary treatment he became a “different child” according to his mother and started to learn as evidenced by the ability to start toilet training. After 1 year of a lysine-restricted diet, both fine and gross motor skills had improved to age-appropriate levels (Peabody scale); his communication, attention span, and repetitive behaviors also showed impressive progress. His expressive speech delay improved but remained mildly delayed with near normalization of receptive skills (Clinical Evaluation of Language Fundamentals). When the diet was shortly interrupted due to adherence difficulty, he experienced a relapse of seizures and a behavioral deterioration. At age 6 years he experienced a single focal seizure, and after addition of clobazam to his treatment he remained seizure free. The lysine-restricted diet was again interrupted at 8 years of age as the family ran out of the lysine-free amino acid mixture. During this interruption, he became dazed, less responsive and clumsy, but returned to baseline once the diet was re-initiated.

Arginine supplementation was added to his current therapy at 8 years of age and his mother reported that his speech and language development accelerated. Neurodevelopmental testing was repeated 6 months after initiation of triple therapy and his mild to moderate intellectual disability was unchanged from the previous assessment at age 6 years. He was age-appropriate for basic receptive and expressive language skills, but below age level for advanced expressive language, visual motor integration, and visual discrimination. There has been a decrease in his fine motor skills compared to his previous assessment, with an improvement in anxiety and in adaptive behavior with respect to home living, health and safety. He also has significant difficulties with inattention, impulsivity and executive function. His working memory is below age level; comparison with previous testing is not possible. Routine EEG was repeated at 8.5 years and demonstrated a moderately reactive posterior dominant rhythm of 8 Hz, absence of sleep spindles and vertex waves with sleep, slow wave activity with intermixed paroxysmal theta activity and frequent multifocal sharp wave activity all consistent with diffuse brain dysfunction. No seizures were captured. Brain MR imaging was performed 2.5 months after adding supplemental arginine to his therapy (*i.e.*, triple therapy) and was normal with no change compared to previous evaluation at age 4 years. Plasma pipercolic acid and urine  $\alpha$ -AASA decreased with initiation of dietary therapy and continued to decline with triple therapy (Supplemental Fig. 7), also the CSF  $\alpha$ -AASA and pipercolic acid showed further decrease on triple therapy (Supplemental Table III).

### 3.2. Metabolite results

The plasma  $\alpha$ -AASA, P6C, pipercolic acid and urine  $\alpha$ -AASA decreased on therapy with pyridoxine supplementation and a lysine restricted diet

to 52% ( $\pm 0.13$ ), 49% ( $\pm 0.13$ ), 40% ( $\pm 0.18$ ), and 22% ( $\pm 0.18$ ) of pre-treatment values respectively (Fig. 4). Addition of arginine to triple therapy further significantly decreased the levels of plasma  $\alpha$ -AASA to 33% ( $\pm 0.15$ ) ( $p = 0.03$ ), and of P6C to 29% ( $\pm 0.12$ ) ( $p = 0.03$ ). Plasma pipercolic acid and urine  $\alpha$ -AASA also decreased to 34% ( $\pm 0.12$ ) and 14% ( $\pm 0.08$ ) although they did not reach significance.

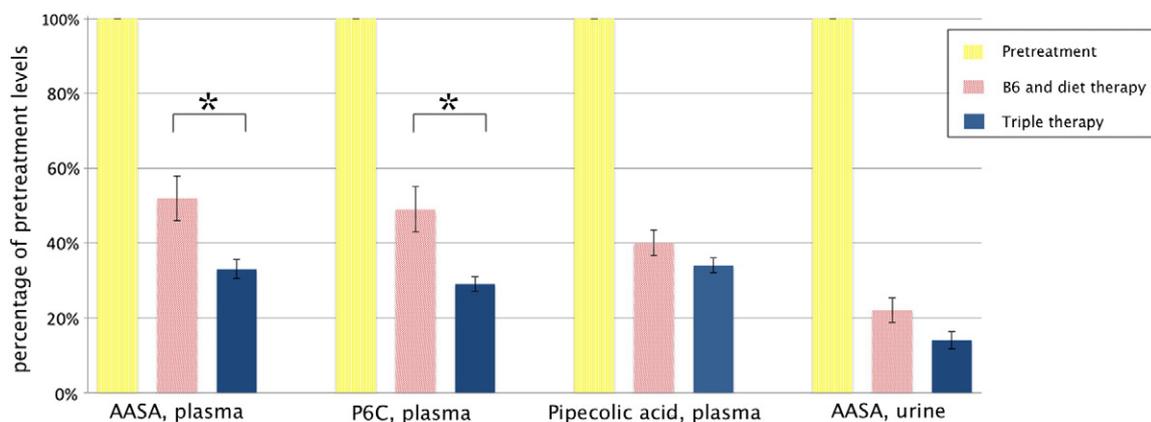
CSF metabolites were available for review in four subjects (1, 2, 5 and 6) (Fig. 5). In subject 1, CSF lysine decreased with initiation of pyridoxine supplementation and a lysine restricted diet and decreased further with triple therapy. A corresponding decrease in CSF  $\alpha$ -AASA was not observed (Supplemental Table I). In subject 2, CSF lysine again decreased after initiation of triple therapy without a decrease in CSF  $\alpha$ -AASA (Supplemental Table II). In subjects 5 and 6 a decrease in CSF  $\alpha$ -AASA was observed following triple therapy (Supplemental Table III).

### 3.3. Brain MR imaging

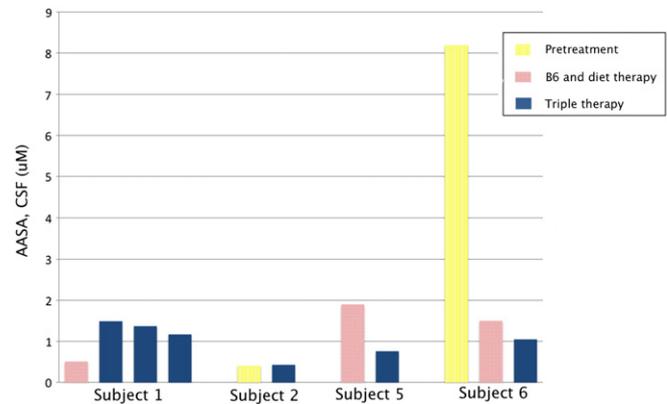
All subjects had brain MR imaging as part of their clinical care. MR imaging was available for systematic review from subjects 1, 2, 5, and 6. Subject 1 had DWI, ADC, FLAIR and T2 images obtained at two time points. At two weeks of age numerous foci of restricted diffusion scattered throughout the bilateral anterior and posterior periventricular white matter were noted. Repeat MR imaging at 2 years of life identified consequent white matter volume loss and gliosis (Fig. 1). Subjects 2, 5 and 6 had FLAIR and T2 images available for review. For subjects 2 and 5 MR imaging was consistent with bilateral mesial temporal sclerosis (Fig. 2). The posterior corpus callosum appeared thin in all four subjects (Supplemental Fig. 2).

## 4. Discussion

Since the initial description of pyridoxine responsive seizures in 1954, PDE has been an important recognized etiology of early infantile epileptic encephalopathy with significant treatment implications. The initial goal of pyridoxine monotherapy has been seizure control as pyridoxine supplementation overcomes the depletion of this important cofactor. As the natural history of PDE has become evident, treatment has focused further on improving the poor cognitive outcome that occurs in 75% of affected individuals on pyridoxine monotherapy [11]. Adjunct therapy has focused on improving the developmental outcome through limitation of putative neurotoxic metabolites. Previous therapies have included lysine-restricted diet and recently arginine supplementation in order to reduce lysine influx into the brain. In this study, all three components (pyridoxine, arginine supplementation and dietary restriction) were combined in a new therapeutic approach.



**Fig. 4.** Reduction of plasma and urine biomarkers in subjects treated with triple therapy. Legend: biomarkers were recorded from those subjects who were treated with dietary lysine restriction (dashed bar) prior to treatment with triple therapy (solid bar). Each biomarker was recorded as a percentage of pre-treatment levels (vertical lines) in order to account for individual variation. Mean values were obtained from plasma  $\alpha$ -AASA (3 subjects), plasma P6C (3 subjects), plasma pipercolic acid (4 subjects) and urine  $\alpha$ -AASA (5 subjects). \* =  $p < 0.05$ .



**Fig. 5.** Serial CSF  $\alpha$ -AASA in subjects treated with triple therapy. Legend: CSF  $\alpha$ -AASA was recorded prior to treatment in two subjects (2, 4) and following dietary therapy in two subjects (1 and 5). All four subjects had CSF  $\alpha$ -AASA recorded following addition of supplemental arginine (triple therapy). The CSF  $\alpha$ -AASA did not decrease in subjects treated within the first year of life (subjects 1 and 2) although CSF  $\alpha$ -AASA was significantly reduced in subjects treated at a later age (subjects 5 and 6).

An amino acid restricted diet is a well-established therapy in many organic acidurias, including disorders of lysine metabolism. Dietary limitation of lysine has previously been reported to reduce the plasma and urinary levels of  $\alpha$ -AASA and P6C in subjects with PDE. Subjects were also reported to have achieved normal developmental milestones or improved development after initiation of dietary therapy [14]. These results suggest that lowering the precursors of the putative neurotoxic metabolites, specifically  $\alpha$ -AASA and P6C, might result in improved neurodevelopmental outcome.

Competitive inhibition of the transport of precursor amino acids has been another therapeutic approach used in some inborn errors of metabolism [24,29–31]. For example, the biochemical effect of competing amino acids on reduction of brain transport has been directly documented in the brain by nuclear magnetic resonance spectroscopy for phenylketonuria [29,32]. In disorders of lysine metabolism, arginine supplementation was added to the treatment regimen in order to further lower putative neurotoxic metabolites through competitive inhibition at the transporter level. The theoretical basis for this approach is based on the characteristics of the dibasic amino acid transporters. The transport system for cationic amino acids in the brain at the blood–brain barrier as well as in neurons and astrocytes is comprised of mCAT1, encoded by *SLC7A1*, and mCAT3, encoded by *SLC7A3*, whereas in the liver it is composed by isoforms of mCATA2, encoded by *SLC7A2* [33–35]. Both mCAT1 and mCAT3 are sodium-independent transporters with a high affinity for L-arginine, L-lysine, and L-ornithine with a Km in

the 70 to 200  $\mu\text{M}$  range, which allows competition between these amino acids at physiological concentrations of these amino acids in plasma [33, 34]. The mCAT2 transporter in the liver has low affinity for cationic amino acids ( $K_m$  in the 2 to 5 mM range), which does not result in effective competition between these amino acids at the concentration found in plasma. The first step of liver lysine catabolism is through the mitochondrial matrix bifunctional enzyme that combines lysine 2-oxoglutarate reductase and saccharopine reductase activities [20]. Mitochondrial import of lysine is required for catabolism and occurs via the mitochondrial ornithine carriers ORNT1 and ORNT2 encoded by *SLC25A15* and *SLC25A2* respectively. L-Arginine reduced mitochondrial L-lysine uptake providing a mechanism for reduced lysine catabolism in the liver in a mouse model of GA I [20]. This should result in reduced formation of the metabolites AASA and P6C, and hence lower plasma concentrations. Thus, the combined effect of reducing the lysine concentration and increasing arginine concentration in plasma can be reduced production of neurotoxic metabolites in liver at the site of mitochondrial entrance, and in the brain at the sites of the blood–brain barrier and uptake in neurons and astrocytes.

This approach has been applied to glutaric aciduria type I, a disorder of lysine metabolism which results in the accumulation of glutaric acid and 3-hydroxyglutaric acid. In a mouse model of GA I, dietary lysine-restriction and arginine supplementation significantly lowered neurotoxic lysine metabolites in the brain, suggesting an additive effect of the two treatment modalities [20]. Translating these theoretical concepts and animal studies to human disorders of lysine metabolism has been done for GA I with recent short-term studies providing evidence for prevention of brain injury [24,31].

This is the first report of subjects with PDE who were treated concurrently with pyridoxine, a lysine-restricted diet, and arginine supplementation. This new triple therapy was effective at lowering plasma lysine levels while increasing plasma arginine levels. To have an effect on brain influx, plasma arginine levels must be increased by a sufficient intake of arginine, which was achieved in our subjects. For competition to occur, plasma arginine levels must increase at the time that plasma lysine levels increase. Therefore, arginine dosing in our cohort was divided in at least three administrations, and preferably taken together with the intake of lysine from food. In our study, the arginine dosing at 150 mg/kg/day in addition to dietary arginine intake resulted in substantially higher intake of arginine than what was reported in the studies in GA1. For example, subjects 1 and 2 had an intake between 230 and 355 mg/kg/day of arginine compared to two reports of patients with GA I, in which total arginine intake was 137 and 119 mg/kg/day respectively [24,31]. Arginine supplementation in this cohort was associated with an increased level of plasma arginine (similar to that seen in urea cycle disorders), and decreased theoretical brain lysine influx values calculated according to Strauss et al. [31] (Supplemental Tables I and II). For safe application of this therapy, plasma levels of lysine and arginine must be monitored. Dietary amino acids should be adjusted to achieve plasma lysine levels at the low end of normal (e.g., < 80  $\mu\text{M}$ ) and plasma arginine levels at the high end of normal or above ( $\geq 120 \mu\text{M}$ ). Using a carefully monitored regimen, no apparent side effects were observed, although initiation and persistence of diet can be challenging in older children.

In those subjects who were treated with dietary lysine restriction prior to triple therapy, triple therapy was able to significantly lower plasma  $\alpha$ -AASA and P6C compared to pyridoxine supplementation and a lysine restricted diet alone (Fig. 4). Despite the recognized limitations, plasma and urinary biomarkers are often used as surrogates for cerebral concentrations of the putative neurotoxic metabolites.  $\alpha$ -Amino adipic semialdehyde exists *in vivo* in a reversible equilibrium with the cyclic Schiff base, P6C; these compounds are useful biomarkers for both pyridoxine-dependent seizures, and potentially sulfite oxidase or molybdenum cofactor defects [36]. Measurement *in vitro* involves protein precipitation with acetonitrile, and treatment with borate buffer (pH 8.5) and fluorenylmethoxycarbonyl chloride (Fmoc-chloride) to

make Fmoc derivatives, followed by analysis by LC–MS/MS [37]. The *in vitro* relationship of  $\alpha$ -AASA and P6C is pH dependent, and it is unknown if the *in vivo* relationship reflects *in vitro* measurement conditions. Plasma  $\alpha$ -AASA and P6C are also unstable compounds at refrigerator or room temperature, and must be processed rapidly and frozen at  $-80 \text{ }^\circ\text{C}$  [37]. If handled properly, measurement of plasma has proved reliable and reproducible, and is most likely a better surrogate for cerebral concentrations of the putative neurotoxic metabolites than urine. Interpretation of urine data alone is complicated due to the natural decline of these metabolites with age in urine [38,39]. Although serial studies on untreated individuals have not been performed, the overall plasma levels measured in presenting patients do not vary significantly with age when measured in the same laboratory under the same conditions. Therefore, the observed decreases in  $\alpha$ -AASA and P6C seen in affected patients on dietary treatment and triple therapy are believed to be unrelated to age, but rather reflect the therapeutic intervention.

Both a lysine restricted diet and arginine supplementation are intended to lower brain lysine metabolism, and levels of CSF lysine were consistently reduced on therapy (Supplemental Tables I and II). In subjects 5 and 6, CSF  $\alpha$ -AASA and CSF pipercolic acid were lower after dietary therapy and were further reduced following initiation of triple therapy (Supplemental Table III). This suggested that therapy also resulted in the lowering of CSF neurotoxic metabolites. Unfortunately the effect on CSF  $\alpha$ -AASA was inconsistent as CSF  $\alpha$ -AASA was not decreased in subjects 1 and 2 (Fig. 5). The inconsistent effect of therapy on CSF  $\alpha$ -AASA may be a result of multiple factors. Subject 6's pretreatment CSF  $\alpha$ -AASA (8.2  $\mu\text{M}$ ) was 20 fold of subject 2's pretreatment CSF  $\alpha$ -AASA (0.41  $\mu\text{M}$ ) and subject 5 and 6's CSF  $\alpha$ -AASA on dietary therapy was three fold of subject 1's CSF  $\alpha$ -AASA on dietary therapy. The  $\alpha$ -AASA CSF levels in patient 1 may reflect the high usage of lysine by the brain for synthesis over catabolism in the early neonatal period, which decreases during the first months of infancy as documented by Strauss et al. [31]. A lack of a decrease in CSF metabolites does not necessarily indicate that lysine catabolism was not decreased in brain tissue. There may also be discrepancies between concentrations of  $\alpha$ -AASA and P6C in plasma, urine, and even CSF as compared to the brain. In the limited number of cases where neuropathology was available in individuals with GA I, brain concentration of GA and 3OHGA did not correlate with plasma or urine concentration [40]. Such neuropathology is not available from individuals with PDE and further studies are required to determine the effect of therapeutic interventions on CSF metabolites, brain concentrations of  $\alpha$ -AASA and neurodevelopment.

Triple therapy for PDE is aimed at improving developmental outcome; in this limited case series, there are some indications that combined triple therapy was associated with improved cognitive outcome, although it is often difficult to identify the contribution of each of the individual therapeutic components. Also antiepileptic medications, and in the case of subject 6 pyridoxine, were lowered with the initiation of therapy, which may have a positive impact on development. Subject 1 achieved normal developmental milestones following the initiation of triple therapy within the first four months of life. In subject 2 there were dramatic developmental gains following initiation of triple therapy, although the subject continues to have significant developmental delay. Subjects 3 and 4 had neurologic improvement following the initiation of triple therapy with significant and sustained developmental improvement as documented by serial developmental assessments (Fig. 3). The benefit of lysine reduction was very clear but the added benefit of the addition of arginine at such late age was less evident in subjects 5 and 6. Formal developmental assessments following triple therapy did not confirm cognitive improvements in subject 5, although both of the subject's caregivers reported a subjective benefit of triple therapy. These cases illustrate positive therapeutic benefits of each component in certain patients, and of added reductions in neurotoxic metabolites from the combined therapy, which represents an optimized therapeutic approach.

The benefit of therapeutic interventions must be evaluated on the background of preexisting brain damage. Past studies have identified possible structural brain defects in individuals with PDE [11,41]. In addition, several subjects in our study showed radiological evidence of brain injury likely caused by the seizures, and possibly the metabolic disturbances such as hypoglycemia, prior to diagnosis. This is best exemplified in subject 1, who showed T2 hyperintense lesions in the white matter in the same distribution as the diffusion restriction noted prior to any therapy. The radiological findings are similar in appearance to those seen in white matter injury of the premature and neonatal infant, and reflect acute brain disturbances occurring immediately prior to therapy, and can be directly related to the neurological sequelae observed. Subjects 2 and 5 had mesial temporal sclerosis with hippocampal injury, and both subjects had memory and concentration impairments [42,43]. Hippocampal sclerosis is most commonly associated with chronic epilepsy and has been associated with temporal lobe epilepsy and a history of febrile seizures [44,45], although it has been infrequently reported in subjects with PDE [41]. These observations link persisting neurologic dysfunction to brain damage occurring prior to the initiation of triple therapy, either the acute neurologic findings in subject 1 or the mesial sclerosis in subjects 2 and 5. Of note, subjects 1, 2, 5 and 6 all had relatively thin posterior corpus callosum (Supplemental Fig. 2).

Multiple factors influence ultimate neurodevelopmental outcome in PDE. Past studies on monotherapy with pyridoxine failed to show an impact of early initiation of therapy. Early initiation of triple therapy may result in improved outcome, similar to other disorders of organic acid metabolism. An impact of age at therapy initiation may be evident on triple therapy, as the best developmental outcome in this cohort was obtained in the subject treated from the first month of life. All neonates and infants presenting with seizures should have biochemical testing including  $\alpha$ -AASA which may identify this treatable condition as early as possible [13]. It also appears that treatment should commence prior to severe episodes of prolonged seizures, which may cause lasting neurological sequelae. Early intervention may require detection through newborn screening which is technically feasible [46]. Early diagnosis, through newborn or neonatal screening, and early treatment with triple therapy may ameliorate cognitive impairment in PDE, providing hope for patients affected with this condition. An ideal confirmatory study would identify patients by newborn screening prior to symptom onset, and be treated with triple therapy to show if neurocognitive outcome can be improved or even normalized.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ymgme.2015.05.011>.

## Acknowledgements

We are grateful to Tim A. Benke for his input and advice on clinical aspects of this paper. We are also grateful to the patients who participated in this study and for the support of the US-based patient organization: "Pyridoxine Dependent Epilepsy Foundation." We acknowledge funding from the BC Children's Hospital Foundation, Vancouver, Canada (TIDE-BC 1st Collaborative Area of Innovation). CvK is the recipient of the Michael Smith Foundation for Health Research Scholar Award.

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