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Epileptic encephalopathy caused by ARV1 deficiency: refinement of the genotype-phenotype spectrum and functional impact on GPI-anchored proteins

Running title: ARV1 and epileptic encephalopathy

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**Conflict of Interest**

The authors declare no potential conflict of interest.

**Data availability**

Provided upon request.
Abstract

Early infantile epileptic encephalopathy 38 (EIEE38, MIM #617020) is caused by biallelic variants in ARV1, encoding a transmembrane protein of the endoplasmic reticulum with a pivotal role in glycosylphosphatidylinositol (GPI) biosynthesis. We ascertained seven new patients from six unrelated families harboring biallelic variants in ARV1, including five novel variants. Affected individuals showed psychomotor delay, hypotonia, early-onset refractory seizures followed by regression, and specific neuroimaging features. Flow cytometric analysis on patient fibroblasts showed a decrease in GPI-anchored proteins on the cell surface, supporting a lower residual activity of the mutant ARV1 as compared to the wildtype. A rescue assay through the transduction of lentivirus expressing wild type ARV1 cDNA effectively rescued these alterations. This study expands the clinical and molecular spectrum of the ARV1-related encephalopathy, confirming the essential role of ARV1 in GPI biosynthesis and brain function.

Keywords: ARV1; early-infantile epileptic encephalopathy; GPI-anchored proteins; lentiviral gene rescue.
**Introduction**

*ARV1* (ARV1 Homolog, Fatty Acid Homeostasis Modulator, MIM *611647*) encodes a transmembrane protein that is localized to the endoplasmic reticulum (ER). This protein contains an N-terminal zinc-binding motif, which is localized to the cytosol and followed by several domains spanning the ER membrane, and a C-terminus ending at the ER lumen. In *Saccharomyces cerevisiae* (*S. cerevisiae*), ARV1 is essential for pheromone-induced mitogen-activated protein kinase signalling and functions as a key component in sphingolipid and sterol homeostasis, as well as in the glycosylphosphatidylinositol (GPI) biosynthesis.\(^1\)\(^2\) The knockdown of *ARV1* in *S. cerevisiae* has been associated with increased cholesterol and decreased neutral lipid levels.\(^2\)

The essential role of ARV1 in the nervous system was suggested by the observation that neuronal deletion of ARV1 in mice results in seizures and sudden death.\(^3\) In humans, biallelic pathogenic variants in *ARV1* cause early infantile epileptic encephalopathy 38 (EIEE 38, MIM #617020), a severe condition characterized by developmental delay (DD), severe intellectual disability (ID), and early-onset refractory seizures.\(^3\)\(^7\) The homozygous missense variant NM_022786.2:c.565G>A; p.(Gly189Arg) was first described in three patients from a consanguineous family with severe DD, early-onset epilepsy, ataxia, and poor head control.\(^4\) Subsequently, the homozygous splice site variant NC_000001.11 (NM_022786.2):c.294+1G>A; p.(Lys59_Asn98del) was identified in a subject with a profound encephalopathy.\(^3\) Four additional families with similar phenotype have been reported since then.\(^5\)\(^7\)

In this study, we present seven new subjects harboring biallelic variants in *ARV1* and reviewed the previously reported patients, refining the molecular and phenotypic spectrum of
ARV1-related encephalopathy. We also investigated the effect of ARV1 variants on GPI biosynthesis through flow cytometry analysis and a lentiviral-based rescue assay.

Materials and methods
We identified five previously unreported subjects from five unrelated families with biallelic variants in ARV1. These individuals were evaluated at different Institutions and ascertained through GeneMatcher for distributed casemaching.8 We also re-evaluated a patient initially reported by Palmer et al.3 (patient 2).

The study protocol was approved by the Institutional Ethics Committee. Samples were collected from patients and families after written informed consent was obtained from the parents or legal guardians. Detailed clinical presentations, family history were recorded and brain magnetic resonance imaging (MRI) findings were reviewed. Exome sequencing (ES) was performed in all cases.9 Sanger sequencing was performed for candidate variants validation and parental segregation.

To assess the impact of ARV1 variants on the levels of GPI-anchored proteins (GPI-APs) on the cell surface, we performed flow cytometry analysis on the fibroblasts from patients 1 and 2 and a lentiviral rescue assay (Supplementary Information).

Results
Our cohort consisted of 5 affected children (patients 1-4) and two antenatally terminated fetuses (patients 5 and 6) (Table 1). Prenatal abnormal findings were reported in patient 4 (hydronephrosis) and in the two fetuses. The pregnancy of a fetus (patient 5) was terminated at gestational week 22, due to megaureter, small measures of femora and humeri, and a narrow
thorax detected on ultrasound. In the following pregnancy (patient 6), an increased nuchal translucency of 6.3mm was seen at gestational week 14 and bilaterally dilated renal pelvis was noticed at gestational week 16+1. This pregnancy was terminated after ES revealed the presence of the homozygous ARV1 variant in both fetuses. Patients 1-4 showed psychomotor delay in the first six months of life and presented with a profound cognitive impairment at the time of last clinical examination, whereas the DD was moderate in patient 7. Symptoms at onset included abnormal eye movements, infantile hypotonia, and seizures. Early-onset epilepsy was present in all cases and was refractory in patients 1 and 2. These individuals experienced status epilepticus and EEG showed slow background and multifocal epileptiform abnormalities, suggestive of severe encephalopathy (Supplementary Figure 1). Neurological involvement was variable, ranging from isolated hypotonia to limb spasticity, dystonic movements, and nystagmus. In patients 3 and 4, gastrointestinal symptoms and genitourinary alterations were also observed (Supplementary Figure 2). Mild skeletal abnormalities were identified in patient 1, 4, 5, and 7. Less common features included facial dysmorphism in patient 1 (Figure 1a) and 7, and hearing impairment in patient 3 (Supplementary Table 2).

Brain MRI revealed supratentorial white matter involvement in all patients, characterized by variable combination of delayed myelination, reduced volume, and T2 signal abnormalities, with secondary thinning of the corpus callosum, ventricular dilatation, and mild subarachnoid spaces enlargement. In one subject, global cerebellar atrophy evolving more rapidly in the first months after clinical onset was noted (Figure 2 and Supplementary Figure 3). In two cases, diffusion-weighted images revealed a peculiar pattern of restricted diffusion at the level of the central tegmental tracts, superior cerebellar peduncles, red nuclei, and subthalamic regions.
Additional involvement of the corticospinal tracts and frontal subcortical regions was also noted (Figure 2).

ES revealed five novel ARV1 variants in our cohort (Figure 1i,j): the homozygous frameshift variant NM_022786.2:c.363_364del, p.(Ser122Glnfs*7) in patient 1, the compound heterozygous variants NM_022786.2:c.518dupA; p.(Pro174Alafs*14) and NM_022786.2:c.101G>A; p.(Cys34Tyr) in patient 3, the missense variant NM_022786.2:c.182G>A; p.(Cys61Tyr) in patient 4, and the splicing variant NC_000001.11 (NM_022786.2):c.674-1G>A in patients 5 and 6. The NM_022786.2:c.518dupA; p.(Pro174Alafs*14) was also detected in homozygous state in patient 7. Parental segregation through Sanger sequencing confirmed a recessive mode of inheritance. All variants were rare in population frequency databases, never reported in the homozygous state in healthy individuals, and consistently predicted as pathogenic by in silico analysis (Supplementary Table 1).

Flow cytometry revealed a ~50% decrease in CD73 and CD109 levels in the fibroblasts from patient 1 in comparison to the healthy control, which was completely rescued by transduction with lentivirus expressing wild type ARV1 cDNA. No decrease in FLAER was observed. In the fibroblasts from patient 2, FLAER, CD73, and CD59 levels were found to be decreased to 50%, 75%, and 75% respectively compared to the healthy controls. The transduction with lentivirus expressing wild type ARV1 cDNA partially rescued FLAER cell surface detection, while completely rescued CD73 and CD59 cell surface levels (Figure 1k).

Discussion

To date, three biallelic ARV1 variants have been identified in 15 individuals with EIEE38 from five unrelated families.3–7 We report seven new subjects harboring biallelic variants in ARV1,
including five novel likely pathogenic variants, likely leading to impaired protein function, supporting the loss-of-function pathogenic model in ARV1 encephalopathy and expand the phenotypic characterization of the subject initially reported by Palmer et al.²

All the affected individuals present with a severe epileptic encephalopathy characterized by profound global DD and ID, hypotonia, variable neurological symptoms, early-onset seizures, and premature lethality. Variable skeletal abnormalities were observed in four cases (patients 1, 4, 5, and 7) (Supplementary Material) and genitourinary involvement in our cohort was more common than expected based on the previous reports. Indeed, nephrocalcinosis was observed in patient 4, whereas a combination of congenital anomalies of the ureters and renal pelvis was observed in patient 3, 5, and 6. So far, skeletal abnormalities and proximal tubulopathy were only reported in a single individual by Davids et al.⁵ Similarly, abnormal cardiac morphology was observed in patient 5, in line with the cardiac involvement very recently reported in two subjects by Segel et al.⁷ These observations suggest that the EIEE 38 phenotypic spectrum might be wider than expected and that these additional clinical features should be investigated in affected children, supporting a central role for clinical surveillance.

We observed a prevalent supratentorial white matter involvement variably associated with delayed myelination, reduced periventricular white matter volume, and T2 signal abnormalities. These abnormalities often lead to corpus callosum hypoplasia and enlargement of the lateral ventricles and subarachnoid spaces. In one subject, we observed rapid progression of cerebellar atrophy. Moreover, in two patients there was a peculiar and partially reversible pattern of restricted diffusion in the brainstem tegmentum, superior cerebellar peduncles, subthalamus, ventral striatum, corticospinal tracts and subcortical white matter. These features are remarkably similar to those of phosphatidylinositol glycan biosynthesis class A protein (PIGA)
The identification of similarities in the lesion distribution and MRI appearance in ARV1 and PIGA deficiencies represents an important step in the pattern recognition of inherited GPI deficiency disorders.

In *S. cerevisiae*, ARV1 deficiency leads to increased glucosaminy1-acyl-PI (GlcN-acylPI), suggesting that ARV1 is crucial for the first mannosylation of the GPI. It has been proposed that ARV1 functions either by guiding GlcN-acylPI into ER lumen from cytosol or presenting it to mannosyltransferase. The results of the flow cytometry analysis of GPI-APs in patient cells and rescue assay confirm the essential role of ARV1 in GPI biosynthesis. The GPI biosynthesis pathway is a multi-step process in which GPI-anchored proteins (GPI-APs) are synthesized in the ER, moved to the Golgi system, and eventually transported to cell surface (Supplementary Figure 4). Variants affecting the function of the proteins involved in this complex pathway are associated with a heterogeneous group of recessive disorders known as Inherited GPI Deficiency disorders (IGDs). Despite a remarkable clinical variability, core neurological phenotypes recur in most IGDs, including DD/ID, epilepsy, hypotonia, spasticity, and movement disorders (ataxia, dystonia, tremors, choreiform movements), with a variable degree of severity depending on the functional impact of the underlying genetic variants. In this context, ARV1 deficiency affects a crucial step of the GPI biosynthesis process and leads to a severe EIEE overlapping the core IGDs-related neurological phenotype. Additionally, patients with EIEE38 also shows distinctive syndromic features (i.e., mild skeletal features and genitourinary abnormalities) which can help differentiate this condition from other IGDs.

In summary, our study expands the molecular and phenotypic spectrum of *ARV1*-related encephalopathy, refining the neuroradiological characterization of this condition and suggesting potentially relevant clues for its early diagnosis. We also suggest that multisystem clinical
manifestations should be investigated in patients with ARV1-related encephalopathy, with clinical surveillance playing a pivotal role in their early detection and prompt management. We confirmed the pivotal role of ARV1 in GPI biosynthesis, supporting an underlying loss-of-function pathogenic model in EIEE38.
References


Table 1. Summary of electroclinical, and neuroimaging features of ARV1 patients.

Figure. 1 Phenotypic, molecular, and functional characterization of patients 1 and 2. (a) Pedigrees of the reported Families. (b) Clinical pictures of patients 1 (5 years) and 3 (4 months and 2 years). Patient 1 has facial dysmorphic features consisting of bitemporal narrowing, hypotelorism, shallow orbits, open bite with high arched palate, tooth agenesis. In detail, the right foot has a short fifth toe associated with a congenital overlapping fifth toe deformity. Patient 2 has frontal bossing, high anterior hairline, upslanted palpebral fissures, depressed nasal bridge, long philtrum, tent-shaped mouth, and uplifted eralobes. (c-e) Brain MRI performed in Patient 2 at the age of 9 months. Axial T2-weighted images (e), diffusion-weighted images (DWI) (d), b=1000s/mm2, and ADC maps (e) reveal T2 hyperintensity and high signal on DWI with reduced ADC values at the level of the central tegmental tracts (arrows), superior cerebellar peduncles (arrowheads), ventral midbrain (dotted arrows), subthalamus, and inferior striatum (empty arrows). (f-h) Brain MRI of Patient 1: Axial T2-weighted images (f), diffusion-weighted images (DWI) (g), b=1000s/mm2, and ADC maps (h) show a similar pattern of restricted diffusion in the central tegmental tracts (arrows), superior cerebellar peduncles (arrowheads), subthalamus, and inferior striatum (empty arrows). (i) Schematic representation of ARV1 protein and gene with variants identified from the previous reports indicated in black, and novel and known variants identified in the present study indicated in red and blue, respectively. Drawing made with Protter (https://wlab.ethz.ch/protter/start/) and ProteinPaint (https://pecan.stjude.cloud/proteinpaint). (j) Multiple sequence alignment showing conservation of amino acids. (k) Flow cytometry analysis of fibroblasts derived from patient 1 (c.363_364del) and patient 2 (c.294+1G>A) compared to healthy controls. The patient fibroblasts were further
transduced with lentiviruses expressing a wild type ARVI cDNA. CD59, CD73 and CD109 antibodies, as well as FLAER, were stained for GPI-APs. The histograms are representatives of at least two separate experiments using three different controls.

**Figure 2. Evolution of brain MRI abnormalities in patient 1.** Brain MRI performed in Patient 1 at the age of 9 months (a, b), 2 years (c, d), 4 years (e, f) and 5 years (g, h). Upper row, T2-weighted images; bottom row, diffusion-weighted images (DWI), b=1000 s/mm². Axial T2-weighted (a) and DWI (b) reveal T2 hyperintensity and high signal on DWI at the level of the central tegmental tracts, superior cerebellar peduncles (arrows), ventromedial midbrain (arrowheads), subthalamus, and inferior striatum (thick arrows). At 2 years of age, corresponding T2-weighted (c) and DWI (d) images demonstrate persistent, slightly reduced, restricted diffusion in previously affected regions, prominent enlargement of the cerebellar CSF spaces (empty arrows), and new areas of restricted diffusion in the posterior limbs of internal capsules (PLIC) (arrows). Mild enlargement of cerebral CSF spaces and diffuse cerebral white matter T2 hyperintensity reflecting abnormal myelination are also present. At 4 years of age, T2-weighted (e) and DWI (f) images reveal resolution of the restricted diffusion in the brainstem, extension of the restricted diffusion along the cortico-spinal tracts (arrows), and presence of restricted diffusion in the frontal subcortical regions (empty arrows). Last follow-up brain MRI (g, h), performed at 5 years of age, shows slowly progressive cerebellar atrophy (notably with increased size of cerebellar CSF spaces, e.g. the fissures between the cerebellar folia) and almost complete resolution of DWI changes, only persisting in the posterior portions of PLIC (arrows).
Family A
I 1 2
[363_364del];[+]
II
Patient 1
[363_364del];[363_364del]

Family B
I 1 2
[294+1G>A];[+]
II
Patient 2
[294+1G>A];[294+1G>A]

Family C
I 1 2
[518dup];[+]
II
Patient 3
[518dup];[101G>A]

Family D
I 1 2
[182G>A];[+]
II
Patient 4
[363_364del];[363_364del]

Family E
I 1 2
[674-1G>A];[+]
II
Patient 5
[674-1G>A];[674-1G>A]

Family F
I 1 2
[518dup];[+]
II
Patient 7
[518dup];[518dup]

Pt1
Pt3

Cytosol
ER lumen

p.Cys347Tyr
p.Cys653Tyr
p.Ser122Gln*7
p.Pro174Ala*14
p.Gly189Arg

p.Cys347Tyr
p.Lys99 Arg100del
p.Ser122Gln*7
p.Gly189Arg

CD73
CD109

FLAER
CD73
CD59

Patient 1

Patient 2

Patient 3

Normal control
Vector control
Patient

Empty lentiviral vector-transduced patient cells

ARV1 lentiviral vector-transduced patient cells

CGE_14033_Figure 1.tif
ARV1 biallelic variants lead to early infantile epileptic encephalopathy 38 with multisystem clinical manifestations. Introns not drawn to scale.

White matter involvement with peculiar and partially reversible restricted diffusion pattern in the brainstem, basal ganglia, and subcortical white matter.

Flow cytometry analysis and a lentiviral-based rescue assay highlights the role of ARV1 in GPI biosynthesis.
Table 1. Summary of electroclinical, and neuroimaging features of ARV1 patients.

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient ID</th>
<th>A Patient 1</th>
<th>B Patient 2</th>
<th>C Patient 3</th>
<th>D Patient 4</th>
<th>E Patient 5</th>
<th>E Patient 6</th>
<th>F Patient 7</th>
<th>5 Families, 15 patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancestry</td>
<td>Italian</td>
<td>Australian-Lebanese</td>
<td>American-Mexican</td>
<td>American-Mexican</td>
<td>Afghan</td>
<td>Afghan</td>
<td>Algerian</td>
<td>American-Mexican (2), Lebanese (5), Palestinian (2), Saudi (3), Middle East (3)</td>
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</tr>
<tr>
<td>Consanguinity</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ (13/15)</td>
<td></td>
</tr>
<tr>
<td>History of miscarriages</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gender, age at FU</td>
<td>F, 5y</td>
<td>F, 12m</td>
<td>F, 13m</td>
<td>M, 11y</td>
<td>F, NA</td>
<td>F, NA</td>
<td>M, 9y</td>
<td>8M and 7F, 10 alive (mean 11.8y)</td>
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<tr>
<td>Premature death</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+ (5/15), mean 2.8y (AP, GI infection)</td>
<td></td>
</tr>
<tr>
<td>Age at onset</td>
<td>3m</td>
<td>6w</td>
<td>6w</td>
<td>6m</td>
<td>NA</td>
<td>NA</td>
<td>6m??</td>
<td>Regular (12), N/A</td>
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<td>Clinical onset</td>
<td>DD, hypotonia, nystagmus</td>
<td>REM, hypotonia</td>
<td>Seizures</td>
<td>DD</td>
<td>NA</td>
<td>NA</td>
<td>DD, hypotonia?</td>
<td>Abnormal eye movements (2), hypotonia (2), seizures (3), DD (8)</td>
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<tr>
<td>Progressive microcephaly</td>
<td>+ (-3.2 SD)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>+ (5) up to -2.6 SD, 6 NA</td>
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<tr>
<td>Seizures</td>
<td>+ (profound)</td>
<td>+ (profound)</td>
<td>+ (profound)</td>
<td>+ (profound)</td>
<td>NA</td>
<td>NA</td>
<td>9??</td>
<td>+ (15/15)</td>
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<tr>
<td>Type</td>
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<td>Focal</td>
<td>Multifocal</td>
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<td>NA</td>
<td>Absence, Focal</td>
<td>FS, focal, GTCS</td>
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<tr>
<td>Status epilepticus</td>
<td>+, electrical</td>
<td>-</td>
<td>+</td>
<td>+ (PHE, LEV)</td>
<td>+ (VPA, LEV)</td>
<td>NA</td>
<td>-</td>
<td>- (3), 6 NA</td>
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<tr>
<td>Response to AEDs</td>
<td>Intractable</td>
<td>Intractable</td>
<td>Seizure free</td>
<td>Partial control</td>
<td>NA</td>
<td>Intractable</td>
<td>(11, 5 died), 4 NA</td>
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<tr>
<td>EEG</td>
<td>Slow background</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>NA</td>
<td>-</td>
<td>Bilateral slow epileptic discharges (1), anomalies suggestive of EE (3), 11</td>
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<td>NA</td>
<td>-</td>
<td>+ (7), 5 NA</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>+ (6)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
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<td>+ (2)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>+ (1), with chorea</td>
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<td>Ataxia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ (5)</td>
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<td>Other</td>
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<td>REM</td>
<td>-</td>
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<td>- (Chamunis) (2), REM (3), interictal decerebration (1)</td>
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<td>Behavioural anomalies</td>
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<td>Irritability</td>
<td>-</td>
<td>Irritability</td>
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<td>NA</td>
<td>Aggressive</td>
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<td>+</td>
<td>+</td>
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<td>NA</td>
<td>NA</td>
<td>-</td>
<td>+ (2), 11 NA</td>
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<tr>
<td>Abnormal vision (CVI)</td>
<td>+</td>
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<td>GU anomalies</td>
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<td>(ectopic ureters, hydronephrosis, inguinal hernia)</td>
<td>+ (nephrocalcinosis)</td>
<td>+ (megareters)</td>
<td>+ (pelvic dilatation)</td>
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<td>Proximal tubulopathy (1)</td>
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<td>GI symptoms</td>
<td>Dysphagia</td>
<td>Reflux</td>
<td>Gastric distention</td>
<td>Dysphagia, reflux, constipation</td>
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<td>Encopresis, constipation</td>
<td>Nasogastric tube feeding (6)</td>
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<td>Skeletal alterations</td>
<td>Short fifth toes, pectus excavatum, prominent heels</td>
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<td>kyphoscoliosis, pectus excavatum, hip subluxation</td>
<td>Short femur and humerus, narrow thorax</td>
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<td>Short hands and fingers</td>
<td>Skull and limbs dysplasia, vertebral malformations, contractures, scoliosis</td>
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<td>Abnormal heart morphology</td>
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<td>Dilated cardiomyopathy (2), ↑IgE, serum iron (2)</td>
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<td>Anterior pituitary hypoplasia, thin pituitary stem</td>
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Abbreviations: AEDs, anti-epileptic drugs; AP, aspiration pneumonia; CCH, corpus callosum hypoplasia; CVI, cortical visual impairment; EE, epileptic encephalopathy; FS, febrile seizures; FU, follow-up; GI, gastrointestinal; GTCS, tonic-clonic seizures; GU, genitourinary; LEV, levetiracetam; m, months; MFED, multifocal epileptic discharges; NA, not available; NT, nuchal translucency; OFC, occipito-frontal circumference; PHB, phenobarbital; REM, roving eye movements; SS, subarachnoid spaces; VPA, valproic acid; w, weeks; WM, white matter; y, years. † PMID 27270415