The molecular and phenotypic spectrum of CLCN4-related epilepsy

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Abstract

Objective: This study was undertaken to expand the phenotypic and genetic spectrum of CLCN4-related epilepsy and to investigate genotype–phenotype correlations.

Methods: We systematically reviewed the phenotypic and genetic spectrum of newly diagnosed and previously reported patients with CLCN4-related epilepsy. Three novel variants identified in four patients reported in this study were evaluated through in silico prediction and functional analysis by Western blot, immunofluorescence, and electrophysiological measurements.

Results: Epilepsy was diagnosed in 54.55% (24/44) of individuals with CLCN4-related disorders and was drug-resistant in most cases. Of 24 patients, 15 had epileptic encephalopathy and four died at an early age; 69.57% of patients had seizure onset within the first year of life. Myoclonic seizures are the most common seizure type, and 56.25% of patients presented multiple seizure types. Notably, seizure outcome was favorable in individuals with only one seizure type. All patients showed intellectual disability, which was severe in 65.22% of patients. Additional common features included language delay, behavioral disorders, and dysmorphic features. Five patients benefitted from treatment with lamotrigine. Most variants, which were mainly missense (79.17%), were inherited (70.83%). Whereas frameshift, intragenic deletion, or inherited variants were associated with milder phenotypes, missense or de novo variants led to more severe phenotypes. All evaluated CLCN4 variants resulted in loss of function with reduced ClC-4 currents. Nonetheless, genotype–phenotype relationships for CLCN4-related epilepsy are not straightforward, as phenotypic variability was observed in recurrent variants and within single families.

Significance: Pathogenic CLCN4 variants contribute significantly to the genetic etiology of epilepsy. The phenotypic spectrum of CLCN4-related epilepsy includes drug-resistant seizures, cognitive and language impairment, behavioral disorders, and congenital anomalies. Notably, the mutation type and the number of seizure types correlate with the severity of the phenotype, suggesting its use for clinical prognosis. Lamotrigine can be considered a therapeutic option.
INTRODUCTION

The CLC gene family contains nine members in mammals, four of which encode plasma membrane chloride channels (CIC-1, CIC-2, CIC-Ka, CIC-Kb) and five intracellular 2Cl\(^-\)/H\(^+\) exchangers (CIC-3–7). \(^1\)\(^\)\(^2\) Dysfunction of some of these leads to severe neurological disorders, including leukodystrophy, neurodegeneration, intellectual disability (ID), epilepsy, and lysosomal storage disease for CIC-2, CIC-3, CIC-4, CIC-6, and CIC-7, \(^3\)\(^\)–\(^16\) highlighting the pivotal role of CLC proteins in the central nervous system. CIC-4, which is encoded by the CLCN4 (Online Mendelian Inheritance in Man database #302910) gene located on human chromosome Xp22.2, is a voltage-dependent 2Cl\(^-\)/H\(^+\) exchanger. \(^17\)\(^\)–\(^19\) Its precise physiological function is unclear, but CIC-4 is likely to be involved in the ion homeostasis of endosomes and intracellular trafficking. \(^20\)\(^\)–\(^23\) CIC-4 is broadly expressed across tissues, including brain, skeletal muscle, liver, kidney, intestine, and heart. In brain, it is prominently found in hippocampus and cerebellum. \(^11\)\(^\)\(^24\) Mutations in the CLCN4 gene have been found to underlie X-linked ID, epilepsy, behavior disorder, and dysmorphic features. \(^6\)\(^\)\(^\)\(^8\)\(^\)\(^12\)\(^\)\(^15\) In 2013, a de novo loss-of-function mutation (p.Gly544Arg) in CLCN4 was first reported to cause early onset epileptic encephalopathy (EE) and severe developmental delay (DD). \(^12\) Subsequently, five new CLCN4 variants were detected in five families with ID and/or epilepsy. \(^6\) As massive parallel sequencing has expanded, increasingly more variants in this gene have been reported in association with supplementary phenotypic features and a range of severity. To date, there is a lack of systematic investigation of the spectrum of seizure semiology and genotype–phenotype correlations in subjects with CLCN4-related epilepsy. In this study, we characterized the functional effects of newly identified variants. Furthermore, we assessed the clinical data of four unreported and all previously reported patients with CLCN4-related epilepsy to delineate the clinical features and molecular genetic findings and to assess genotype–phenotype relationships.

MATERIALS AND METHODS

2.1 SUBJ EcTS

Four subjects from four unrelated families with epilepsy of unknown etiology were analyzed. All patients were clinically evaluated and underwent whole exome sequencing (WES). This study was approved by the Ethics Committee of Xiangya Hospital of Central South University, China (human study/protocol # 201603205) and performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Written informed consent was obtained from the parents of all subjects.

2.2 Sequencing and bioinformatic analysis of novel CLCN4 variants

The sequencing of genomic DNA from peripheral blood and the bioinformatic analysis of the CLCN4 variants are described in detail in the Supplementary Materials. Clinical significance of novel variants was determined using the American College of Medical Genetics and Genomics (ACMG) standard guidelines. \(^25\)

2.3 Systematic literature review

We conducted a systematic literature review focused on CLCN4-related epilepsy by searching the PubMed database using the keywords “CLCN4”, "CIC-4", and "epilepsy". All publications were reviewed for case series or cohort studies that contained clinical data on patients with CLCN4 variants.
Individuals were included if they had a pathogenic $CLCN4$ variant and their phenotype included epileptic seizures. We rechecked all the variants with nucleotide and amino acid numbering according to the $CLCN4$ reference transcript NM_001830.3 (reference protein NP_001821.2). We systematically reviewed the detailed clinical, molecular genetics, and functional characterization of the previously reported and newly identified individuals with $CLCN4$-related epilepsy.

### 2.4 In vitro functional assessment of novel $CLCN4$ variants

To assess the effect of the three novel ClC-4 variants, we performed Western blot analysis of expression levels, immunofluorescence imaging of subcellular localization, and electrophysiological measurements upon heterologous expression, as previously described and detailed in the Supplementary Materials.

### 3 RESULTS

### 3.1 Identification of $CLCN4$ variants in four families

Using trio-based WES, we identified three novel (p.Val455Ile, p.Thr532Lys, p.Leu625Phe) and one previously reported (p.Arg718Trp) $CLCN4$ variants. All probands suffered from severe ID and intractable EE. No pathogenic variation was found in other epilepsy or ID candidate genes (detailed clinical information is given in the Supplementary Materials). The p.Thr532Lys variant affects a highly conserved residue of the helical intramembrane domain, the p.Leu625Phe variant substitutes a conserved residue in the first cystathionine-β-synthase (CBS) domain, and the p.Val455Ile variant alters a less conserved residue of the helical transmembrane domain (Figure 1 and Figure S1). The p.Thr532Lys and p.Leu625Phe variants were not present in any database (Table 1). The pathogenicity of p.Thr532Lys and p.Leu625Phe variants was classified as likely pathogenic in accordance with the ACMG guideline (Table 1). Although the p.Val455Ile variant had a very low frequency in gnomAD, ExAC, 1000 Genomes, and ESP6500, it is predicted to be benign (Table 1). The p.Arg718Trp variant was recently reported in two patients.

### 3.2 Phenotypic spectrum of patients with $CLCN4$ variants

To date, a total of 40 individuals with $CLCN4$-related disorders have been reported. Twenty of them had epilepsy and are the subjects of this study. Therefore, with our four patients, the total number of patients with $CLCN4$-related epilepsy known to date is 24. Thus, epilepsy was...
diagnosed in 54.55% (24/44) of individuals with \textit{CLCN4}-related disorders at the time of reporting. The clinical features of the 24 patients with epilepsy are summarized in Table 2. As expected, males are more often affected than females, with 87.5% (21/24) of individuals being male and 12.5% (3/24) female. However, female patients can be as severely affected as the male counterparts. The seizure onset, ranging from neonatal to 14 years (data available for 23), was detected within the first year of life in 69.57% of patients. The majority (62.5%, 15/24) of affected individuals developed EE. Of the 15 patients with EE, four developed infantile spasms (IS), and one developed epilepsy of infancy with migrating focal seizures (EIMFS). The seizure types were available for review in 16 patients. Seven (43.75%) individuals had only one type of seizure, and nine (56.25%) patients showed multiple seizure types. Seizure semiology is variable, including myoclonic, absence, IS, tonic, complex partial, tonic-clonic, focal, and secondary generalized seizures. Myoclonic seizures, occurring in seven patients, were the most common seizure type.

In addition to the variability of the epilepsy phenotype, seizure outcome varied. One patient had brief absence seizures that did not require treatment. Twenty-three patients received antiseizure therapy. Thirteen of 23 (56.52%) patients were drug-refractory with at least two drugs failing that were appropriately applied. Only 30.43% (7/23) were controlled on monotherapy, and three (13.04%) remitted after polytherapy. Seizures were refractory in all individuals with EE, and four patients died at an early age (2.75, 12, 16, and 19 years). In the EE group, four had IS, and three of them received adrenocorticotropic hormone therapy, which showed transient effectiveness in two of the three patients. Four patients were treated with a ketogenic diet in combination with other antiepileptic drugs (AEDs), which showed transient or no effectiveness on seizures. Of note, seizure outcome was favorable in individuals with only one seizure type, as seizures were controlled well with monotherapy or even without pharmacotherapy in all patients except one, whose seizures were controlled on polytherapy. Conversely, all nine individuals with multiple seizure types required two or more AEDs, and only two patients remitted. There is no established therapy for \textit{CLCN4}-related epilepsy, and no superior medication was identified. Two (p.Val455Ile, p.Leu625Phe) patients with IS in this study had a trial of lamotrigine with good clinical response. One patient became seizure-free, and one showed dramatic improvement after other drugs had failed. Three previously reported individuals (one p.Gly544Arg, two p.Val536Met) also showed significant improvement with the addition of lamotrigine. One patient was well controlled on carbamazepine monotherapy. By contrast, treatment with valproic acid was not effective for our four patients.
3.3 Developmental, behavioral, neurologic, and facial features

Cognitive development has been monitored in all 24 cases with CLCN4-related epilepsy, and five patients showed developmental regression after seizure onset. Cognition before seizure onset varied from normal to severely delayed, and was abnormal during follow-up in all patients. Fifteen of 23 patients (65.22%) for whom information on cognition was available had severe ID; ID was moderate in six (26.08%) and mild in two cases (8.7%). Cognitive outcome was worse in patients with epilepsy than in those without epilepsy. Severe ID was observed in 15 of 23 (65.22%) patients with CLCN4-related epilepsy and in five of 20 (25%) patients carrying pathogenic CLCN4 variants without epilepsy. Moreover, patients with severe seizures showed more severe cognitive impairment. However, cognition did not improve in most patients after seizures were controlled. Language delay was observed in 10 patients (41.67%, 10/24), and 25% (6/24) were nonverbal.

Neurologic examination showed anomalies in most patients. Microcephaly, defined as occipitofrontal circumference in the 3rd percentile or smaller, was observed in seven individuals (30.43%, 7/23). Six of them (85.71%, 6/7) developed EE. Infantile hypotonia, often more prominent in the limbs, was found in seven patients (29.17%). Additional neurologic features included hyperreflexia, nystagmus, spastic quadriplegia, extrapyramidal signs, and cerebellar symptoms. Other growth parameters, such as weight and height, were in the normal range for most patients. Behavioral disorders occurred in 10 patients (43.48%, 10/23), including aggression (30.43%, 7/23), autistic features (8.7%), hyperactivity (8.7%), self-abusive behavior (4.35%), irritability (4.35%), depressive disorder (4.35%), apathy and social disinhibition (4.35%), and bipolar disorder (4.35%). A variety of subtle facial dysmorphic features were noted in 15 patients (65.22%, 15/23). Notably, although most patients showed facial dysmorphisms, and the common facial features were long face, broad nasal bridge, and prominent chin, there was no recognizable facial pattern nor any facial features typical for pathogenic CLCN4 variants.

3.4 Electroencephalographic and magnetic resonance imaging features

Electroencephalography (EEG) and neuroimaging were also variable with no consistent findings. EEG data were available for 14 patients; 11 individuals (78.6%, 11/14) displayed epileptiform discharges, and three of them remitted. Three patients without epileptiform discharges were controlled on monotherapy. The spectrum of EEG abnormalities was wide, and no specific EEG abnormalities were found. Slow background activity and multifocal spike-and-slow waves were the most common patterns observed on EEG. Three patients with IS showed an hypsarrhythmia EEG, and one patient showed IS without hypsarrhythmia. Neuroimaging was available for review in 15 patients and showed brain structural abnormalities in 12 (80%, 12/15) individuals. The most common abnormalities were corpus callosum hypoplasia (46.67%, 7/15) and white matter changes (46.67%). Ventriculomegaly was observed in four cases. Cerebellar atrophy was evident in three cases and cerebral atrophy in two cases. Delayed myelination was evident in two patients who were imaged after 3 months of age. Other findings include small perivascular spaces, small hippocampus, enlarged subarachnoid spaces, and subcortical fluid-attenuated inversion recovery/T2 hyperintensity.

3.5 Molecular genetics

Nine different variants were reported previously for the 20 patients with CLCN4-related epilepsy. In this study, we identified three novel variants (p.Val455Ile, p.Asp15Serfs*18), and one previously reported variant (p.Arg718Trp). Thus, a total of 12 distinct CLCN4 variants have been identified in 24 patients from 15 independent families (Table 3). Most variants (70.83%, 17/24) were inherited, and seven variants arose de novo, including in one patient who was mosaic. The most common types of variants are missense variants (79.17%, 19/24), followed by frameshift variants (12.5%, 3/24) and a small intragenic copy number deletion (8.33%, 2/24). In total, only two variants were recurrent, including p.Gly544Arg in two and p.Arg718Trp in three patients. The CIC-4 protein is a dimer with two identical subunits, each of which contains a pore responsible for the selective coupling of the Cl− flux to H+ countertransport. Each subunit contains 18 α-helices and two CBS domains. Four variants mapped to the helical intramembrane domain (H-IM) (p.Ile626Asnfs*135, chrX: g.[10182428_10187807]_10189796_10201480) delete maternal, p.Arg718Trp; Figure 1). The CLCN4 gene is composed of 13 exons, and variants were identified in Exons 11 (50%), 12 (16.67%), 3 (8.33%), 7 (8.33%), 8 (8.33%), and 9 (8.33%), whereas no variants were reported for Exons 1, 2, 4, 5, 6, and 10. To date, functional studies have been performed for six missense variants (p.Leu221Val, p.Val275Met, p.Arg718Trp, p.Phe750Ser, p.Thr532Lys, p.Thr532Ile, p.Val536Met), one to the helical transmembrane domain (H-TM) (p.Val455Ile, p.Arg718Trp, p.Ser534Leu, p.Arg718Trp), one to the helical intramembrane domain (H-IM) (p.Leu625F, p.Ile626Asnfs*135, chrX: g.[10182428_10187807]_10189796_10201480) delete maternal, p.Arg718Trp; Figure 1). The CLCN4 gene is composed of 13 exons, and variants were identified in Exons 11 (50%), 12 (16.67%), 3 (8.33%), 7 (8.33%), 8 (8.33%), and 9 (8.33%), whereas no variants were reported for Exons 1, 2, 4, 5, 6, and 10. To date, functional studies have been performed for six missense variants (p.Leu221Val, p.Val275Met, p.Arg718Trp, p.Ser534Leu, p.Val536Met, p.Gly544Arg, p.Arg718Trp). All evaluated CLCN4 variants resulted in loss of function with significantly reduced current amplitude.6,8
### Table 2 Clinical details of patients with CLCN4-related epilepsy

| Variants       | Country of origin                  | Gender          | Age at case report          | Affected individuals | Affected families | Head circumference |
|----------------|-----------------------------------|-----------------|-----------------------------|----------------------|------------------|-------------------|
| Asp15Serfs*18  | Belgian                           | Male            | 52 years                    | 1                    | 1                | 50th–97th centile |
| Leu221Val      | Anglo-Australian                  | Male            | 16 years                    | 1                    | 1                | 50th centile      |
| Val275Met      | Anglo-American                    | Female          | 10 years                    | 1                    | 1                | 25th centile      |
| Val455Ile      | Chinese                           | Male            | 3 years 7 months            | 1                    | 1                | 50th–75th centile |
| Thr532Lys      | Chinese                           | Male            | 2 years 9 months            | 1                    | 1                | <3rd centile      |
| Asp15Serfs*18  | Northern European                 | Male            | 3 years                     | 8                    | 1                | 50th–98th centile |
| Leu536Met      | 2 male, 1 female                  | 12 years, 16 years, 19 years, 42 years, 66 years, NR (3) | 2                    | 2                | 2nd centile       |
| Gly544Arg      | Dutch (1), North American (1)     | 2 male          | 14 months, 10 years         | 2                    | 2                | 2nd centile       |
| Leu625Phe      | Chinese                           | Male            | 5 years                     | 1                    | 1                | 75th–98th centile |
| Ile626Asnfs*135| Kurdish                           | Male            | 24 years, 22 years          | 2                    | 1                | 2nd–50th centile  |
| chrX: g(10189796_10201480) del mat | Hispanic               | 2 male          | 14 years, 15 years          | 2                    | 1                | 3rd–>98th centile |
| Arg718Trp      | Scottish (1), Chinese (2)         | 2 male, 1 female| 1 year 6 months, 3 years, 15 years | 3                    | 3                | <3rd–75th centile (2), NR (1) |

| Variants       | Level of ID                      | Regression | Infantile hypotonia | Hypertonia |
|----------------|---------------------------------|------------|---------------------|------------|
| Asp15Serfs*18  | Moderate                        | No         | No                  | No         |
| Leu221Val      | Moderate                        | No         | Yes                 | No         |
| Val275Met      | Severe                          | Yes        | No                  | Yes        |
| Val455Ile      | Severe                          | No         | Yes                 | No         |
| Thr532Lys      | Severe                          | No         | No                  | Yes        |
| Ser534Leu      | Severe                          | No         | Yes                 | Yes        |
| Val536Met      | Mild (1), moderate (2), severe (5) | Yes (1), no (7) | Yes (1), no (7) | No (8)    |
| Gly544Arg      | Severe                          | No (2)     | Yes (1), no (1)     | No (2)     |
| Leu625Phe      | Severe                          | Yes        | No                  | No         |
| Ile626Asnfs*135| Moderate (2)                    | No (2)     | No (2)              | No (2)     |
| chrX: g(10182428-10187807)_(10189796_10201480) del mat | Severe (1), mild (1) | Yes (1), no (1) | Yes (1), no (1) | No (2) |
| Arg718Trp      | Severe (2), NR (1)              | Yes (1), no (2) | Yes (1), no (2) | Yes (1), no (2) |
| Variants                  | Country of origin | Gender | Age at case report | Height | Weight | Seizure onset | Seizure types | EE | Seizure-related death | Speech abilities |
|--------------------------|-------------------|--------|--------------------|--------|--------|---------------|---------------|-----|------------------------|------------------|
| Asp15Serfs*18            | Belgian           | Male   | 52 years           | 1st−97th centile | 98th−25th centile | NR            | Absence       | No  | No                     | LD               |
| Leu221Val                | Anglo- Australian | Male   | 16 years           | 50th centile | 25th−50th centile | Infancy      | CP            | No  | No                     | Severe LD         |
| Val275Met                | Anglo- American   | Female | 10 years           | <3rd centile | 25th−75th centile | 13 months    | IS, M, GT, FS | Yes, IS | No                     | NV               |
| Val455Ile                | Chinese           | Male   | 3 years, 7 months  | <3rd centile | <3rd centile       | Neonatal     | IS, M, GT, FS | Yes, IS | No                     | Severe LD         |
| Thr532Lys                | Chinese           | Male   | 2 years, 9 months  | <3rd centile | <3rd centile       | <3rd centile | IS, M, GT, FS | Yes, IS | 2 years, 9 months      | Normal (2)       |
| Ser534Leu                | Northern European | Male   | <3rd centile       | <3rd centile | 3rd−15th centile  | 3 months     | CP, GTC, atonic | Yes (5), no (3) | No                     | LD (4), normal (4) |
| Val536Met                | Anglo- Australian | Male   | 7, 12, 16, 19, 42, | 50th−98th centile | 25th−>98th centile | Infancy/early childhood (8) | CP, GTC, atonic | Yes (5), no (3) | No                     | NV (1)            |
| Gly544Arg                | Dutch, North American | Male, Female | 1, 1 | <1st centile | 75th−90th centile | <1st centile | Absence, GTC (1), CP, secondary GS (1) | Yes (2) | No (2) | NV (1), NR (1) |
| Leu625Phe                | Chinese           | Male   | 14 months, 10 years| 3rd−15th centile | 98th−75th centile | 6 months, 14 years (1) | IS, M, GT, GTC | Yes (2), EIMFS (1), no (1) | No (3) | NV (2), NR (1) |
| Ile626Asnfs*135          | Kurdish           | Male, Female | 1, 3 | 2nd−50th centile | 50th−75th centile | 4 months, 1 year (1) | Absence, GTC (1), CP, secondary GS (1) | Yes (2) | No (2) | No (2) |
| chrX: g.(10189796_10201480) del mat | Hispanic | Male, Female | 14 years, 15 years | 3rd−>98th centile | 50th−95th centile | 6 months, 1 year (1) | IS, M, T, absence | Yes (5), no (3) | No (2) | Severe LD (2) |
| Arg718Trp                | Scottish, Chinese | Male, Female | 1, 3, 15 years | 85th−75th centile | 75th−75th centile | 6 months, 14 years (1) | Absence, GTC (1), CP, secondary GS (1) | Yes (2), EIMFS (1), no (1) | No (3) | NV (2), NR (1) |

**Other neurological features**

| Behavioral issues | Facial dysmorphism | Other issue |
|-------------------|-------------------|-------------|
| Shuffling gait    | Aggression        | No          |
| Clumsiness and fatigue on walking | Depressive disorder | Gastroesophageal reflux, asthma |
| Increased peripheral tone, brisk patella reflexes, ABS | No | No |
| Tendon hyporeflexia, ABS | Repetitive patting on the head | No |
| Nystagmus         | No                | Right inguinal hernia, congenital laryngeal chondrodysplasia |
| CVI, evolving upper limb hypertonia and spasticity | No | FD, recurrent aspiration, capillary hemangioma |
| PS, unsteady gait (2), diplegia (2), ataxia (1) | Aggression (3), hyperactivity (1), bipolar disorder (1), no (5) | Long face, PC, slightly "coarse" facial features, DNB (4), no (4) |
| Dystonic posturing (1), PS, unsteady gait (1) | Apathy, social disinhibition, motor stereotypes (1), no (1) | Round face, downslowing palpebral fissures, open mouth (1), NR (1) |
| Unsteady gait     | Aggression, hyperactivity, irritability | Congenital left upper eyelid ptosis |
| No (2)            | Aggression (2)    | No (2)      |
| Gait abnormality, CVI, nonprogressive upper limb choreiform movements (1), no (1) | No (2) | No (2) |
| ABS (1), NR (2)   | Drooling, some self-abusive behavior (1), no (1), NR (1) | NS, deep set eyes, wide philtrum, full lower lip (1), no (2) |

(Continues)
### TABLE 2 (Continued)

| Variants           | EEG                                                                 |
|--------------------|----------------------------------------------------------------------|
| Asp15Serfs*18      | No epileptiform activity                                           |
| Leu221Val          | Mildly abnormal due to SB                                           |
| Val275Met          | High-amplitude discharges with excessive generalized SB and absence of posterior rhythm |
| Val455Ile          | Multifocal spike wave, SSW, PSSW, AH, SB                           |
| Thr532Lys          | Multifocal spike wave, SSW, PSSW, AH                               |
| Ser534Leu          | Focal activity evolving to intermittent multifocal spikes and generalized SB |
| Val536Met          | Potentially epileptogenic activity throughout recording (1), not tested (7) |
| Gly544Arg          | SB, temporal spikes (1), high-amplitude spikes, generalized spike and polyspike waves (1) |
| Leu625Phe          | Multifocal or generalized sharp waves, spike waves, SSW, polyspike waves IH |
| Ile626Asnfs*135    | Not tested (2)                                                     |
| chrX: g.(10182428_10187807)_(10189796_10201480) del mat | Bioccipital spikes, generalized spikes, generalized SB (1), normal (1) |
| Arg718Trp          | SB, IH, multifocal spike waves, PSSW, SSW (1), multifocal discharges (1), not tested (1) |

Abbreviations: ABS, abnormal Babinski signs; AH, atypical hypsarrhythmia; BSF, broad square forehead; CA, cerebellar atrophy; CCH, corpus callosum hypoplasia; CP, complex partial; CVI, cortical visual impairment; DM, delayed myelination; DNB, depressed nasal bridge; EE, epileptic encephalopathy; EEG, electroencephalogram; EIMFS, epilepsy of infancy with migrating focal seizures; FD, feeding difficulties; FLAIR, fluid-attenuated inversion recovery; FS, focal seizures; GS, generalized seizures; GT, generalized tonic; GTC, generalized tonic–clonic; ID, intellectual disability; IH, intermittent hypsarrhythmia; IS, infantile spasms; LD, language delay; M, myoclonic; NA, not available; NR, not reported; NV, nonverbal; PC, prominent chin; PS, progressive spasticity; PSSW, polyspike and slow waves; SB, slow background; SSW, spike and slow waves; T, tonic; TCC, thin corpus callosum; WML, white matter loss.

### 3.6 Genetic–phenotype correlations

Frameshift or intragenic deletion variants are associated with milder epileptic phenotypes, and seizures were easily controlled compared with most individuals with missense variants. The majority (4/5) of individuals with frameshift or intragenic deletion variants were controlled on monotherapy, whereas only 21.05% (4/19) of patients with missense variants achieved this with monotherapy or no treatment, and four of them died before the age of 19 years. In addition, the degree of ID was greater in individuals with missense variants. Severe ID was observed in 20% (1/5) of cases with frameshift or intragenic deletion variants and in 77.78% (14/18) of cases with missense variants. Hence, phenotypes of individuals with frameshift or intragenic deletion variants were relatively mild compared with the majority of individuals with missense variants. In comparison to inherited variants, de novo variants were more prone to cause more severe phenotypes. All patients with de novo variants had severe ID, and 85.71% (6/7) of these had intractable EE. In contrast, inherited variants are less frequently found among patients with severe ID (52.94%, 9/17) and EE (52.94%, 9/17). We observed no definite correlation between the location of the variants and the severity of the associated phenotype. Even with the same variant, a large phenotypic variance is observed. This is exemplified by the recurrent p.Arg718Trp variant, which has now been described in three unrelated patients, two in previous studies and one here. Of the three patients, one had brief absence seizures with onset at 8 years of age and no need of treatment. In contrast, two suffered from intractable EE starting at the age of 1 month. This variability was illustrated by a considerable intrafamilial variability in disease severity. For example, eight patients from one family carrying the same variant (p.Val536Met) had distinct phenotypes. Drug-resistant epilepsy was present in six individuals and early death occurred in three individuals, possibly because of limited antiepileptic treatment options. However, seizures were controlled on monotherapy in two individuals. In addition, they also showed different degrees of ID, which was evaluated as severe in five patients, moderate in two, and mild in one.

### 3.7 Functional findings of three novel CLCN4 variants

To investigate the functional consequences of the three novel CIC-4 variants, we first tested their protein levels by Western blot analysis. The three mutant proteins were expressed at levels similar to wild-type (WT) CIC-4-FLAG in HEK293
| Neuroimaging                                                                 | Response to treatment                                      |
|-----------------------------------------------------------------------------|------------------------------------------------------------|
| Not tested                                                                  | Well controlled on monotherapy, medication ceased by 7 years |
| Small perivascular spaces seen in the inferior aspects of the putamen       | Well controlled on carbamazepine monotherapy              |
| Persistently enlarged 3rd ventricle, nonspecific subcortical FLAIR/T2        | Refractory to polytherapy                                  |
| hyperintensity left frontal region                                           |                                                            |
| Periventricular leukomalacia                                                  | Refractory to polytherapy                                  |
| CCH white matter reduction in posterior horn of lateral ventricle           | Refractory to polytherapy                                  |
| Periventricular WML with compensatory dilation of lateral and third         | Refractory to polytherapy                                  |
| ventricles                                                                   |                                                            |
| Marked supra- and infratentorial atrophy, TCC, CA (2), ventriculomegaly (1), | Well controlled on monotherapy (2), refractory to polytherapy (6) |
| cortical atrophy, WML (1), not tested (6)                                   |                                                            |
| CCH, increased FLAIR signal in white matter (1), normal (1)                 | Refractory to polytherapy until lamotrigine added (1), refractory (not fully responsive to medications) at 14 months (1) |
| Normal (2 years), CA (4 years)                                               | Refractory to polytherapy until lamotrigine added          |
| Not tested (2)                                                              | Controlled on monotherapy medication ceased by 7–10 years of age (2) |
| DM, TCC, global cerebral atrophy, particularly affecting the cerebral       | Refractory to polytherapy (1), controlled on levetiracetam |
| white matter (1), normal (1)                                                 | monotherapy (1)                                            |
| TCC (2), white matter reduction, small left hippocampus, left               | Refractory to polytherapy, but one finally remitted (2), not treated (1) |
| ventriculomegaly, DM (1), normal (1)                                        |                                                            |

4 | DISCUSSION

Pathogenic CLCN4 variants have been reported in patients with X-linked ID, epilepsy, behavioral disorder, and dysmorphic features. As expected for an X-linked disease, females are less often affected. However, the affected females can develop as severe phenotypes as males. The underlying complexity of X-chromosome inactivation in cells and tissues hinders a prediction of the outcome. So far, CLCN4 variants have been reported in 40 individuals, and 20 of them presented with epilepsy. Here, we identified three novel and one previously reported CLCN4 variant. All four patients displayed global DD and EE. Bioinformatics analysis and functional study supported the deleterious effect in p.Thr532Lys and p.Leu625Phe variants. For p.Val455Ile, the prediction tools suggest a benign variant. However, this variant segregated in the family, and WES showed no other likely pathogenic variants. Importantly, electrophysiological measurements showed that this variant caused a marked reduction of CIC-4 currents, supporting the pathogenicity of this variant. Combining our series with previously published cases, epilepsy was diagnosed in 54.55% of the subjects with CLCN4 variants, suggesting that all individuals with pathogenic CLCN4 variants should be evaluated for epilepsy due to the high prevalence of seizures in this population. Our

cells (Figure 2A). To test for the functionality of the CIC-4 variants, we performed electrophysiological recordings of WT and mutant CIC-4 in HEK293 T cells. All three novel variants yielded drastically reduced outwardly rectifying currents compared to WT, demonstrating loss of function (Figure 2B,C). We next assessed the subcellular localization of the CIC-4 variants upon heterologous expression in HEK293T cells. In agreement with previous reports, WT CIC-4 localized predominantly to the endoplasmic reticulum (ER), which was positive for the coexpressed marker calnexin (Figure 2D). CIC-4, seemingly devoid of endosomal localization signals, can heterodimerize with its paralogue CIC-3, enabling the transport of CIC-3/CIC-4 dimers from the ER to endosomal–lysosomal compartments. When we coexpressed WT CIC-4 with CIC-3b in HEK293T, both proteins expectedly colocalized to slightly enlarged endosomal compartments (Figure 2D). The three CIC-4 variants colocalized with CIC-3b, demonstrating that the ability to heterodimerize was not impaired. However, in addition to the vesicular localization, we observed a slightly more pronounced residual reticular pattern of CIC-3b/CIC-4 for CIC-4 variants than for WT (Figure 2E). This might be due to stronger ER retention of mutant CIC-4 and contribute to the loss of function observed in the electrophysiological measurements.
| Location | Nucleotide change | AA change    | Exon | Mutation type | Inheritance | Protein domain   | Functional effect | Consequence | References |
|----------|-----------------|--------------|------|---------------|-------------|------------------|------------------|-------------|------------|
| chrX: 10153111-10153123 | del13 bp         | Asp15Serfs*18 | 3    | Frameshift    | Inherited   | Cytoplasmic region | NA              | LOF         | Palmer et al. 2018 |
| chrX: 10174503   | c.661C>G          | Leu221Val    | 7    | Missense      | Inherited   | Loop             | Reduced current   | LOF         | Hu et al. 2016, Palmer et al. 2018 |
| chrX: 1017479    | c.823G>A          | Val275Met    | 8    | Missense      | De novo     | H-ID             | Reduced current   | LOF         | Palmer et al. 2018 |
| chrX: 10176604   | c.1363G>A         | Thr532Lys    | 11   | Missense      | De novo     | H-ID             | Reduced current   | LOF         | This study |
| chrX: 10181745   | c.1601C>T         | Ser534Leu    | 11   | Missense      | De novo     | H-ID             | Reduced current   | LOF         | Palmer et al. 2018 |
| chrX: 10181750   | c.1606G>A         | Val536Met    | 11   | Missense      | Inherited   | H-ID             | Reduced current   | LOF         | Hu et al. 2016, Palmer et al. 2018 |
| chrX: 10181774   | c.1630G>C or G>A | Gly544Arg    | 11   | Missense      | De novo     | Loop             | Reduced current   | LOF         | Veeramah et al. 2013, Hu et al. 2016, Palmer et al. 2018 |
| chrX: 10182017   | c.1873C>T         | Leu625Phe    | 11   | Missense      | Inherited   | CBS1             | Reduced current   | LOF         | This study |
| chrX: 10182019-10182020 | Insert A       | Ile626Asnfs*135 | 11   | Frameshift    | Inherited   | CBS1             | NA              | LOF         | Palmer et al. 2018 |
| chrX: deleted at maternal | DELETE maternal | NA | 12 | Intragenic copy number deletion | Inherited | CBS1 | NA | LOF | Palmer et al. 2018 |
| chrX: 10188877   | c.2152C>T         | Arg718Trp    | 12   | Missense      | Inherited   | CBS2             | Reduced current   | LOF         | Zhou et al. 2018, Palmer et al. 2018, this study |

Abbreviations: AA, amino acid; CBS, cystathionine-β-synthase domain; H-ID, helical intramembrane domain; H-TD, helical transmembrane domain; LOF, loss of function; NA, not applicable.
results further indicate that \textit{CLCN4} variants are associated with a wide phenotypical spectrum varying from mild brief absence seizures to severe EE. The seizure onset was within the first year of life in 69.57\% of individuals, indicating an infantile onset epilepsy. EE was diagnosed in 62.5\% of patients, including IS, EIMFS, and unclassifiable EE. All patients presented ID, which was more severe in individuals with more severe seizure phenotypes. Moreover, cognitive outcome was worse in patients with \textit{CLCN4}-related epilepsy than in those without epilepsy. This suggests that ID is a common comorbidity of \textit{CLCN4}-related epilepsy and severe epilepsy may negatively influence cognitive outcome. However, cognition and development did not improve in most patients after seizure control. This suggests that cognitive impairment was partially irreversible, possibly due to the negative effect of CIC-4 dysfunction on cognition. Our data also underline that epilepsy outcome is highly variable between patients with \textit{CLCN4}-related epilepsy. Only one patient did not undergo any treatment, and 43.48\% of patients remitted. Seizure-related death was observed in four patients, suggesting a relevant risk for premature and unexpected death with \textit{CLCN4}-related epilepsy. Seizure semiology was variable, including myoclonic, absence, tonic, complex partial, tonic–clonic, focal, and secondary generalized seizures and IS, notably at the time of reporting, as it is not possible to capture the full seizure spectrum. Of note, there was a trend for patients with multiple seizure types to be more pharmacoresistant than those with a single seizure type. To date, there are few data on treatment response in \textit{CLCN4}-related epilepsy. Causal treatment is not available. The limited data suggest that \textit{CLCN4}-related epilepsy is difficult to treat but has responded relatively well to lamotrigine, a blocker of voltage-sensitive sodium channel that reduces neuronal excitability, suggesting that the targets of lamotrigine and CIC-4 are coexpressed in the same brain areas. Interestingly, our patients did not react to valproic acid, which exerts various pharmacodynamic effects, for example on ionic currents and \(\gamma\)-aminobutyric acid levels.\textsuperscript{34} Lamotrigine abrogated or dramatically reduced seizures in five patients. Therefore, lamotrigine should be considered as a therapeutic option for \textit{CLCN4}-related epilepsy in the future. However, these beneficial effects were seen in few patients, and larger prospective studies are needed to identify more effective therapeutic regimens. We also observed behavioral problems in almost half of the cases, with aggression being the most common. Neurological disturbances included microcephaly and infantile hypotonia as the most prominent features. The facial dysmorphisms found in most patients did not provide a recognizable pattern or any facial features suggestive for \textit{CLCN4} variants. In addition, most individuals demonstrated structural brain abnormalities as detected by neuroimaging, but with no apparent pattern among the affected individuals. Nevertheless, corpus callosum abnormalities and white matter change were recurrent findings. The relationship of both white matter impairment and callosal abnormalities to epilepsy or DD will be important for future studies. However, no obvious changes were observed in white matter or corpus callosum of CIC-4-deficient mice.\textsuperscript{8,23,35}

Although mutations were found throughout the gene or the protein, a significant number of mutations were identified in Exon 11 of the \textit{CLCN4} gene, or located in the H-IM and CBS domains of the CIC-4 protein, underlining the crucial importance of these regions. However, no obvious correlation between the position of mutations on the gene or protein and severity of the phenotype was observed. We found two recurrent missense variants, p.Gly544Arg and p.Arg718Trp, suggesting these areas are possible mutational “hot spots” in \textit{CLCN4}. Remarkably, all \textit{CLCN4} variants lead to loss of function, whereas diseases associated with \textit{CLCN6} and \textit{CLCN7} can also be caused by gain-of-function mutations that increase the current amplitude or alter voltage-dependent activation kinetics.\textsuperscript{10,36–39} For \textit{CLCN4}, it is notable that frameshift or intragenic deletion variants cause rather mild phenotypes, whereas missense variants cause more severe phenotypes. Moreover, early death occurred in four patients with missense variants, whereas it did not occur in patients with frameshift or intragenic deletion variants, which may suggest that perturbation of normal CIC-4 protein function is more deleterious than a reduction in its abundance or a complete absence. Such reduced protein levels are expected for the frameshift and intragenic deletion variants, because the alteration is located before the last exon (Figure 1) and even production of a truncated CIC-4 version will be largely prevented through nonsense-mediated decay. A possible explanation is that defective proteins caused by missense mutations could be more deleterious, whereas loss of function due to reduced protein may be compensated by other proteins. In addition, CIC-4 mutants within CIC-3/CIC-4 heteromers may impinge on CIC-3, for example, by affecting the common subcellular trafficking or ion transport by the other subunit, as shown for CIC-7 dimers.\textsuperscript{40,41}

Whereas de novo variants were associated with more severe ID and epileptic phenotype, patients with inherited variants had milder ID and epileptic phenotype. Notably, phenotypic variability was seen in both recurrent variants and within single families. A remarkable phenotypic variability was found for the recurrent p.Arg718Trp variant, with one carrier showing brief absence seizures without the need of treatment, whereas others had more severe EE. Particularly noteworthy were the discrepancies in seizure phenotype within single families in which different individuals presented with either well-controlled seizures on monotherapy or severe EE and early death. \textit{CLCN4}-related epilepsy thus displays a remarkable phenotypic heterogeneity, indicating that the genotype–phenotype relationship remains complex. This presents challenges for genetic counseling. The
Functional findings of the three novel CLCN4 variants identified in this study. (A) Western blot analysis of HEK293 cells expressing wild-type (WT) CIC-4 and CIC-4 variants. WT and mutant CIC-4 proteins showed similar protein expression levels. β-actin was used as loading control. NC, negative control (mock-transfected). Values are expressed as mean ± SEM of five independent experiments. (B) Representative current traces recorded from HEK293T cells expressing either WT or mutant CIC-4. (C) Comparison of current–voltage relationships between WT (black solid squares, n = 12) and the three CLCN4 variants (p.V455I [red solid circles, n = 11], p.T532K [blue solid triangles, n = 13], L625F [inverted green solid triangles, n = 12]). (D) Subcellular localization of human CIC-4 variants heterologously expressed in HEK293T cells. CIC-4WT and the respective mutant proteins localize mainly to the endoplasmic reticulum (ER) and show strong colocalization with the coexpressed ER marker calnexin (WT CIC-4, n = 22; p.V455I CIC-4, n = 23; p.T532K CIC-4, n = 26; or L625F CIC-4, n = 20). (E) Representative images of HEK293T cells heterologously coexpressing CIC-4 variants with CIC-3b. Colocalization of CIC-3b and WT or all the three CIC-4 mutants in cytoplasmic vesicles of HEK293T cells (WT CIC-4, n = 20; V455I CIC-4, n = 20; T532K CIC-4, n = 17; or L625F CIC-4, n = 19). Scale bars = 5 μm.

mechanisms underlying phenotypic heterogeneity are unclear but may be explained by individual genetic background, genetic modifiers, epigenetic effects, or environmental factors. Further studies are required to precisely define the correlation between genotype and phenotype.

Electrophysiological studies revealed that all these missense variants reduced or abolished CIC-4 currents,6,8,12 consistent with loss of function underlying the epilepsy and cognitive defects. The number of dendritic branches and dendritic length are remarkably reduced in primary hippocampal neurons from Clcn4−/− mice and in Clcn4 knockdown mouse hippocampal or cortical neurons.3,42 Therefore, CIC-4 showed a significant effect on neuronal differentiation, which corroborates the notion that loss of CIC-4 function may be the major pathogenic mechanism. However, this conclusion appears to conflict with an apparent lack of neurological or behavioral phenotype, or altered ClC-4 function may be the major pathogenic mechanism. Therefore, ClC-4 showed a significant effect on neuronal

In conclusion, this study expands the number of reported patients with CLCN4-related epilepsy and describes three novel loss-of-function variants. It provides an overview of all CLCN4-related epilepsy cases, comparing the epilepsy and developmental findings in 24 subjects. The phenotypic spectrum of CLCN4-related epilepsy includes medication-resistant seizures, ID, behavioral disorders, and congenital anomalies. Notably, patients with multiple seizure types, or missense or de novo variants showed more severe phenotype, whereas single seizure type, frameshift or intragenic deletion, or inherited variants were associated with milder phenotypes. However, genotype–phenotype relationships for CLCN4-related epilepsy are not straightforward. Phenotypic variability was observed in recurrent variants and within single families. Loss of CIC-4 function is the most likely underlying disease mechanism. Further elucidation of disease mechanisms may facilitate the development of targeted treatments, which are much needed for this drug-resistant genetic epilepsy.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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