Missense and truncating variants in the X-chromosome-linked CLCN4 gene, resulting in reduced or complete loss-of-function (LOF) of the encoded chloride/proton exchanger ClC-4, were recently demonstrated to cause a neurocognitive phenotype in both males and females. Through international clinical matchmaking and interrogation of public variant databases we assembled a database of 90 rare CLCN4 missense variants in 90 families: 41 unique and 18 recurrent variants in 49 families. For 43 families, including 22 males and 33 females, we collated detailed clinical and segregation data. To confirm causality of variants and to obtain insight into disease mechanisms, we investigated the effect on electrophysiological properties of 59 of the variants in the publicly available database ClinVar, 73% (111) were classified to be of uncertain significance. Without clear establishment of pathogenicity, families remain on a diagnostic odyssey, cannot make fully informed reproductive choices, or benefit from advances in condition-specific management guidelines or targeted therapies.

The first CLCN4 variant was reported in an infant male with developmental and epileptic encephalopathy and suggested CLCN4 as a novel candidate disease gene [1]. Three years later,
as part of an X chromosomal exome sequencing study, our group demonstrated that truncating and missense variants were associated with a neurocognitive phenotype in males in five unrelated families [2]. Two families had linkage intervals including Xp22: A two generation French family with five affected males with severe to profound intellectual disability (ID) and variable behavioral difficulties was reported by Raynaud et al., in 1996 [3] and a Belgian family with five males spanning two generations with ID, challenging behaviors and autistic features described by Claes et al. [4]. Heterozygous females in those families were neurotypical or had a mild neurocognitive/psychiatric phenotype. Therefore, a phenotypic entity of X-linked recessive ID (Raynaud-Claes Syndrome) was proposed (MIM *300114).

Subsequently, we reported 10 additional families consisting of 29 hemizygous males and 23 heterozygous females [5]. We clarified that all males had a core phenotype of mild to severe ID, with considerable intrafamilial heterogeneity. For the first time, we reported the phenotype in females with de novo variants, which overlapped in severity with that of males. Other common clinical features included epilepsy, subtle white matter changes on neuroimaging, autism spectrum disorder, challenging behaviors, and mental health complications including bipolar disorder, depression, and anxiety. More recently, an additional six males with CLCN4-related neurodevelopmental condition were reported confirming the core feature of ID and common comorbidities of epilepsy and challenging behaviors [2, 6, 7]. Xu et al., reported on a female with ID, autistic features and brain abnormalities, with a maternally inherited CLCN4 missense variant where the mother had mild ID [8]. We recently summarized the published genotypic and phenotypic spectrum [9], noting that, to date, all CLCN4 variants studied in the Xenopus expression system demonstrated partial or complete loss-of-function (LOF) [1, 10, 11].

CIC-4 is one of the nine members of the CLC gene family encoding anion-transporting membrane proteins [12]. CLC proteins are divided into two groups: four members (CIC-1, CIC-2, CIC-Ka, and CIC-Kb) are CI− channels localized in the plasma membrane, while the remaining CLCs (CIC-3 to -7) are secondary Cl−/H+ antiporters physiologically localized in intracellular endo-/lysosomal membranes; the latter are also called vesicular CLCs (vCLCs). Among the vCLCs, CIC-3 to -5 are highly homologous and are localized to endosomes, while the more divergent CIC-6 and CIC-7 are localized to late endosomes and lysosomes, respectively [12]. The vesicular Cl−/H+ antiporter activity is important for ionic homeostasis of endo-/lysosomes by assisting in vesicular acidification and increasing intracellular Cl− concentration. The function of CIC-4 critically depends on the highly related CIC-3 transporter, with which it forms heterodimers [13, 14]. While most CLCs are physiologically homodimeric, CIC-4 appears to preferentially associate with CIC-3, whereas CIC-4 homodimers are biochemically relatively unstable [13, 14].

CIC-4, and other members of this protein family, CIC-3, CIC-6, CIC-7, and Ostm1, an obligatory subunit of CIC-7, are implicated in neurological disorders [2, 5, 12, 15, 16]. This could be postulated to be related to the postmitotic nature of neurons and their heavy reliance on vesicular trafficking. For example, mice lacking late endosomal CIC-6 transporters show signs of lipofuscin accumulation [17], and lacking lysosomal CIC-7 exhibit a severe lysosomal storage phenotype, respectively [18]. Recently a recurrent gain-of-function (GOF) variant reported in CLCN6 caused the severe neurodegenerative disease CONRIBA (Neurodegeneration, child-hood-onset, hypotonia, respiratory insufficiency and brain imaging abnormalities CONRIBA; MIM 619173) [15] while a variant found in a patient with clinical features of late-onset neuronal ceroid lipofuscinosis [17] was found to have greatly reduced functional activity [19]. LOF of CIC-3 in mice leads to neurodegeneration [20] and both GOF and LOF CLCN3 variants in humans cause severe global developmental delay [16]. Conversely, knock-out mouse models of CIC-4 have no overt phenotype [21], implying a complex causative mechanism that requires further exploration to understand the pathophysiological basis of CLCN4-related neurodevelopmental condition.

Understanding the pathogenicity of missense variation in CLCN4 both clinically and functionally is therefore the next step [6]. We firstly undertook a collaborative study aiming to further characterize the genotypic and phenotypic spectrum of CLCN4-related neurodevelopmental condition in both males and females. Secondly, we studied the functional impact of novel and previously reported missense variants in heterologously expressing Xenopus oocytes by employing electrophysiological measurements using extended voltage-protocols.

SUBJECTS AND METHODS

Subjects
We collected de-identified detailed clinical data on 55 individuals from 43 previously unreported families with (presumed) CLCN4-related neurodevelopmental condition, including individuals from three families where the proband had a blended clinical phenotype with a second genetic diagnosis. Data were obtained through an international collaborative process wherein clinicians and diagnostic laboratories with variants identified in CLCN4 contacted our team, and we also contacted the laboratory or clinician who had deposited variants in CLCN4 in the public databases DECIPHER, ClinVar, and LOVD [22–24]. In each participating center, written informed consent was obtained from the individual’s legal guardians before genetic testing as approved by relevant local ethical committees. Clinical information was obtained by review of medical records and examination of affected individuals. Written informed consent for the publication of clinical data and photographs was also obtained from the participants’ legal guardians.

Expression construct
The human CIC-4 cDNA was cloned in the pT7N expression vector [25], in which the disease-associated variants were introduced using standard restriction-free mutagenesis. All constructs were verified by Sanger sequencing.

Expression in oocytes
RNA was transcribed using the SP6 mMessageMachine kit (ThermoFisher, Milan, Italy) after linearization with MluI. Following linearization with MluI, RNA was injected into Xenopus laevis oocytes were injected with ~6 ng of RNA and incubated at 18°C for 2–5 days prior to measurements as described previously [26].

Two electrode voltage clamp recordings
Recording pipettes were filled with 3 M KCl (resistance about 0.6 MOhm) and currents were recorded using a TEAC3 two electrode voltage clamp amplifier (npi electronics, Tamnn, Germany). Ground electrodes were connected to the bath via agar bridges. The standard extracellular solution contained 100 mM NaCl, 5 mM MgSO4, 10 mM HEPES (pH 7.3). For solutions at pH 6.3 and 5.3, HEPES buffer was replaced by MES (2-(N-morpholino)ethanesulfonic acid) buffer, pH was adjusted with NaOH. Currents were acquired using the custom GePulse acquisition program and an itc-16 interface (Instrutech, Colorado, USA), filtering at 5 kHz and sampling at 50 kHz. Two types of stimulation protocols were applied from a holding potential of −30 mV. The first consisted of 10 ms pulses to voltages ranging from −160 to −120 mV (in 20 mV steps) without leak subtraction. The second protocol consisted of steps ranging from +170 to −10 mV (in 10 mV steps), applying linear leak and capacity subtraction using a ‘P/4’ leak subtraction protocol from the holding potential −30 mV. For this procedure 4 pulses of ¼ of the regular amplitude were applied towards negative voltages, their response was averaged, adequately scaled, and subtracted. This procedure approximately eliminates linear capacitive currents and ‘leak’, assuming that CIC-4 is inactive at negative voltages.

Data analysis
To evaluate the relative expression levels of mutant compared to wild-type (WT) CIC-4, currents were measured for >5 oocytes for each batch of injection of each construct, and the average current-voltage relationship was obtained using the P/4 subtracted protocol. Average currents
RESULTS

Detailed clinical data were analyzed on 55 previously unreported individuals, 22 hemizygous males and 33 female heterozygotes, from 43 previously unreported families, as well as updated clinical information on one previously reported female who was now recognized to have a recurrent variant [5]. The 44 families were divided into five groups (A-E). This includes families with missense variants, who were divided into groups A-D based on the functional results obtained in the Xenopus laevis oocyte model for the CLCN4 missense variants as described below, as well as three additional patients with novel truncating variants (Group E). Similar to the “right-shifted” variants, we interpret the emergence of inward currents at acidic as a partial disruption of the gating process that in WT keeps the transporter inactive at negative voltages, similar to what was described for CLCN3 variants [16]. Error bars in all figures represent SEM. Statistical significance was assessed by Student’s unpaired two-tailed t-test. Variance is similar between all groups because the same batches of oocytes were utilized for WT and variant measurements.

For data analysis of currents measured at various external pH values, the following leak-subtraction was performed. For each oocyte, currents measured at pH 7.3 were fitted in the range −120 mV ≤ V ≤ 0 mV with straight line. The line was extrapolated to all voltages and subtracted from the current-voltage relationships (IVs) measured in the various conditions, and normalized to the current at pH 7.3, 160 mV. This is because for WT ClC-4 and for most variants, at pH 7.3, currents recorded at voltages V = −0 mV are very small and indistinguishable from currents in un-injected oocytes and represent a mixture of leak and endogenous currents. Similar to the “right-shifted” variants, we interpret the emergence of inward currents at acidic as a partial disruption of the gating process that in WT keeps the transporter inactive at negative voltages, similar to what was described for CLCN3 variants [16]. Error bars in all figures represent SEM. Statistical significance was assessed by Student’s unpaired two-tailed t-test. Variance is similar between all groups because the same batches of oocytes were utilized for WT and variant measurements.

Some variants, for example p.(Val92Met), showed current levels that were barely above those seen in un-injected oocytes (Fig. 4A–C). A similar near complete loss or reduced function was observed for other variants e.g., p.(Lys62Arg), p.(Ser278Arg), p.(Gly342Glu), and p.(Gly484Arg) (for full list see Table 1, Supplementary Fig. 1). As the variant p.Gly731Val affected the last amino acid of exon 12, we analyzed if this variant impacted splicing (Supplementary Fig. 2), but this could not be demonstrated. Little mechanistic insight can be obtained from these LOF variants as we did not analyze for example if protein stability was affected.

Other variants, for example p.(Asn309Ser), showed a reduced expression level, but no sign of altered voltage-dependence (Fig. 4A–C). The lack of altered voltage-dependence is highlighted in Fig. 4C, which shows that the ratio of currents mediated by variant p.(Asn309Ser) and currents of WT ClC-4 has a practically voltage-independent value of ~0.25. A similar, partially reduced function was observed for variants p.(Ile374Thr) and p.(Gln489Lys) (Table 1, Supplementary Fig. 1). In contrast to the voltage-independent reduction seen in the variants described above, several other variants, including p.(Leu276Phe), showed a “right-shifted” voltage dependence. This is difficult to appreciate by just comparing the raw current traces (Fig. 4A) or the average current-voltage relationship (Fig. 4B) but is clear in Fig. 4C. For p.(Leu276Phe), the ratio of currents compared to WT is small for V = −20 mV but progressively enlarged at more positive voltages. The reduction of currents at “physiological” voltages is overcome by sufficiently large positive voltages. This essential LOF phenotype likely reflects an effect on the gating process of ClC-4, as detailed in Subjects and Methods. Similar LOF by apparently right-shifted gating was observed to various degrees for variants p.(Gly78Ser), p.(Val212Gly), p.(Gly269Asp), p.(Ile272Gly), p.(Val275Leu), p.(Val275Met), p.(Glu319Ser), and p.(Arg718Trp) (Table 1, Supplementary Fig. 1).

Group B. This group includes nine missense CLCN4 variants from 17 independent families: 14 were previously unreported, one, a female with the de novo variant p.(Ala555Val), was previously reported by our group [5], and four families with the same variants or amino acid mutated, that were included in public databases but for whom we could not obtain detailed clinical data. These variants were grouped, as they showed compelling clinical evidence for pathogenicity (rarity, de novo status, matching clinical phenotype, and recurrence across unrelated families), without gross effect at the regular recording conditions at pH 7.3. However, p.(Ile549Asn) and some other variants exhibited a characteristic alteration (Fig. 4C): the ratio of currents compared to WT became progressively larger towards more negative voltages. This behavior is reminiscent of a GOF effect described for CLCN3 variants [16]. Indeed, closer inspection of this and other variants revealed a dramatic GOF that is apparent particularly at acidic...
Table 1. Summary of rare CLCN4 variants reported in this study and, if recurrent, in previous literature or public databases.

| Families | Genomic position and variant, (GRCh38), NC_000023.10 | Exon number | c.DNA change, NM_001830.4(CLCN4): | Protein change, NP_001821 | Protein domain* for missense variants | Source : This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | Gender of proband. Others with variant in family. | Inheritance | Recurrent in unrelated families | Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | SIFT (dbNSFP version 4.2); converted rankscore | PolyPhen | CADD | REVEL | SpliceAI | Frequency in heterozygotes (gnomAD) | Frequency in hemizygotes (gnomAD) | Functional impact in Xenopus oocyte model | Severe functional impact in Xenopus oocyte model | Blended phenotype? | Genetic test |
|----------|----------------------------------------------------|--------------|---------------------------------|---------------------------|-------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------|---------------------|----------------------|-----------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------|---------------------|-----------------|------------------|-------------------|-----------------|-------------------------------|---------------------------|----------------------|----------------------|------------------------|
| A1       | X:10187555A>G                                      | 4            | c.185A>G                        | A> G232                  | N-term, intracellular               | This study; ClinVAR SCV002525716                                                             | 1 affected male, mother unaffected                | Maternally inherited | No                   | VOUS P VOUS LP P VOUS                                                                 | Tolerated (0.48) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Benign (0.005) Possibly damaging (0.817) Benign (0.03) Probably damaging (0.992) Probably damaging (0.925) Probably damaging (0.997) | 21.2              | 0.466              | ≤ 0.2               | 0                   | 0                   | 0               | LOF                           | No                      | No                   | No                   | No                     |
| A2       | X:10187602 G>A                                      | 4            | c.232G>A                        | A                     | Helix B, transmembrane              | This study; ClinVar SCV000920556.1                                                          | 3 affected males                                  | Maternally inherited | No                   | De novo                                                                 | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | 25.4              | 0.937              | ≤ 0.2               | 0                   | 0                   | 0               | LOF by shift of voltage dependence | No                      | No                   | No                   | No                     |
| A3       | X:10194940 G>A                                      | 5            | c.274G>A                        | A                     | Helix B, transmembrane              | This study; ClinVAR SCV002525716                                                             | 1 affected female proband, 1 affected male (father) | Paternally inherited | No                   |                | Tolerated (0.48) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | 21.8              | 0.608              | ≤ 0.2               | 0                   | 0                   | 0               | Almost complete LOF | No                      | No                   | No                   | No                     |
| A4       | X:10206410 C>T                                      | 7            | c.608C>T                        | T                     | Helix E, intramembrane              | This study; ClinVAR SCV000245780.1                                                          | 1 affected male, mother unaffected                | Maternally inherited | No                   |                | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | 25.1              | 0.969              | ≤ 0.2               | 0                   | 0                   | 0               | Almost complete LOF | No                      | No                   | No                   | No                     |
| A5       | X:10206437 T>G                                      | 7            | c.63ST>G                        | G                     | Helix E, intramembrane              | This study; ClinVAR SCV002525717                                                             | 2 affected males (3 other males in family with ID not tested). | Maternally inherited | No                   |                | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | 25.1              | 0.944              | ≤ 0.2               | 0                   | 0                   | 0               | LOF by shift of voltage dependence | No                      | No                   | No                   | No                     |
| A6       | X:10206479 C>T                                      | 7            | c.677C>T                        | G                     | Helix F, intramembrane              | This study; ClinVAR SCV002525717                                                             | 1 affected male                                    | De novo              | No                   |                | Tolerated (0.48) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | 0                 | 0.967              | ≤ 0.2               | 0                   | 0                   | 0               | LOF                           | No                      | No                   | No                   | No                     |
| Families | Genomic position and variant, (GRCh38), NC_000023.10 | Exon number | c.DNA change, NM_001830.4 (CLCN4): | Protein change, NP_001821 | Source: This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR, LOVD and/or DECIPHER |
|----------|-----------------------------------------------|-------------|---------------------------------|---------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| A7       | X:10206739 G>A                                | 8           | c.806G>A                        | p.(Gly269Asp)             | Helix G, intramembrane                                                        |
| A8       | X:10206739 G>A                                | 8           | c.806G>A                        | p.(Gly269Asp)             | ClinVAR, SCV000582636.4                                                     |
| A9       | X:10206747 A>G                                | 8           | c.814A>G                        | p.(Ile272Val)             | Helix H, intramembrane                                                        |
| A10      | X:10206756 G>C                                | 8           | c.823G>C                        | p.(Val275Leu)             | ClinVar, SCV000742044.2                                                     |
| A11      | X:10206756 G>A                                | 8           | c.823G>A                        | p.(Val275Met)             | This study; ClinVAR, SCV002525718                                            |
| A12      | X:10206756 G>A                                | 8           | c.823G>A                        | p.(Val275Met)             | ClinVar, SCV000577686.4                                                     |

| Gender of proband. Others with variant in family. | 1 affected female | NR | NR | 1 affected male, mother unaffected | 1 affected female |
| Inheritance | De novo | NR | NR | Maternally inherited. De novo in mother | De novo |
| Recurrent in unrelated families | Yes | Yes | No | No | Yes |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | LP | LP | VOUS | VOUS | P | P |
| SIFT (dbNSFP version 4.2); converted rankscore | Deleterious (0) | Deleterious (0) | Tolerated (0.16) | Deleterious (0) | Deleterious (0) | Deleterious (0) |
| PolyPhen | Probably damaging (1) | Probably damaging (1) | Benign (0.173) | Probably damaging (0.919) | Probably damaging (0.971) | Probably damaging (0.971) |
| CADD | 26.2 | 26.2 | 18.6 | 25.7 | 26.7 | 26.7 |
| REVEL | 0.946 | 0.946 | 0.564 | 0.92 | 0.925 | 0.925 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| Frequency in heterozygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Frequency in hemizygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Functional impact in Xenopus oocyte model | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence | LOF by shift of voltage dependence | LOF by shift of voltage dependence | LOF by shift of voltage dependence | LOF by shift of voltage dependence |
| Severe functional impact in Xenopus oocyte model | Yes | Yes | No | No | No | No |
| Blended phenotype? | No | No | NR | No | No | NR |
| Genetic test | Trio exome sequencing | NR | NR | Singleton exome sequencing | Trio exome | Exome |
| Genomic position and variant, (GRCh38), NC_000023.10 | A13 | A14 | A15 | A16 | A17 | A18 |
|-------------------------------------------------|-----|-----|-----|-----|-----|-----|
| Exon number | 8   | 8   | 8   | 8   | 8   | 9   |
| c.DNA change, NM_001830.4(CLCN4): | c.826C>T | c.832A>C | c.832A>C | c.835C>G | c.840A>T | c.848G>A |
| Protein change, NP_001821 | p.(Leu276Phe) | p.(Ser278Arg) | p.(Ser278Arg) | p.(Leu279Val) | p.(Glu280Asp) | p.(Ser283Asn) |
| Protein domain* for missense variants | Helix H, intramembrane | Helix H, intramembrane | Helix H, intramembrane | Helix H, intramembrane | Helix H, intramembrane | Loop H-I, intracellular |
| Source: This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | This study; ClinVAR SCV002525719 | ClinVar SCV000549940.2 | ClinVar SCV001542314.1 | This study; ClinVAR SCV002525720 | This study; ClinVAR SCV002525721 | This study; ClinVAR SCV002525722 |
| Gender of proband. Others with variant in family. | 1 affected male, mother unaffected | NR | NR | 1 affected female | 1 affected male | 1 affected female |
| Inheritance | Maternally inherited | NR | NR | De novo | De novo | De novo |
| Recurrent in unrelated families | No | Yes | Yes | No | No | No |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | VOUS | VOUS | VOUS | LP | LP | VOUS |
| SIFT (dbNSFP version 4.2); converted rankscore | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) |
| PolyPhen | Probably damaging (0.94) | Probably damaging (0.99) | Probably damaging (0.99) | Probably damaging (0.964) | Probably damaging (0.998) | Probably damaging (0.964) |
| CADD | 26.2 | 27.1 | 27.1 | 23.8 | 23.5 | 25.4 |
| REVEL | 0.945 | 0.985 | 0.985 | 0.801 | 0.888 | 0.915 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| Frequency in heterozygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Frequency in hemizygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Functional impact in Xenopus oocyte model | LOF by shift of voltage-dependence | Almost complete LOF | Almost complete LOF | LOF | LOF | LOF |
| Severe functional impact in Xenopus oocyte model | Yes | No | No | Yes | No | No |
| Blended phenotype? | No | NR | NR | No | No | No |
| Genetic test | Trio exome sequencing | NR | NR | Trio exome sequencing | Trio exome sequencing | Trio whole genome sequencing |
| Genomic position and variant, (GRCh38), NC_000023.10 | A19 | A20 | A21 | A22 | A23 | A24 |
|-----------------------------------------------------|-----|-----|-----|-----|-----|-----|
| **Families**                                        |     |     |     |     |     |     |
| Exon number                                         | 9   | 9   | 9   | 9   | 9   | 9   |
| c.DNA change, NM_001830.4 (CLCN4):                  |     |     |     |     |     |     |
| Protein change, NP_001821                           |     |     |     |     |     |     |
| Protein domain* for missense variants               |     |     |     |     |     |     |
| Source :This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER |     |     |     |     |     |     |
| Gender of proband. Others with variant in family.   |     |     |     |     |     |     |
| Inheritance                                         |     |     |     |     |     |     |
| Recurrent in unrelated families                     | Yes | Yes | No  | Yes | Yes | No  |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | VOUS| VOUS| VOUS| VOUS| VOUS| VOUS|
| SIFT (dbNSFP version 4.2); converted rankscore       |     |     |     |     |     |     |
| PolyPhen                                            |     |     |     |     |     |     |
| CADD                                                |     |     |     |     |     |     |
| REVEL                                               |     |     |     |     |     |     |
| SpliceAI                                            |     |     |     |     |     |     |
| Frequency in heterozygotes (gnomAD)                 |     |     |     |     |     |     |
| Frequency in hemizygoty (gnomAD)                    |     |     |     |     |     |     |
| Functional impact in Xenopus oocyte model           |     |     |     |     |     |     |
| Severe functional impact in Xenopus oocyte model     |     |     |     |     |     |     |
| Blended phenotype?                                  |     |     |     |     |     |     |
| Genetic test                                        |     |     |     |     |     |     |
| Families | A25 | A26 | A27 | A28 | A29 | A30 |
|----------|-----|-----|-----|-----|-----|-----|
| Genomic position and variant, (GRCh38), NC_000023.10 | X:10208322 T>C | X:10208322 T>C | X:10212527 G>A | X:10212653 G>A | X:10212653 G>A | X:10212653 G>A |
| Exon number | 9 | 9 | 10 | 10 | 10 | 10 |
| c.DNA change, NM_001830.4(CLCN4): | c.1121T>C | c.1121T>C | c.1450G>A | c.1465C>A | c.1576G>A | c.1576G>A |
| Protein change, NP_001821 | p.(Ile374Thr) | p.(Ile374Thr) | p.(Gly484Arg) | p.(Gln489Lys) | p.(Gly526Ser) | p.(Gly526Ser) |
| Protein domain* for missense variants | Helix K, intramembrane | Helix K, intramembrane | Helix N, intramembrane | Helix N, intramembrane | Helix O, intramembrane | Helix O, intramembrane |
| Source : This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/ or DECIPHER | ClinVar SCV000577573.3 | ClinVar SCV002200551.1 | LOVD#0000346105 | This study; ClinVar SCV000589760.3 | This study; ClinVar SCV000693819.1 | ClinVar SCV000942548.4 |
| Gender of proband. Others with variant in family. | NR | NR | NR | 1 affected female | 1 affected male, 1 affected brother and maternal uncle (not tested) | NR |
| Inheritance | NR | NR | NR | De novo | Maternally inherited | NR |
| Recurrent in unrelated families | Yes - but also present in gnomAD | Yes - but also present in gnomAD | No | No | Yes | Yes |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | VOUS | VOUS | LP | LP | LP | VOUS |
| SIFT (dbNSFP version 4.2); converted rankscore | Tolerated | Tolerated | Deleterious (0) | Tolerated (0.06) | Deleterious (0.03) | Deleterious (0.03) |
| PolyPhen | Benign (0.04) | Benign (0.04) | Probably damaging (0.999) | Benign (0.221) | Possibly damaging (0.73) | Possibly damaging (0.73) |
| CADD | 21.2 | 21.2 | 28.1 | 22.3 | 33 | 33 |
| REVEL | 0.733 | 0.733 | 0.975 | 0.806 | 0.913 | 0.913 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ΔS donor gain 0.54 | ΔS donor gain 0.54 |
| Frequency in heterozygotes (gnomAD) | 1.02 x 10-5 | 1.02 x 10-5 | 0 | 0 | 0 | 0 |
| Frequency in hemizygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Functional impact in Xenopus oocyte model | Reduced function | Reduced function | LOF | Reduced function | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence |
| Severe functional Impact in Xenopus oocyte model | No | No | No | No | No | No |
| Blended phenotype? | NR | NR | NR | No | No | NR |
| Genetic test | NR | NR | NR | Trio exome sequencing | Exome | NR |
| Families | A31 | A32 | A33 | A34 | A35 | A36 |
|----------|-----|-----|-----|-----|-----|-----|
| Genomic position and variant, (GRCh38), NC_000023.10 | X:10213701 G>A | X:10213710 G>A | X:10213710 G>A | X:10213734 G>C | X:10213734 G>C | X:10213738 G>A |
| Exon number | 11 | 11 | 11 | 11 | 11 | 11 |
| c.DNA change, NM_001830.4(CLCN4): | c.1597G>A | c.1606G>A | c.1606G>A | c.1630G>A | c.1630G>A | c.1633G>A |
| Protein change, NP_001821 | p.(Val533Met) | p.(Val536Met) | p.(Val536Met) | p.(Gly544Arg) | p.(Gly544Arg) | p.(Gly545Ser) |
| Protein domain* for missense variants | Helix P, intramembrane | Helix P, intramembrane | Helix P, intramembrane | Loop P-Q, intramembrane | Loop P-Q, intramembrane | Loop P-Q, intramembrane |
| Source: This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | This study; ClinVAR SCV002525727 | Hu et al., 2016; Palmer et al., 2018; ClinVar SCV000297914.2 | ClinVar SCV001847703.1 | Veeramah et al., 2013; ClinVar SCV000120005.3 | Palmer et al., 2018; ClinVar SCV000245787.1 | ClinVar SCV000570417.4 |
| Gender of proband. Others with variant in family. | 1 affected male | 7 affected males, two affected females (one severely affected) | NR | 1 affected male | 1 affected male | NR |
| Inheritance | Maternally inherited | Maternally inherited | Maternally inherited | De novo | Mosaic de novo | NR |
| Recurrent in unrelated families | No | Yes | Yes | Yes | Yes | No |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | VOUS | P | P | P | P | VOUS |
| SIFT (dbNSFP version 4.2); converted rankscore | Deleterious (0.02) | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) | Tolerated (0.17) |
| PolyPhen | Probably damaging (0.924) | Probably damaging (0.997) | Probably damaging (0.997) | Probably damaging (0.999) | Probably damaging (0.999) | Benign (0.402) |
| CADD | 26.3 | 26.8 | 26.8 | 27.1 | 27.1 | 22.8 |
| REVEL | 0.871 | 0.907 | 0.907 | 0.884 | 0.884 | 0.648 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| Frequency in heterozygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Frequency in hemizygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Functional impact in Xenopus oocyte model | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence | Almost complete LOF |
| Severe functional impact in Xenopus oocyte model | No | No | No | Yes | Yes | No |
| Blended phenotype? | No | No | No | No | NR | NR |
| Genetic test | Exome | X-chromosome exome | NR | X-chromosome exome | NR | NR |
| Genomic position and variant, (GRCh38), NC_000023.10 | A37 | A38 | A39 | A40 | A41 | A42 |
|-----------------------------------------------------|-----|-----|-----|-----|-----|-----|
| X:10181778 G>A                                      |     |     |     |     |     |     |
| X:10213749A>C                                      |     |     |     |     |     |     |
| X:10213782A>G                                      |     |     |     |     |     |     |
| X:10214008 C>G                                      |     |     |     |     |     |     |
| X:10214010 G>A                                      |     |     |     |     |     |     |
| X:10220837 C>T                                      |     |     |     |     |     |     |
| Exon number                                         | 11  | 11  | 11  | 11  | 11  | 12  |
| c.DNA change, NM_001830.4(CLCN4):                  |     |     |     |     |     |     |
| p.(Gly545Asp)                                       |     |     |     |     |     |     |
| p.(Ile549Leu)                                       |     |     |     |     |     |     |
| p.(Lys560Glu)                                       |     |     |     |     |     |     |
| p.(Pro635Arg)                                       |     |     |     |     |     |     |
| p.(Val636Met)                                       |     |     |     |     |     |     |
| Source :This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/ or DECIPHER |     |     |     |     |     |     |
| Protein domain* for missense variants               |     |     |     |     |     |     |
| Loop P-Q, intramembrane                             |     |     |     |     |     |     |
| Helix Q, transmembrane                              |     |     |     |     |     |     |
| Helix Q, transmembrane                              |     |     |     |     |     |     |
| CBS1, intracellular                                 |     |     |     |     |     |     |
| CBS1, intracellular                                 |     |     |     |     |     |     |
| CBS1, intracellular                                 |     |     |     |     |     |     |
| CBS1, intracellular                                 |     |     |     |     |     |     |
| Protein change, NP_001821                           |     |     |     |     |     |     |
| p.(Gly545Asp)                                       |     |     |     |     |     |     |
| p.(Ile549Leu)                                       |     |     |     |     |     |     |
| p.(Lys560Glu)                                       |     |     |     |     |     |     |
| p.(Pro635Arg)                                       |     |     |     |     |     |     |
| p.(Val636Met)                                       |     |     |     |     |     |     |
| Frequency in heterozygotes (gnomAD)                 | 0   | 0   | 0   | 0   | 0   | 0   |
| Frequency in hemizygotes (gnomAD)                   | 0   | 0   | 0   | 0   | 0   | 0   |
| Functional impact in Xenopus oocyte model           |     |     |     |     |     |     |
| LOF by shift of voltage-dependence                  |     |     |     |     |     |     |
| LOF by shift of voltage-dependence                  |     |     |     |     |     |     |
| LOF                                                  |     |     |     |     |     |     |
| Reduced function                                    |     |     |     |     |     |     |
| LOF by shift of voltage-dependence                  |     |     |     |     |     |     |
| Severe functional impact in Xenopus oocyte model    | Yes | Yes | No  | No  | No  | No  |
| Blended phenotype?                                  | NR  | NR  | NR  | NR  | Exome sequencing | Targeted MPS gene panel |
| Genetic test                                        | NR  | NR  | NR  | NR  | Exome sequencing | Targeted MPS gene panel |
| Gender of proband. Others with variant in family.   | NR  | NR  | NR  | 1 affected female, mother mildly affected | 1 affected female, mother unaffected | 1 affected female |
| Inheritance                                         | NR  | NR  | NR  | Maternally inherited. De novo in mother | Maternally inherited | De novo |
| Recurrent in unrelated families                     | No  | No  | No  | No  | No  | Yes |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | LP  | VOUS | VOUS | VOUS | VOUS |
| SIFT (dbNSFP version 4.2): converted rankscore       | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0.02) | Deleterious (0.01) | Deleterious (0) |
| PolyPhen                                           | Possibly damaging (0.877) | Benign (0.17) | Probably damaging (0.993) | Probably damaging (0.998) | Probably damaging (0.914) | Probably damaging (0.999) |
| CADD                                                | 25.7 | 23.4 | 26.8 | 24.9 | 26.2 | 26 |
| REVEL                                               | 0.828 | 0.675 | 0.962 | 0.954 | 0.9 | 0.874 |
| SpliceAI                                            | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| Exome sequencing                                    |     |     |     |     |     |     |
| Targeted MPS gene panel                             |     |     |     |     |     |     |
| Families | A43 | A44 | A45 | A46 | A47 | A48 |
|----------|-----|-----|-----|-----|-----|-----|
| Genomic position and variant, (GRCh38), NC_000023.10 | X:10220837 C>T | X:10220837 C>T | X:10220837 C>T | X:10220837 C>T | X:10220837 C>T | X:10220837 C>T |
| Exon number | 12 | 12 | 12 | 12 | 12 | 12 |
| c.DNA change, NM_001830.4 (CLCN4): | c.2152C>T | c.2152C>T | c.2152C>T | c.2152C>T | c.2152C>T | c.2152C>T |
| Protein change, NP_001821 | p.(Arg718Trp) | p.(Arg718Trp) | p.(Arg718Trp) | p.(Arg718Trp) | p.(Arg718Trp) | p.(Arg718Trp) |
| Protein domain* for missense variants | CBS2, intracellular | CBS2, intracellular | CBS2, intracellular | CBS2, intracellular | CBS2, intracellular | CBS2, intracellular |
| Source: This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/ or DECIPHER | Palmer et al., 2018; ClinVar SCV00245785.1 | He et al., 2021 | Zhou et al., 2018 | ClinVar SCV002069088.1 | ClinVar SCV002058687.1 | ClinVar SCV001976771.1 |
| Gender of proband. Others with variant in family. | 1 affected female | 1 affected male, unaffected mother | 1 affected male | NR | 1 affected female | NR |
| Inheritance | De novo | Maternal (n.b. mother has a karyotype 47,XXX/46,XX) | De novo | NR | NR | NR |
| Recurrent in unrelated families | Yes | Yes | Yes | Yes | Yes | Yes |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | P | P | P | P | P | P |
| SIFT (dbNSFP version 4.2); converted rankscore | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) |
| PolyPhen | Probably damaging (0.999) | Probably damaging (0.999) | Probably damaging (0.999) | Probably damaging (0.999) | Probably damaging (0.999) | Probably damaging (0.999) |
| CADD | 26 | 26 | 26 | 26 | 26 | 26 |
| REVEL | 0.874 | 0.874 | 0.874 | 0.874 | 0.874 | 0.874 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| Frequency in heterozygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Frequency in hemizygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Functional impact in Xenopus oocyte model | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence | LOF by shift of voltage-dependence |
| Severe functional impact in Xenopus oocyte model | No | No | No | No | No | No |
| Blended phenotype? | No | No | No | No | No | No |
| Genetic test | WGS | Trio exome | Targeted MPS gene panel | NR | NR | NR |
| Families | Genomic position and variant, (GRCh38), NC_000023.10 | Exon number | c.DNA change, NM_001830.4(CLCN4): | Protein change, NP_001821 | Protein domain* for missense variants | Source :This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | Gender of proband. Others with variant in family. | Inheritance | Recurrent in unrelated families | Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | SIFT (dbNSFP version 4.2); converted rankscore | PolyPhen | CADD | REVEL | SpliceAI | Frequency in heterozygotes (gnomAD) | Frequency in hemizygotes (gnomAD) | Functional impact in Xenopus oocyte model | Severe functional impact in Xenopus oocyte model | Blended phenotype? | Genetic test |
|----------|---------------------------------------------------|-------------|---------------------------------|--------------------------|-------------------------------------|--------------------------------------------------------------------------------|------------------------------------------------|----------------|------------------|-------------------------------------------------|---------------------------------|----------------|----------|----------|-----------|-----------------|-----------------|----------------|-----------------|------------------|-----------------|-----------------|
|          | A49: X:10220837 C>T A50: X:10220876 G>A A51: X:10220877 G>T B1: X:10194931 G>A B2: X:10194931 G>A B3: X:10206737 T>G | 12          | c.2152C>T                      | p.(Arg718Trp)            | CBS2, intracellular                 | ClinVar SCV000957439.2 Palmer et al., 2018; ClinVar SCV001582304.2 This study; ClinVar SCV002525730 This study; ClinVar SCV000569027.4 ClinVar SCV001468990.1 This study; ClinVar SCV000589740.2 | NR 3 affected males 1 affected male 1 affected female NR 1 affected female | De novo Maternally inherited Maternally inherited De novo NR De novo | Yes No No Yes Yes No | Yes No No NR No No | Deleterious (0) Deleterious (0.03) Deleterious (0) Deleterious (0) Deleterious (0) Deleterious (0) | Probable damaging (0.999) Possibly damaging (0.798) Possibly damaging (0.993) Probably damaging (0.918) Probably damaging (0.918) Probably damaging (0.996) | ≤ 0.2 ≤ 0.2 ≤ 0.2 ≤ 0.2 ≤ 0.2 ≤ 0.2 | 0 0 0 0 0 0 | 0 0 0 0 0 | LOF by shift of voltage-dependence LOF LOF GOF GOF GOF | No No No NR No No | No No No NR Exome sequencing |
| Families | B4 | B5 | B6 | B7 | B8 | B9 |
|----------|----|----|----|----|----|----|
| Genomic position and variant, (GRCh38), NC_000023.10 | X:10208129 C>T | X:10208150 G>A | X:10208150 G>A | X:10208150 G>T | X:10208386 C>G |
| Exon number | 9 | 9 | 9 | 9 | 9 |
| c.DNA change, NM_001830.4(CLCN4): | c.928C>T | c.949G>A | c.949G>A | c.949G>A | c.949G>T | c.1185C>G |
| Protein change, NP_001821 | p.(Pro310Ser) | p.(Val317Ile) | p.(Val317Ile) | p.(Val317Ile) | p.(Val317Phe) | p.(Ser395Arg) |
| Protein domain* for missense variants | Loop I-J, extracellular | Loop I-J, extracellular | Loop I-J, extracellular | Loop I-J, extracellular | Loop I-J, extracellular | Loop K-L, intramembrane |
| Source:This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | This study; ClinVar SCV002525731 | This study; ClinVar SCV001437777.1 | This study; ClinVar SCV000572387.4 | This study, DECIPHER Patient 279296 | ClinVar SCV000621815.2 | This study; ClinVar SCV002525733 |
| Gender of proband. Others with variant in family. | 1 affected female | 1 affected male | 1 affected male, mother mildly affected | 1 affected male | NR | 1 affected female |
| Inheritance | De novo | De novo | Maternally inherited. Mosaic in mother | De novo | NR | De novo |
| Recurrent in unrelated families | No | Yes | Yes | Yes | No | No |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | VOUS | VOUS | VOUS | LP | VOUS | VOUS |
| SIFT (dbNSFP version 4.2); converted rankscore | Deleterious (0) | Tolerated (0.15) | Tolerated (0.15) | Tolerated (0.15) | Deleterious (0) | Deleterious (0) |
| PolyPhen | Possibly damaging (0.883) | Possibly damaging (0.612) | Possibly damaging (0.612) | Possibly damaging (0.612) | Probably damaging (0.993) | Probably damaging (0.933) |
| CADD | 24.5 | 22.8 | 22.8 | 22.8 | 25 | 14.46 |
| REVEL | 0.931 | 0.525 | 0.525 | 0.525 | 0.924 | 0.732 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| Frequency in heterozygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Frequency in hemizygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Functional impact in Xenopus oocyte model | GOF | GOF | GOF | GOF | GOF | Reduced outward currents, and slight GOF |
| Severe functional impact in Xenopus oocyte model | No | No | No | No | No | No |
| Blended phenotype? | No | No | No | No | NR | No |
| Genetic test | Exome sequencing | Singleton exome sequencing | Duo exome sequencing (proband and mother) | DDD project (whole genome sequencing) | NR | Targeted MPS gene panel |
| Families | B10 | B11 | B12 | B13 | B14 | B15 |
|----------|-----|-----|-----|-----|-----|-----|
| Genomic position and variant, (GRCh38), NC_000023.10 | X:10213750 T>A | X:10213752 G>C | X:10213768 C>T | X:10213768 C>T | X:10213768 C>T | X:10213768 C>T |
| Exon number | 11 | 11 | 11 | 11 | 11 | 11 |
| c.DNA change, NM_001830.4(CLCN4); | c.1646T>A | c.1646G>C | c.1664C>T | c.1664C>T | c.1664C>T | c.1664G>C |
| Protein change, NP_001821 | p.(Ile549Asn) | p.(Val550Leu) | p.(Ala555Val) | p.(Ala555Val) | p.(Ala555Val) | p.(Ala555Val) |
| Protein domain* for missense variants | Helix Q, transmembrane | Helix Q, transmembrane | Helix Q, transmembrane | Helix Q, transmembrane | Helix Q, transmembrane | Helix Q, transmembrane |
| Source :This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | This study; ClinVar SCV002525734 | This study; ClinVar SCV002525735 | Palmer et al., 2018; ClinVar SCV000245784.1 | This study; ClinVar SCV002525736 | This study; ClinVar SCV000511380.1 | This study; ClinVar SCV000490472.1 |
| Gender of proband. Others with variant in family. | 1 affected female | 1 affected female | 1 affected female | 1 affected female | 1 affected female | 1 affected female |
| Inheritance | De novo | De novo | De novo | De novo | De novo | De novo |
| Recurrent in unrelated families | No | No | Yes | Yes | Yes | Yes |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | VOUS | VOUS | P | P | P | P |
| SIFT (dbNSFP version 4.2); converted rankscore | Deleterious (0) | Tolerated (0.29) | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) |
| PolyPhen | Probably damaging (0.964) | Benign (0.279) | Possibly damaging (0.627) | Possibly damaging (0.627) | Possibly damaging (0.627) | Possibly damaging (0.627) |
| CADD | 26.3 | 21.7 | 23.1 | 23.1 | 23.1 | 23.1 |
| REVEL | 0.928 | 0.694 | 0.734 | 0.734 | 0.73 | 0.734 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| Frequency in heterozygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Frequency in hemizygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| Functional impact in Xenopus oocyte model | GOF | GOF | GOF | GOF | GOF | GOF |
| Severe functional impact in Xenopus oocyte model | No | No | No | No | No | No |
| Blended phenotype? | No | No | No | No | No | No |
| Genetic test | Trio exome sequencing | Targeted MPS gene panel (intellectual disability) | Trio exome sequencing | Targeted MPS gene panel (intellectual disability) | Targeted MPS gene panel (epilepsy) | Trio exome sequencing |

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| Families | Genomic position and variant, (GRCh38), NC_000023.10 | GROUP C: Missense variants LOF: blended phenotype |
|----------|----------------------------------------------------|--------------------------------------------------|
| gen     | B16  | B17  | C1   | C2   | C3   | C4   |
| exon     | 11   | 11   | 3    | 4    | 9    | 9    |
| c.DNA change, NM_001830.4(CLCN4): | c.1664C>T | c.1664C>T | c.100G>A | c.206C>T | c.1106C>T | c.1106C>T |
| protein change, NP_001821 | p.(Ala555Val) | p.(Ala555Val) | p.(Asp34Asn) | p.(Ser69Leu) | p.(Pro369Leu) | p.(Pro369Leu) |
| protein domain* for missense variants | Helix Q, transmembrane | Helix Q, transmembrane | N term, intracellular | N term, intracellular | Helix K, intramembrane | Helix K, intramembrane |
| source: This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | ClinVar SCV000740693.2 | ClinVar SCV002242006.1 | This Study: DECIPHER Patient 277726; ClinVar SCV002525737 | This study: ClinVar SCV002525738 | This study: ClinVar SCV002525739 | ClinVar SCV001503010.2 |
| gender of proband. Others with variant in family. | NR | NR | 1 affected male, mother and sister unaffected neurodevelopmentally | 1 affected male, mother unaffected neurodevelopmentally | 1 affected male | NR |
| maternally inherited. | NR | NR | Maternally inherited. | Maternally inherited | De novo | NR |
| recurrent in unrelated families | Yes | Yes | No | No | Yes | Yes |
| assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | VOUS | P | VOUS | VOUS | VOUS | VOUS |
| SIFT (dbNSFP version 4.2); converted rankscore | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) | Deleterious (0) |
| polyphen | Possibly damaging (0.627) | Possibly damaging (0.627) | Probably damaging (1) | Benign (0.332) | Probably damaging (0.925) | Probably damaging (0.925) |
| CADD | 23.1 | 23.1 | 27.7 | 24.2 | 25.2 | 25.2 |
| REVEL | 0.734 | 0.734 | 0.778 | 0.869 | 0.896 | 0.896 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| frequency in heterozygotes (gnomAD) | 0 | 0 | 1 | 0 | 0 | 0 |
| frequency in hemizygotes (gnomAD) | 0 | 0 | 0 | 0 | 0 | 0 |
| functional impact in Xenopus oocyte model | GOF | GOF | LOF | Almost complete LOF | LOF | LOF |
| severe functional impact in Xenopus oocyte model | No | No | No | No | No | No |
| blended phenotype? | No | No | Yes with Desbuquois syndrome | Yes with SOX11-related condition | Yes a clinical diagnosis of Donnai-Barrow syndrome | NR |
| genetic test | NR | NR | DDD project (WGS) | Trio exome sequencing | Trio whole genome sequencing | NR |
### GROUP D: Functional studies like wild type

| Families | Genomic position and variant, (GRCh38), NC_000023.10 | Exon number | c.DNA change, NM_001830.4(CLCN4): | Protein change, NP_001821 | Protein domain* for missense variants | Source :This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/ or DECIPHER | Gender of proband. Others with variant in family. | Inheritance | Recurrent in unrelated families | Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | SIFT (dbNSFP version 4.2); converted rankscore | PolyPhen | CADD | REVEL | SpliceAI | Frequency in heterozygotes (gnomAD) | Frequency in hemizygotes (gnomAD) | Functional impact in Xenopus oocyte model | Severe functional impact in Xenopus oocyte model | Blended phenotype? | Genetic test |
|----------|---------------------------------|-------------|---------------------------------|--------------------------|---------------------------------|-------------------------------------------------------------------------------------------------|---------------------------------|-------------|----------------|-------------------------------------------------|--------------------------|----------------|--------|------|--------|----------------|----------------|------------------------|------------------------|------------------|----------------|
|          | X:10185119 C>G                  | 3           | c.87C>G                          | p.(Asp29Glu)             | N-term                          | This study; ClinVar SCV002525740; ClinVar SCV000549937.4                                    | 2 affected males, 1 mildly affected female | Maternally inherited  | No          | VOUS                                      | Deleterious (0.04)           | Probably damaging (0.983) | 23.9 | 0.852 | ≤ 0.2 | 0              | 0              | WT                  | No                     | No               | No |
|          | X:10194980 C>G                  | 5           | c.314C>G                          | p.(Ser105Cys)            | Loop B-C, extracellular         | ClinVar SCV002003533.1; ClinVar SCV000570777.4; This study; ClinVar SCV001480412.1          | NR                              | NR           | Yes - but also present in gnomAD | VOUS                       | Tolerated (1) | 22.6 | 0.494 | ≤ 0.2 | 1              | 0              | WT                  | No                     | No               | No |
|          | X:10194980 C>G                  | 5           | c.712T>C                          | p.(Phe238Leu)            | Loop B-C, extracellular         | This study; ClinVar SCV001480412.1                                                          | NR                              | NR           | Yes - but also present in gnomAD | VOUS                       | Tolerated (1) | 22.6 | 0.494 | ≤ 0.2 | 1              | 0              | WT                  | No                     | No               | No |
|          | X:10206514 T>C                  | 7           | c.944G>A                          | p.(Arg315His)            | Helix F, intramembrane          |                                                                                             | NR                              | NR           | No - but also present in gnomAD | VOUS                       | Tolerated (1) | 20.9 | 0.583 | ≤ 0.2 | 1              | 0              | WT                  | No                     | No               | No |
|          | X:10208145 G>A                  | 9           | c.944G>A                          | p.(Arg315His)            | Loop I-J, extracellular         |                                                                                             | NR                              | NR           | No - but also present in gnomAD | VOUS                       | Tolerated (1) | 20.5 | 0.575 | ≤ 0.2 | 1              | 0              | WT                  | No                     | No               | No |
|          | X:10208145 G>A                  | 9           | c.944G>A                          | p.(Arg315His)            | Loop I-J, extracellular         |                                                                                             | NR                              | NR           | No - but also present in gnomAD | VOUS                       | Tolerated (1) | 20.5 | 0.575 | ≤ 0.2 | 1              | 0              | WT                  | No                     | No               | No |
| Families | **D7** | **D8** | **D9** | **D10** | **D11** | **D12** |
|----------|--------|--------|--------|--------|--------|--------|
| Genomic position and variant, (GRCh38), NC_000023.10 | X:10208145 G>A | X:10208291 A>G | X:10208496 G>A | X:10213966 A>G | X:10213990 C>T | X:10214041 T>C |
| Exon number | 9 | 9 | 9 | 11 | 11 | 11 |
| c.DNA change, NM_001830.4(CLCN4): | c.944G>A | c.1090A>G | c.1295G>A | c.1862A>G | c.1886C>T | c.1937T>C |
| Protein change, NP_001821 | p.(Arg315His) | p.(Arg364Gly) | p.(Arg432Gln) | p.(Asp621Gly) | p.(Thr629Ile) | p.(Ile646Thr) |
| Protein domain* for missense variants | Loop I-J, extracellular | Loop J-K, intracellular | Loop L-M, extracellular | CBS1, intracellular | CBS1, intracellular | CBS1, intracellular |
| Source: This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | ClinVar SCV002250535.1 | This study; ClinVar SCV000594131.1 | ClinVar SCV000620779.1 | ClinVar SCV002525742 | ClinVar SCV000741977.2 |
| Gender of proband. Others with variant in family. | NR | 1 affected male | NR | NR | 1 affected female | NR |
| Inheritance | NR | NR | NR | NR | Maternally inherited | NR |
| Recurrent in unrelated families | Yes - but also present in gnomAD | No | No | No | No | Yes - but also present in gnomAD |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | VOUS | VOUS | VOUS | VOUS | VOUS | VOUS |
| SIFT (dbNSFP version 4.2): converted rankscore | Tolerated (1) | Tolerated (0.18) | Tolerated (0.23) | Deleterious (0.05) | Deleterious (0.01) | Deleterious (0.03) |
| PolyPhen | Benign (0.01) | Benign (0.062) | Benign (0.037) | Benign (0.044) | Possibly damaging (0.851) | Benign (0.158) |
| CADD | 20.5 | 18.99 | 22.7 | 23.1 | 24.4 | 23.8 |
| REVEL | 0.575 | 0.366 | 0.576 | 0.72 | 0.888 | 0.888 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| Frequency in heterozygotes (gnomAD) | 0 | 0 | 2 | 0 | 0 | 11 |
| Frequency in hemizygotes (gnomAD) | 1 | 0 | 0 | 0 | 0 | 4 |
| Functional impact in Xenopus oocyte model | WT | WT | WT | WT | WT | WT |
| Severe functional impact in Xenopus oocyte model | No | No | No | No | No | No |
| Blended phenotype? | No | No | NR | NR | NR | NR |
| Genetic test | NR | Intellectual disability gene panel off exome backbone | NR | NR | Singleton exome sequencing | NR |
| Families | **D13** | **D14** | **D15** | **D16** | **D17** | **D18** |
|----------|---------|---------|---------|---------|---------|---------|
| Genomic position and variant, (GRCh38), NC_000023.10 | X:10214041 T>C | X:10214059 G>C | X:10214067 A>G | X:10220838 G>A | X:10220838 G>A | X:10220838 G>A |
| Exon number | 11 | 11 | 11 | 12 | 12 | 12 |
| c.DNA change, NM_001830.4 (CLCN4): | c.1937T>C | c.1955G>C | c.1963A>G | c.2153G>A | c.2153G>A | c.2153G>A |
| Protein change, NP_001821 | p.(Ile646Thr) | p.(Arg652Thr) | p.(Ile655Val) | p.(Arg718Gln) | p.(Arg718Gln) | p.(Arg718Gln) |
| Protein domain* for missense variants | CBS1, intracellular | CBS1, intracellular | CBS1, intracellular | CBS2, intracellular | CBS2, intracellular | CBS2, intracellular |
| Source:This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | | | | | | |
| Gender of proband. Others with variant in family. | NR | NR | NR | NR | 1 affected male | NR |
| Inheritance | NR | NR | NR | NR | Maternally inherited | NR |
| Recurrent in unrelated families | Yes - but also present in gnomAD | No | No | Yes - but also present in gnomAD | Yes - but also present in gnomAD | Yes - but also present in gnomAD |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | Benign | VOUS | VOUS | VOUS | LP | VOUS |
| SIFT (dbNSFP version 4.2); converted rankscore | Deleterious (0.03) | Deleterious (0.04) | Tolerated (0.37) | Tolerated (0.09) | Tolerated (0.09) | Tolerated (0.09) |
| PolyPhen | Benign (0.158) | Benign (0.224) | Benign (0) | Possibly damaging (0.658) | Possibly damaging (0.658) | Possibly damaging (0.658) |
| CADD | 23.8 | 23.7 | 17.72 | 25.2 | 25.2 | 25.2 |
| REVEL | 0.868 | 0.857 | 0.271 | 0.779 | 0.779 | 0.779 |
| SpliceAI | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 | ≤ 0.2 |
| Frequency in heterozygotes (gnomAD) | 11 | 0 | 1 | 3 | 3 | 3 |
| Frequency in hemizygotes (gnomAD) | 4 | 0 | 0 | 2 | 2 | 2 |
| Functional impact in Xenopus oocyte model | WT | WT | WT | WT | WT | WT |
| Severe functional impact in Xenopus oocyte model | No | No | No | No | No | No |
| Blended phenotype? | NR | NR | NR | NR | NR | NR |
| Genetic test | NR | NR | NR | NR | NR | NR |
| Families | E1 | E2 | E3 |
|----------|----|----|----|
| Genomic position and variant, (GRCh38), NC_000023.10 | X:10208122 CATCA>C | X:10220667 CCAGA>C | X:10220710 C>G |
| Exon number | 9 | 9 | 9 |
| c.DNA change, NM_001830.4(CLCN4): | c.925_928del | c.1987_1990del | c.2025C>G |
| Protein change, NP_001821 | p.(Asn309Profs) | p.(Gln663Glyfs) | p.(Tyr675Ter) |
| Protein domain* for missense variants | FRAMESHIFT | FRAMESHIFT | NONSENSE |
| Source: This study = families previously unreported in the medical literature with detailed clinical data; ClinVAR; LOVD and/or DECIPHER | This study; ClinVar SCV002525743 | This study; ClinVar SCV002525744 | This study; ClinVar SCV002525745 |
| Gender of proband. Others with variant in family. | 1 affected male, mother unaffected | 1 affected male, 1 affected maternal uncle, mother unaffected | 1 affected male |
| Inheritance | Maternally inherited | Maternally inherited. De novo |
| Recurrent in unrelated families | No | No | No |
| Assessment of pathogenicity according to ACMG criteria on ClinVAR, or as assessed by authors using Varsome | P | LP | P |
| SIFT (dbNSFP version 4.2); converted rankscore | NA-frameshift | NA-frameshift | NA-nonsense |
| PolyPhen | NA-frameshift | NA-frameshift | NA-nonsense |
| CADD | NA-frameshift | NA-frameshift | ≤ 0.2 |
| REVEL | NA-frameshift | NA-frameshift | NA-nonsense |
| SpliceAI | NA-frameshift | NA-frameshift | ≤ 0.2 |
| Frequency in heterozygotes (gnomAD) | 0 | 0 | 0 |
| Frequency in hemizygotes (gnomAD) | 0 | 0 | 0 |
| Functional impact in Xenopus oocyte model | Frameshift | Frameshift | Nonsense |
| Severe functional impact in Xenopus oocyte model | Not tested | Not tested | Not tested |
| Blended phenotype? | No | No | No |
| Genetic test | Intellectual disability gene panel of exome backbone | Singleton whole genome sequencing | Trio exome sequencing |

Data presented include genomic coordinates, reporting laboratory assessment of pathogenicity using ACMG criteria as reported in ClinVar or determined by the authors using VARSOME prior to functional studies were conducted, demographic details, inheritance, recurrence within families, recurrence across families, including public databases as of 25th May 2022, selected in silico pathogenicity scores, frequency in gnomAD database, functional impact in Xenopus oocyte model, and if the individual has more than one genetic diagnosis (blended phenotype). Data which are supportive of pathogenicity is color-coded orange (with darker orange for most supportive data), data which are not supportive of pathogenicity are coded green. ACMG American College of Medical Genetics and Genomics, CBS cystathionine β-synthase, NA not applicable, NR not reported; N-term N terminus, GOF gain of function, LOF loss-of-function, LOVD Leiden Open Variation Database, MPS massively parallel sequencing, WT wild type. In silico scores include PolyPhen, CADD, REVEL and SpliceAI.
extracellular pH (Fig. 4E–G). While outward currents of WT ClC-4 were slightly inhibited at acidic pH and inward currents remained undetectable, comparably large inward currents became visible at pH 6.3 and 5.3 for variant p.(Ile549Asn) (Fig. 4D, E). A quantitative analysis revealed that similarly large inward currents were seen for variants p.(Phe268Leu) and p.(Ala555Val) (Fig. 4B, C). Smaller, but highly significant inward currents were also detected for variants p.(Asp89Asn), p.(Pro310Ser), p.(Val317Ile), p.(Val317Phe), p.(Ser395Arg), and p.(Ala550Leu) (Fig. 4F, G and Supplementary Fig. 1). Evidently, these variants partially disrupted the gating process of ClC-4 that normally prevents inward currents even at very acidic pH. For variants p.(Phe268Leu) and p.(Ile549Asn) inward currents were large enough to estimate reversal potentials at pH 6.3 and pH 5.3. The fact that the reversal potential in these conditions differed by about 12.5 mV for both variants (Fig. 4H) demonstrates that the inward currents carried by these variants are at least partially mediated by H\(^+\) transport. However, the difference falls short of the expected value of ~20 mV for a coupled 2Cl\(^-\)/1H\(^+\) antiporter [32], suggesting that currents mediated by the variants are at least partially uncoupled. More detailed studies will however be needed to determine precise transport stoichiometry for these variants as well as for WT ClC-4.

Group C consisted of families with variants p.(Asp34Asn), p.(Ser69Leu) and p.(Pro369Leu). Although these variants all showed a functional LOF similar to those in Group A (Supplementary Fig. 1), the affected individuals had more complex clinical presentations (Supplementary Fig. 3) and an additional genetic condition was proven or strongly suspected, consistent with a blended phenotype. Consequently, they were separated from the other groups, and not included in the clinical summary in Table 2.
Fig. 2  Clinical photographs of individuals with previously unreported variants in CLCN4, and representative neuroimaging. A Clinical photographs demonstrate that some males and females have progressive lengthening of their face and ‘squaring’ of the jaw with age. LOF loss-of-function, GOF gain-of-function, ROF reduction of function, m months, y years. B Neuroimaging (T1 mid-sagittal view) from affected probands. In all individuals there are abnormalities of the corpus callosum. The proband of Family A10 has a dysplastic corpus callosum: it is of normal length but globally hypoplastic. Family A19: two affected brothers both display complete agenesis of the corpus callosum (affecting the posterior part and splenium), colpocephaly and mild dilatation of the 3rd ventricle. Family B6: the proband has a dysplastic corpus callosum, and mildly small optic chiasm and optic nerves bilaterally.
Fig. 3  Mapping of all CLCN4 variants functionally investigated in this study. A Schematic of the CLCN4 gene and ClC4-protein with position of variants from newly identified families with clearly affected males and females depicted above the schematic, and position of variants published to date shown below the schematic. B Position of the investigated missense variants in a CLC topology model. Altered residues are shown as circles and functional effects are color-coded as indicated in the figure. C Three-dimensional homology model of the human ClC-4 protein based on the structure of the CmClC homodimer (Protein Data Bank: 3ORG). The view from within the membrane delimited by dashed lines. The two subunits forming the homodimer are shown in dark and light grey. Mutated residues are shown as spheres colored as in B. Right 3D model viewed from the extracellular site.
Group D consisted of 18 families with rare missense variants with supportive in silico pathogenicity scores and/or clinical features suggestive of CLCN4-related condition, but for which no functional impact in the Xenopus expression system could be demonstrated. This group included variants p.(Asp29Glu), p.(Ser105Cys), p.(Phe238Leu), p.(Arg315His), p.(Arg364Gly), p.(Arg432Gln), as well as variants located in the intracellular CBS1 and CBS2 domains: the variants p.(Asp621Gly), p.(Thr629Ile), p.(Ile646Thr), p.(Arg652Thr), p.(Ile655Val), and p.(Arg718Gln) (Table 1, Supplementary Fig. 1). It is plausible that these variants...
are pathogenic by a mechanism not modelled in our cellular system, but given the lack of evidence on their pathogenicity, the families with Group D variants were not included in the summary Table 2.

Group E consisted of three individuals with a frameshift or nonsense variant in CLCN4 for whom detailed clinical data were available.

This study thus brings the total number of individuals with (likely) pathogenic variants in CLCN4 to a total of 122: 58 males and 64 females. For 20 of the females, parental studies demonstrated the variant to be de novo, while the other 44 females were identified as being heterozygous for a CLCN4 variant only after a relative (usually a son, but on two occasions a daughter) was identified in their family to have CLCN4-related condition [1, 2, 5, 7, 11, 31]. The clinical features of this expanded cohort are summarized in Table 2.

DISCUSSION

Our study addresses the interpretation of novel missense variants, a common clinical conundrum across clinical genetic practice [33]. We robustly demonstrate a much wider range of functional impacts of CLCN4 variants in the Xenopus oocyte model than had been previously demonstrated. In addition, we provide new insights into the common clinical features of CLCN4-related neurodevelopmental condition, which have enabled us to provide updated clinical management advice to clinicians [9], and improved patient and family education via the patient advocacy group CureCLCN4.

We confirm that cognitive disability is the most common clinical feature in males, most commonly in the moderate or severe/ profound range (Table 2 and Supplementary Table 1). For the first time, however, we report a male with a verbal IQ in the normal range. This 12-year-old male (Family A21; p.(Phe319Ser)mat; functional studies: LOF by shifted voltage dependence) had a verbal IQ of 90 on formal psychometric testing (WISC-II-NL) but did have a lower performance IQ (61: within the mild ID range) and significant comorbidities with delayed language acquisition, articulation difficulties, severe treatment-resistant epilepsy, autistic features, and hyperactivity. We also report the first observation of a male with CLCN4-related condition and mild ID who has had a family (Family A3; p.(Val92Met); functional studies: almost complete LOF). He has two daughters who are obligate heterozygotes, one with mild ID and the other with specific learning disabilities.

Phenotypic prediction of cognitive function in females with CLCN4-related condition is very difficult with a wide spectrum of severity of neurodevelopmental and medical issues, including about half of heterozygous female carriers being apparently completely unaffected (Table 2). In general, females with a de novo variant had a more severe phenotype than those with an inherited variant. However, this observation is far from absolute, as evidenced by several female individuals in the cohort. For example, in families A10 and A24, the mother of a severely affected male had a de novo variant and yet was completely unaffected. On the other hand, we report females with inherited variants who have severe phenotypes: for example, the proband in Family A40 had moderate ID and a missense variant (p.(Pro635Arg); functional studies: LOF), which she inherited from a mother with mild ID. The proband had no additional genetic condition identified by WGS, and there was no evidence of mosaicism in the unaffected mother. We have previously reported that X-inactivation status does not correspond to clinical severity [5], and, as demonstrated across this and previous studies [8, 31], female-to-female inheritance from a very mildly, or even apparently non-affected mother does not ensure a mild phenotype in the daughter. Clinically, were a de novo missense CLCN4 variant to be detected on a prenatal exome in a female embryo, there would remain a degree of uncertainty whether there would be a neurodevelopmental phenotype postnatally. From our previous study, it was apparent that females with a CLCN4 frameshift or nonsense variant or a small intragenic chromosomal deletion of CLCN4 are typically unaffected [5]. This was confirmed in the current study: both female carriers in the two families with inherited truncating variants in Group E were unaffected (Family E1 and E2). This observation may signify that the impact of missense variants in females could lead to a toxic gain-of-function or a LOF that could be at least partially imparted also on ClC-3/ClC-4 heterodimers.

Behavioral and mental health disorders are the next most common clinical features. The four most common conditions were attention deficit hyperactivity disorder (ADHD) or significant hyperactivity, impulsiveness, or restlessness affecting 59% of all males and 46.7% of females with de novo variants; autism spectrum disorder (or autistic behavior) affecting 54.5% of all males and 40% of females with de novo variants; angry outbursts or challenging behaviors, affecting 36.4% of males and 26.7% of females, and lastly anxiety, affecting 27.2% of all males, 53% of females with de novo variants and 10.5% of females with inherited variants or variants with unknown inheritance. The mental health conditions were reported to significantly impact the affected individual's ability to learn and their quality of life. Less frequent mental health disorders included obsessive compulsive disorder and depression/bipolar disorder, which commonly had onset in late teenanoe years or early adulthood and caused a significant deterioration in quality of life. This highlights the need for close monitoring of all individuals for psychiatric complications, with appropriate referral to a psychiatrist skilled in the management of individuals with neurodevelopmental conditions.

Epilepsy is also confirmed as a significant feature of CLCN4-related neurodevelopmental condition, affecting 59% of all males and 20% of females. Most individuals with epilepsy had seizure onset within the first three years of life, although two were diagnosed at age 13, highlighting the need for ongoing seizure surveillance beyond childhood. Seizure semiologies were broad, including generalized absence and tonic-clonic seizures and focal onset seizures, as evidenced by EEG showing focal onset in some and generalized onset in others. Epilepsy can be severe, consistent
Table 2. Summarized clinical features, presented with HPO (Human Phenotype Ontology) nomenclature, of all individuals with a CLCN4-related neurodevelopmental condition from this study and from previous reports (in the case that detailed clinical data were available).

| Feature                                      | HPO term          | Males and females total | This study | Previously informative/ positive | Previously positive/ total | This study | Previously informative/ positive | Previously informative/ total | This study | Previously informative/ positive | Previously informative/ total |
|----------------------------------------------|-------------------|-------------------------|-----------|---------------------------------|---------------------------|-----------|---------------------------------|-----------------------------|-----------|---------------------------------|-----------------------------|
| Neurodevelopment                             |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Borderline intellectual disability           |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Mild intellectual disability                 |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Moderate intellectual disability             |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Specific learning disability                 |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Treatment-resistant epilepsy                 |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Progressive neurological abnormalities       |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Abnormality of white matter (e.g., white matter hyperintensities/periventricular leukomalacia/delayed or abnormal myelination) |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Cerebral and/or cerebellar atrophy          |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Other abnormality of the brain, e.g., Cortical dysplasia/sclerosis/cortical atrophy |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Anxiety                                      |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Depression/bipolar disorder                  |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Obsessive and/or compulsive behaviors        |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Attention deficit/hyperactivity/impulsivity  |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Other abnormality of the brain, e.g., Cortical dysplasia/sclerosis/cortical atrophy |                   |                         |           |                                 |                           |           |                                 |                             |           |                                 |                             |
| Feature                          | HPO term                  | Males | Females | Males and females total | All variants | De novo variants | Inherited or inheritance unknown variants | All variants | Total positive/total informative | Previously reported positive/total informative | This study positive informative/total informative |
|---------------------------------|---------------------------|-------|---------|-------------------------|--------------|------------------|------------------------------------------|--------------|------------------------------------|-----------------------------------------------|-----------------------------------------------|
| **Psychotic disorder**          | HP:0000709                | 0/36 (0%) | 1/22 (4.5%) | 1/58 (1.7%) | 0/5 (0%) | 0/15 (0%) | 0/20 (0%) | 0/19 (0%) | 0/37 (0%) | 0/57 (0%) | 2/115 (1.7%) |
| **Anger outbursts/ aggressive behavior** | HP:0000718                | 8/36 (22.2%) | 8/22 (36.4%) | 16/58 (27.6%) | 2/5 (40%) | 4/15 (26.7%) | 6/20 (30%) | 0/19 (0%) | 1/19 (5.3%) | 1/37 (2.7%) | 7/57 (12.3%) |
| **Gastrointestinal and growth** |                           |       |         |                         |              |                 |                                          |              |                                    |                                               |                                              |
| Gastroesophageal reflux         | HP:0002020                | 1/36* (2.8%) | 8/22 (36.4%) | 9/58 (15.5%) | 0/5 (0%) | 5/15 (33.3%) | 5/20 (25%) | 0/19 (0%) | 1/19 (5.3%) | 1/37 (2.7%) | 6/57 (10.5%) |
| Constipation                    | HP:0002019                | 0/36 (0%) | 8/22 (36.4%) | 8/58 (13.8%) | 1/5 (20%) | 6/15 (40%) | 7/20 (35%) | 0/19 (0%) | 1/19 (5.3%) | 1/37 (2.7%) | 8/57 (14%) |
| Feeding difficulties            | HP:0011968                | 2/36 (5.6%) | 8/22 (36.4%) | 10/58 (17.2%) | 3/5 (60%) | 8/15 (53.3%) | 11/20 (55%) | 0/19 (0%) | 3/19 (15.8%) | 3/37 (8.1%) | 14/57 (24.6%) |
| Secondary microcephaly          | HP:0005484                | 5/28 (17.8%) | 4/20 (20%) | 9/48 (18.7%) | 2/5 (40%) | 9/11 (81.8%) | 11/16 (68.7%) | 0/19 (0%) | 1/19 (5.3%) | 1/37 (2.7%) | 12/57 (21.3%) |
| Failure to thrive               | HP:0001508                | 2/27 (7.4%) | 4/18 (22.2%) | 6/45 (13.3%) | 0/5 (0%) | 5/13 (38.5%) | 5/18 (27.8%) | 0/19 (0%) | 1/19 (5.3%) | 1/37 (2.7%) | 6/55 (10.9%) |
| Short stature                   | HP:0003222                | 1/27 (3.7%) | 6/22 (27.2%) | 7/48 (14.2%) | 1/4 (20%) | 4/13 (30.7%) | 5/17 (29.4%) | 0/19 (0%) | 1/19 (5.3%) | 1/37 (2.7%) | 6/54 (11.1%) |
| **Other**                       |                           |       |         |                         |              |                 |                                          |              |                                    |                                               |                                              |
| Sleep disturbance               | HP:0003860                | 0/36 (0%) | 2/22 (9.1%) | 2/58 (3.4%) | 1/5 (20%) | 3/13 (23.1%) | 4/18 (22.2%) | 0/19 (0%) | 1/19 (5.3%) | 1/37 (2.7%) | 5/55 (9.1%) |
| Scoliosis/ kyphosis             | HP:00010674               | 3/36 (8.3%) | 1/22 (4.5%) | 4/58 (6.9%) | 1/5 (20%) | 2/13 (15.4%) | 1/18 (5.6%) | 0/19 (0%) | 0/19 (0%) | 0/37 (0%) | 1/55 (1.8%) |
| Other skeletal/ joint abnormalities |                           | 1/36 (2.8%) | 7/22 (31.8%) | 8/58 (13.8%) | 2/5 (40%) | 1/13 (7.7%) | 3/18 (16.7%) | 0/19 (0%) | 0/19 (0%) | 0/37 (0%) | 3/55 (5.5%) |
| Hearing impairment              | HP:00004301; HP:00004307  | 0/36 (0%) | 6/22 (27.3%) | 6/58 (10.3%) | 0/5 (0%) | 2/15 (13.3%) | 2/18 (11.1%) | 0/19 (0%) | 1/19 (5.3%) | 0/37 (0%) | 2/55 (3.6%) |
| Vision Impairment               | HP:0000866; HP:0000868; HP:0000345; HP:0000353 | 0/36 (0%) | 6/22 (27.3%) | 6/58 (10.3%) | 0/5 (0%) | 3/18 (16.7%) | 3/18 (16.7%) | 0/19 (0%) | 2/19 (10.3%) | 0/37 (0%) | 3/55 (5.5%) |

This table excludes patients with rare missense variants from Group D, for whom the functional studies were similar to wild type, and patients from Group C that had a more severe phenotype due to an additional monogenic condition.
with a developmental and epileptic encephalopathy as highlighted in recent reports [1, 11]. The severity of epilepsy, however, does not necessarily correlate with the severity of cognitive impairment. Due to \( CLCN4 \) being an antipporter of protons and chloride, which may be important in acid-base balance, acetazolamide has been trialed without any clear evidence of improvement in seizure control. Indeed, no specific anti-seizure medications have been demonstrated to best correlate with epilepsy control.

Neuroimaging showed abnormalities in 58.8% of males and 61.5% of females, most commonly of the white matter. Two brothers and one female had complete agenesis of the corpus callosum. This suggests that \( CLCN4 \) should be added to panels of genes interrogated in individuals with corpus callosus abnormalities [34, 35].

Infantile hypotonia was reported in about half of all males and of females with \textit{de novo} variants in this cohort. Progressive microcephaly was more common in females with \textit{de novo} variants (80.8%) compared to males (20%). 31.8% of males and 40% of females with \textit{de novo} variants had later onset neurological symptoms including tremor, ataxia, hyperkinesis or stereotypical movements, changes in gait such as walking with a stooped posture, or progressive spasticity.

Functional gastrointestinal symptoms, such as gastrointestinal reflux and constipation, were common in females with \textit{de novo} variants (33.3% had gastrointestinal reflux and 40% had constipation) and impacted also a significant proportion of males (36.4% had gastrointestinal reflux and 36.4% had constipation). A small proportion of individuals, particularly those with GOF variants, have a striking growth phenotype. All four females with the recurrent \textit{de novo} p.(Ala555Val) variant, for whom clinical data were available (Families B12-B15) had severe symmetrical growth restriction and feeding difficulties, two requiring gastrostomy feeds. The female proband from Family B13 was investigated by a pediatric endocrinologist, without evidence of growth hormone deficiency. The cause of this growth restriction requires further study but may reflect roles of the \( CLC-4 \) protein in fundamental growth processes or impact on enteric neurological function. Our findings underscore the importance of involving neurogastroenterology specialists in the comprehensive management of children with neurodevelopmental conditions, due to the significant impact on quality of life of underrecognized and untreated functional gastrointestinal comorbidities [36].

Other, less commonly noted clinical features include scoliosis, pes planus and/or lax joints, sleep disorders, otitis media with effusions, and strabismus. However, to date, our and other studies suggest that non-neurological congenital anomalies outside of the neurological system are not core features of \( CLCN4 \)-related condition. With age, as previously described, there is a progressive lengthening of the face in males and females, with some males having a relatively ‘square’ jaw [5] (Fig. 2). Facial features in infancy and childhood are variable, without a recognizable ‘gestalt’.

With a larger cohort now functionally characterized, we examined whether distinct functional impacts of the \( CLC-4 \) variants correlated with phenotypic features. Some early observations could be made. Firstly, the GOF variants (Group B) were commonly associated with a severe growth, feeding and/or functional gastrointestinal component. Secondly, they had a higher female: male ratio; 73% of the affected individuals in Group A (LOF) were male, compared to only 41% in Group B (GOF). Thirdly, all three males with GOF variants had the same variant (p.Val317Ile): in two of these families the variant was \textit{de novo}, in one maternally inherited. These males had similar clinical phenotypes including moderate to severe global developmental delay or ID, visual impairment (two were proven to have optic atrophy) and abnormalities of the corpus callosum. The functional impact of this variant was milder than that of the other GOF variants present in females. A possibility is that a severe GOF variant may not be compatible with life in a hemizygous male.

We cannot yet discount the pathogenicity of variants which performed like WT in our cellular model, as this is far from a complete model of the complexity of \( CLC-4 \) in animals in vivo, and, more specifically in the developing human. For example, variants that behaved like WT included the rare p.Arg315His \textit{de novo} variant in a female (Family D5), who had clinical features entirely consistent with the spectrum seen in \( CLCN4 \)-associated neurodevelopmental condition: however, this variant has also been reported in two other unrelated families in gnomAD. We also could not demonstrate a functional impact for several variants in the distal CBS domain, although it is possible that these variants may impact protein sorting or other mechanisms unable to be evaluated with the current Xenopus oocyte model.

In a structural model of a homodimeric \( CLC-4 \) protein, most variants characterized by a LOF with “rightward shifted voltage dependence” are localized at or near the dimer interface. This observation agrees with the hypothesis that voltage-dependent gating of \( CLC-4 \) is associated with a rearrangement of the dimer interface, as has been proposed for gating of the lysosomal \( CIC-7 \) [37]. Similarly, most GOF variants cluster at the dimer interface, mostly close to the luminal side. These mutants appear to partially destabilize the gate of the transporter that evidently must be tightly closed at negative voltages for proper function in endosomes. Interestingly, the isoleucine mutated in variant p.Ile549Asn (Family B10, severely affected female) that shows a particularly large GOF corresponds with Ile607 in the highly homologous \( CIC-3 \) protein; a variant at this position in \( CLCN3 \) (p.Ile607Thr) similarly caused a dramatic GOF and the affected individual died within the first month of life. It is important to note that \( CIC-4 \) most likely forms heterodimeric complexes with \( CIC-3 \) [13]. Overall, the disease phenotypes caused by \( CLCN3 \) and \( CLCN4 \) variants are quite different, demonstrating that the two genes have overlapping but not identical phenotypes. Our previous investigations on \( CLCN4 \) missense variants which were found in heterozygous females did not support a potential dominant negative effect when equal amounts of WT and mutant \( CLC-4 \) were co-expressed in \textit{Xenopus} oocytes [5]. However, the effect of voltage-gated shifted variants as well as GOF variants in heterodimeric \( CIC-3/CLC-4 \) complexes remains to be investigated [14]. Interestingly, the recurrent GOF variant p.Tyr553Cys in the late-endosomal \( CIC-6 \) causes a marked leftward-shift of the gating process [19, 38]. The corresponding tyrosine residue in \( CLC-4 \) is located just one residue away from Ile549. Both residues are in the linker connecting helices P and Q. The dramatic functional alterations of these variants provide additional evidence for a critical role of the linker P-Q in \( CLC \) transporter gating and corroborate the hypothesis that the GOF variants of vesicular \( CLCs \) are associated with a disrupted gating process.

We attempted to look at the possible impact of mosaicism on the phenotypic severity of \( CLCN4 \) variants, but data are too scarce to robustly conclude that mosaicism for a \( CLCN4 \) variant is predictive of phenotypic expression in females or males. This may be due to the lack of knowledge between the level of mosaicism in blood to that in the brain. For example, the variant p.Arg718Trp, in the CBS2 domain, has now been reported \textit{de novo} in both males and females with a severe phenotype (Table 1, Supplementary information), as well as in one unaffected mother, reported by He et al. [11]. However, we do note that this unaffected mother had a mosaic karyotype (47,XXX/46,XX) and it is possible that the ‘extra’ X chromosome may have somewhat moderated her phenotypic expression, as we considered for the unaffected male with Klinefelter syndrome, with an inherited \( CLCN4 \) variant which resulted in a severe phenotype in his male relatives [5].

We report on four individuals (C1-C4) with a \textit{de novo} or inherited missense \( CLCN4 \) variant and supportive functional
studies, but a more complex clinical phenotype, which we could attribute to a likely, or confirmed, blended genotype due to two monogenic conditions. For example, the male proband in Family C1 (p.Asp34Asn); whose functional studies were consistent with a LOF of CLC-4, has short stature and distinctive skeletal and facial features consistent with a diagnosis of Desbuquois dysplasia (XYLT1-related) that he shares with his sister. However, he has significant ID, epilepsy, and autism spectrum disorder, which are atypical for Desbuquois syndrome, and thus most likely has a blended phenotype of Desbuquois dysplasia and CLCN4-related neurodevelopmental condition. The finding of four patients with a blended phenotype due to suspected or proven multi-locus pathogenic variation in a total cohort of 122 individuals with CLCN4 variants (4/122: 3.3%) is consistent with other studies estimating this phenomenon occurs in about 5% of individuals with ASD [41]. Several research priorities remain. We need to better ascertain the causality of all rare missense variants to elucidate targeted treatments. Establishment of a robust animal model is an urgent priority. This could potentially be a rat model, given that a LOF of ClC-4, has short stature and distinctive skeletal and facial 

In summary, our study considerably expands our knowledge of the range of phenotypic and genotypic variation in CLCN4-related condition and for the first time robustly demonstrates a range of functional impacts, including gain of function. Variant classification still remains a nuanced art, rather than a precise science [40]. Fully informed genetic counselling is required to guide families through the diagnostic limitations and uncertainties inherent in genetic testing for neurodevelopmental conditions [41]. Several research priorities remain. We need to better ascertain the causality of all rare missense variants to elucidate targeted treatments. Establishment of a robust animal model is an urgent priority. This could potentially be a rat model, given that a LOF of ClC-4, has short stature and distinctive skeletal and facial

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AUTHOR CONTRIBUTIONS

CF, MIH, AM, MN, BC, CB, FSA, AC, MOH, HS, SW, AV, BC, SR, KN, SA, MR, CSW-M, KJ, MM, DB, ND, MG, TBB, EC, AMC, DH, ST, MW, LR, CS, GC, LD, RL-M, TD-B, JB, CS, EF, SEC, M-AS, AP, BG, M-TAW, GR, CM, SD, SB, CA, JBM, TTS, GNW, EJS, LM, DL, RS, RM, OM, FC, MC, LR, NHW, CWO, RP, SDK, MF, FERL, AMF, ARS, VM, SN, SG, DDW, LMB, JF, VC, SJ, LP, PMC, MB, EKB, JAR, CB, ZP, KMwC, TB, ET, MmA, SSM, and RA were responsible for compilation of genetic and clinical information and critical review and approval of manuscript. AP, VS, JG, AH, LS, and DK were responsible for performing experimental work and data analysis, and approval of manuscript. EP, MP, and VMK were responsible for conceiving the idea of the study, performing experimental work and data analysis, drafting and finalizing, and approval of the manuscript.

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ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Elizabeth E. Palmer, Michael Pusch or Vera M. Kalscheuer.

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1Centre for Clinical Genetics, Sydney Children’s Hospital Network, Randwick, NSW, Australia. 2Discipline of Paediatrics and Child Health, Faculty of Medicine and Health, University of New South Wales, Randwick, NSW, Australia. 3Istituto di Biofisica, CNR, Genova, Italy. 4Department of Clinical Genetics, Liverpool Hospital, Liverpool, NSW, Australia. 5Max Planck Institute for Molecular Genetics, Group Development and Disease, Berlin, Germany. 6Department of Biomedical and Clinical Sciences, Linköping University of New South Wales, Randwick, NSW, Australia. 7Department of Human Genetics, Gilbert and Rose-Marie Chagoury School of Medicine, Lebanese American University, Byblos, Lebanon. 8Institut Jerome Lejeune, Paris, France. 9Service de Génétique Médicale, CHU de Nantes, Nantes Université, Nantes, France. 10Nantes Université, CNRS, INSERM, Institut du Thorax, Nantes, France. 11Department of Translational Genomics, Center for Genomic Medicine, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia. 12Department of Neurosciences, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia. 13Applied and Translational Neurogenomics Group, VIB Center for Molecular Neurology, VIB, Antwerp, Belgium. 14Neurology Department, Antwerp University Hospital, Antwerp, Belgium. 15Translational Neurosciences, Faculty of Medicine and Health Science, University of Antwerp, Antwerp, Belgium. 16Department of Child Neurology & Metabolism, Ghent University Hospital, Ghent, Belgium. 17Department of Pediatric Neurology, University Hospital Antwerp/University of Antwerp, Edegem, Belgium. 18Children’s Hospital at Westmead, Sydney Children’s Hospitals Network, Westmead, Australia. 19Service de Genétique Clinique, Centre Hospitalier Regional Universitaire de Tours, Tours, France. 20Genetic Center, Akron Children’s Hospital, Akron, OH, USA. 21Center of Medical Genetics, University Hospital Antwerp/University of Antwerp, Edegem, Belgium. 22Department of Pediatric Neurology, University Hospital Antwerp/University of Antwerp, Edegem, Belgium. 23Institute of Human Genetics, Heidelberg University, Heidelberg, Germany. 24Department of Medical Genetics and Applied Genomics, University of Tuebingen, Tuebingen, Germany. 25Department of Clinical Genetics, Great Ormond Street Hospital for Children, London, UK. 26Developmental Neurosciences, UCL Great Ormond Street Institute of Child Health, London, UK. 27Department of Neurology, Great Ormond Street Hospital, London, UK. 28Wessex Clinical Genetics Service, Princess Anne Hospital, Southampton, UK. 29Genetic Services of WA, King Edward Memorial Hospital, Subiaco, WA, Australia.
