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THE IMPACT OF TARGETED NEXT GENERATION SEQUENCING IN THE DIAGNOSTIC WORK-UP OF PEDIATRIC EPILEPSY: A SINGLE CENTRE OBSERVATIONAL COHORT STUDY

Dottorando **Relatore**

Mario Mastrangelo MD, PhD **Ch.mo Prof. Vincenzo Leuzzi**

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SUMMARY

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ABSTRACT

Background Next generation sequencing techniques (targeted gene panels, whole exome sequencing and whole genome sequencing) allowed an increase of molecular diagnosis of genetic epilepsies, an expansion of the phenotypic spectrum of several epileptic syndromes and an optimization of the correlated diagnostic work-up.

Aim of the study: To characterize the epilepsy phenotypes that could be associated with a better detection rate of targeted next generation sequencing for pathogenic/likely pathogenic variants.

Patients and methods: A retrospective cohort analysis was performed on 58 patients (28 males and 30 females; mean age=9,06 ± 6,97 years) who underwent targeted next generation sequencing gene for epilepsy between 2015 and 2018. Data about demographic features, seizures semiology and evolution during follow-up, associated neurological and non-neurological features, EEG and MRI characteristics were collected. These variables were evaluated and compared in: - patients with epileptic encephalopathies (seizures causing developmental impairment- A group); -patients with developmental encephalopathies including epilepsy (developmental impairment preceding epilepsy- B group); - patients with isolated idiopathic epilepsy without signs of encephalopathy (no developmental impairment- C group).

Results: Pathogenic or likely pathogenic variants were assessed in 18/58 patients (13/18 were de novo) with a detection rate of 31,03% in the whole sample (51,6% in the B group; 11,1% in group C and 0% in the A group). Genes with pathogenic/likely pathogenic mutations were represented by: SCN1A (in 3 patients), IQSEC2 (in 2 patients), PRICKLE1, GABRB3, SLC2A1, MFF, SCN1B, KCTD7, CDKL5; FOXG1, SYNGAP1, ATP1A3, GRIN2A, PRRT2 and CACNA1A (one affected patients for each one of these genes). Atypical phenotypes were associated with variants involving SCN1A, KCTD7, PRICKLE1 and PRRT2. Molecular diagnosis addressed positively therapeutic choices in 13/18 patients with an optimization of seizures control.

Conclusions: Patients with developmental encephalopathies including epilepsy should undergo targeted next generation sequencing since the first stages of diagnostic work-up.

BACKGROUND

Genetic epilepsies: general aspects

Epilepsy is the most common neurologic disorder in pediatric age with an incidence of about 70 per 100.000 cases in children under the age of 2[1].

The complex landscape of genetic etiologies of epilepsies was largely expanded in the last decades[2]. About 5000 genes with a presumed pathogenic role and more than 150 genes with a known associated clinical phenotype were reported in the literature (about 30% of the whole diagnosed epilepsies)[2]. Most of the genetic epilepsies were prominently studied in subjects in which an early or very early onset of seizures and a very severe developmental and neurological impairment occurred[3, 4]. Other common associated clinical presentations include facial dysmorphisms, abnormalities of head circumference (mainly microcephaly), movement disorders and malformations in other organs such as heart, eye or kidney[5]. In this context, OMIM database currently reports 67 disorders caused by single gene mutations and classified as “early infantile epileptic encephalopathies” (Table 1-https://www.ncbi.nlm.nih.gov/omim).

Other genetic epilepsies are associated with chromosomal abnormalities including copy number variants (CNVs)[3, 4]. This last mechanism is the pathogenic basis for several chromosomal abnormalities and syndromes[3, 4]. Deletions up to 2MB are the most common chromosomal mutations that are reported in the clinical practice[5]. The most commonly involved chromosomal regions are 15q11.2, 15q13.3, and particularly 16p13.1[5]. In this context, epilepsy is usually associated with different degree of intellectual disability and dysmorphisms[5].

EARLY INFANTILE EPILEPTIC ENCEPHALOPATHIES (EIEE)	DISEASE-CAUSING GENES (function of the encoded protein)	PHENOTYPES
EIEE 1	ARX (Regulator of cellular cycle/signaling)	Infantile spasms, Myoclonic epilepsy, Tonic spasms and other Seizures, Intellectual disability, Generalized spasticity, Dyskinetic movements, Generalized dystonia, Ambigous genitalia, Suppression burst or Hypsarrhythmia on EEG
EIEE 2	CDKL5 (Regulator of cellular cycle/signaling)	Infantile spasms, Intellectual disability, Severe motor impairment, Hypotonia, Poor eye contact Rett-like phenotype (secondary deceleration of head growth, sleep disturbances, hand apraxia, and stereotypies)
EIEE 3	SLC25A22 (Membrane transporter)	Myoclonic seizures, Hypotonia, Microcephaly, Suppression burst pattern on EEG, Abnormal electroretinogram
EIEE 4	STXBP1 (Modulator of vescicular release)	Tonic spasms or tonic-clonic seizures, Dravet syndrome, Intellectual disability, Developmental delay, Hypotonia, Suppression-burst on EEG
EIEE 5	SPTAN1 (Structural protein)	Infantile spasms with hypsarrhythmia, Other Generalized seizures, Developmental delay, Intellectual disability, Spastic quadriplegia, Progressive microcephaly, Hypomyelination and diffuse brain atrophy on MRI
EIEE 6	SCN1A (ion channel subunit)	Dravet syndrome (Febrile or afebrile seizures, Generalized or unilateral clonic seizures, Myoclonic seizures, Atypical absences, Partial seizures, Photosensitivity, Developmental delay or regression, Ataxia); Genetic epilepsy with seizures plus (GEFS+)
EIEE 7	KCNQ2 (ion channel subunit)	Tonic spasms, Infantile spasms, Benign Familial Neonatal Seizures; Developmental delay, Suppression burst or Hypsarrhythmia on EEG, Transient T1 and T2 hyperintensities in the basal ganglia and thalamus
EIEE 8	ARHGEF 9 (Structural protein)	Focal seizures, Status epilepticus during sleep, Developmental delay, Focal epileptic abnormalities or spike and waves during sleep on EEG, Frontal hypoplasia or Polymicrogyria on MRI,
EIEE 9	PCDH 19 (Structural protein)	Febrile and afebrile seizures, Rare myoclonic jerks and atypical absences, Dravet syndrome, Intellectual disability, Motor impairment
EIEE 10	PNKP (Regulator of cellular cycle/signaling)	Polymorphic seizures, Microcephaly, Developmental delay Peripheral neuropathy, Movement disorders, Behavioral disorders
EIEE 11	SCN2A (ion channel subunit)	Polymorphic seizures (Myoclonic, Tonic, Clonic, Atonic, Generalized tonic-clonic), Dravet syndrome, Benign Familial Neonatal Infantile Seizures, Intellectual disability, Autism, Developmental delay, Movement disorders Possible optic atrophy and temperature dysregulation, Possible hypersomnia, Suppression burst or Hypsarrhythmia, Focal or multifocal epileptic discharges, Slow background activity on EEG, Possible brain atrophy or T2 hyperintensities in the basal ganglia or callosal hypoplasia on MRI
EIEE 12	PLCB1 (Regulator of cellular cycle/signaling)	Tonic seizures, Infantile spasms, Developmental delay, Suppression burst or Hypsarrhythmia on EEG
EIEE 13	SCN8A (ion channel subunit)	Polymorphic seizures (Infantile spasms, Migrating partial seizures in infancy, Focal, tonic, clonic, myoclonic and absence Seizures), Developmental delay, Dystonia, Hypotonia, Non

		specific EEG abnormalities (Background slowing Focal or multifocal epileptic discharges, Electrical status epilepticus), Non specific MRI abnormalities (Possible brain or cerebellar Atrophy, Possible callosal Hypoplasia)
EIEE 14	KCNT1 (ion channel subunit)	Malignant migrating partial seizures of infancy Delayed myelination
EIEE 15	ST3GAL3 (Enzyme of intermediate metabolism)	West syndrome
EIEE 16	TBC1D24 (Modulator of vescicular release)	Malignant migrating partial seizures of Infancy, Psychomotor regression, Loss of visual contact, Different EEG abnormalities (Focal theta discharge delta large-amplitude hemispheric discharge, Migrating ictal discharges, Multifocal spikes, Slow background activity), Brain atrophy sparing posterior fossa on MRI
EIEE 17	GNAO1 (Regulator of cellular cycle/signaling)	Tonic seizures, Tonic upgaze, Developmental Delay, Movement disorders, Variable EEG patterns (Suppression burst Diffuse spike and slow waves complex), Non specific MRI abnormalities (Delayed myelination, Cerebral atrophy, Thin corpus callosum)
EIEE 18	SZT2 (Regulator of cellular cycle/signaling)	Tonic or Tonic-clonic seizures, Variable EEG abnormalities (Slow background activity, Focal or multifocal epileptic discharges), Thick corpus callosum,
EIEE 19	GABRA1 (ion channel subunit)	Polymorphic seizures (Febrile or afebrile seizures, Generalized or unilateral clonic, Myoclonic, focal seizures, Atypical absences), Dravet syndrome, Intellectual disability, Motor impairment Non specific EEG abnormalities (Normal EEGat onset Multifocal or focal spikes or spike andwaves discharge, Diffuse slow waves)
EIEE 20	PIGA (Regulator of cellular cycle/signaling)	Multiple congenital anomalies, Hypotonia, Polymorphic seizures
EIEE 21	NECAP1 (Modulator of vescicular release)	Multifocal clonic or tonic seizures, Global developmental Delay, Non specific EEG and MRI abnormalities (Multifocal dischargesSlowed background activity, Possible diffuse brain Atrophy)
EIEE 22	SLC35A2 (Membrane transporter)	Congenital disorder of glycosylation type II m
EIEE 23	DOCK7 (Regulator of cellular cycle/signaling)	Dysmorphisms, Intellectual disability, Focal or tonic-clonic Seizures, Epileptic spasms, Cortical blindness Variable EEG abnormalities (Plurifocal epileptic Discharges, occipital epileptic abnormalities), Variable MRI abnormalities (Marked pontobulbar sulcus, T2 hyperintensities and occipital lobe atrophy)
EIEE 24	HCN1 (ion channel subunit)	Polymorphic seizures (Dravet-like syndrome, Fever-induced seizures, Atypical absences, Myoclonic seizures, Focal seizures, Autistic traits
EIEE 25	SLC13A5 (Membrane transporter)	Polymorphic seizures (Myoclonic, Focal or Tonic seizures), Profound developmental delay, Multifocal epileptic Discharges on EEG
EIEE 26	KCNB1 (ion channel subunit)	Polymorphic seizures (Focal, atonic, Tonic-clonic seizures,I Infantile spasms, Atypical absences, Variable EEG abnormalities (Focal or multifocal epileptic discharges, Hypsarrhythmia), Possible hippocampal volume loss on MRI
EIEE 27	GRIN2B (ion channel subunit)	West syndrome
EIEE 28	WWOX	Polymorphic seizures (Epileptic spasms, Tonic, clonic, or

	(Regulator of cellular cycle/signaling)	myoclonic seizures), Hypotonia, Developmental delay, Retinopathy, Hypokinesia, Microcephaly, Variable EEG abnormalities (Slow background activity, Focal or plurifocal epileptic discharges, Hypsarrhythmia), Variable MRI abnormalities (Delayed myelination Brain atrophy, Corpus callosum, hypoplasia, Hippocampal dysplasia)
EIEE 29	AARS (Regulator of cellular cycle/signaling)	Variable early onset seizures, Developmental delay,Congenital microcephaly, spasticity, vertical tali, movement disorders, peripheral neuropathy, Variable EEG abnormalities (Background slowing, multifocal epileptiform discharges, paroxysmal fast activity), Progressive brain atrophy or hypomielination on MRI
EIEE 30	SIK1 (Regulator of cellular cycle/signaling)	Polymorphic seizures (Epileptic spasms, Tonic, clonic, atonic, or myoclonic seizures), Developmental delay, Movement disorders, Poor visual or auditory responses, Variable EEG abnormalities (Slow background activity, Focal or plurifocal epileptic discharges, Suppression burst pattern or Hypsarrhythmia),
EIEE 31	DNM1 (Modulator of vescicular release)	Polymorphic seizures (Infantile spasms Myoclonic, atonic, tonic or focal seizures, Atypical absences), Variable EEG abnormalities (Hypsarrhythmia, Slow background Diffuse multifocal sharp waves and sharp waves-slow waves, Paroxysmal fast activity), Possible diffuse brain atrophy on MRI
EIEE 32	KCNA2 (ion channel subunit)	Polymorphic seizures (Febrile, Myoclonic, atonic, tonic-clonic, tonic or focal seizures, Atypical absences), Intellectual disability, Delayed speech, Ataxia, Movement disorders, Variable EEG abnormalities (Slow background Diffuse multifocal sharp waves and sharp waves-slow waves),
EIEE 33	EEF1A2 (Regulator of cellular cycle/signaling)	West syndrome, Developmental delay, Autistic traits, Acquired microcephaly, Behavior disorders, Incoordination, Gait instability
EIEE 34	SLC12A5 (Membrane transporter)	Focal, tonic and atonic seizures, Developmental regression, Microcephaly, Variable EEG abnormalities (Slow background Diffuse multifocal sharp waves and sharp waves-slow waves), Brain atrophy or Delayed miyelination on MRI
EIEE 35	ITPA (Enzyme of intermediate metabolism)	Polymorphic neonatal seizures, Mcrocephaly, Hypotonia, No developmental milestones, cardiac or ocular abnormalities, Delayed or diffuse demyelination and progressive brain atrophy on MRI
EIEE 36	ALG13 (Enzyme of intermediate metabolism)	West syndrome, Dysmorphisms, Developmental impairment or regression, Poor eye contact, Diffuse brain atrophy on MRI
EIEE 37	FRRS1L (Regulator of cellular cycle/signaling)	Polymorphic seizures, Severe developmental delay, Hyperkinetic movement disorders, Variable EEG abnormalities (Slow background Diffuse multifocal sharp waves and sharp waves-slow waves),Cortical or cerebellar volume loss and flattening of the caudate heads on MRI
EIEE 38	ARV1 (Membrane transporter)	Polymorphic seizures, Intellectual disability, Ataxia, Visual impairment, Movement disorders, Variable EEG abnormalities (Hypsarrhythmia, Slow background Diffuse multifocal sharp waves and sharp waves-slow waves), Frontal atrophy on MRI
EIEE 39	SLC25A12 (Membrane transporter)	Polymorphic early onset seizures, Developmental arrest or delay, Hypotonia, Poor eye contact, Hypomyelination and diffuse neuronal degeneration (decreased NAA and increased

		lactate on ^{1}HMRS) on MRI
EIEE 40	GUF1 (Regulator of cellular cycle/signaling)	West syndrome, Developmental arrest, Spasticity, Movement diosrders, Possible cortical atrophy on MRI
EIEE 41	SLC1A2 (Membrane transporter)	Glut 1 deficiency syndrome (polymorphic seizures including myoclonic or tonic clonic seizures and early onset atypical absences, movement disorders, symptoms induced by fasting, Mycrocephaly)
EIEE 42	CACNA1A (ion channel subunit)	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, tonic or focal seizures,), Hypo or hypertonia, Ataxia, Hyperkinetivc movement disorders, Abnormal eye movement, Variable EEG abnormalities (Slow background Diffuse multifocal sharp waves and sharp waves-slow waves),
EIEE 43	GABRB3 (ion channel subunit)	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, atypical absences), global developmental delay, behavioral abnormalities, Variable EEG abnormalities (Hypsarrhythmia, Slow background Diffuse multifocal sharp waves and sharp waves-slow waves).
EIEE 44	UBA5 (Regulator of cellular cycle/signaling)	Polymorphic early onset seizures, global developmental delay, Poor eye contact, movement disorders, Hypotonia, Post-natal microcephaly, Spasticity, Variable MRI abnormalities (cortical or cerebellar atrophy, thin corpus callosuum, demyelination)
EIEE 45	GABRB1 (ion channel subunit)	Early onset seizures, Global developmental delay, Hypotonia, Cortical visual impairment, Ataxia, Hypsarrhytmia, Thin corpius callosuum)
EIEE 46	GRIN2D (ion channel subunit)	Early onset seizures, Global developmental delay, Minor dysmorphisms, Cortical visual impairment, Hypotonoia, Gastrointestinal dismotility, Uncoordinated movement, Variable EEG abnormalities (Hypsarrhythmia, Diffuse multifocal sharp waves and sharp waves-slow waves),
EIEE 47	FGF12 (Regulator of cellular cycle/signaling)	Early onset seizures, Developmental regression, Variable EEG abnormalities (Hypsarrhythmia, Slow background Diffuse multifocal sharp waves and sharp waves-slow waves).
EIEE 48	AP3B2 (Modulator of vescicular release)	Early onset seizures, Global developmental delay, Microcephaly, Movement disorders, Poor eye contact, Variable EEG abnormalities (Hypsarrhythmia, Slow background Diffuse multifocal sharp waves and sharp waves-slow waves).
EIEE 49	DENND5A (Regulator of cellular cycle/signaling)	Polymorphic seizures (Myoclonic, tonic-clonic,), Hypo or hypertonia, Spasticity, Global developmental delay, Diffuse multifocal sharp waves and sharp waves-slow waves on EEG, Basal ganglia calcifications or corpus callosuum abnormalities on MRI
EIEE 50	CAD (Enzyme of intermediate metabolism)	Early onset seizures, Global developmental delay or regression, Normocytic anemia, Diffuse multifocal sharp waves and sharp waves-slow waves on EEG, Brain atrophy on MRI
EIEE 51	MDH2 (Enzyme of intermediate metabolism)	Early onset seizures, Global developmental delay, Hypotonia, Movement disorders, Variable MRI abnormalities (cortical or cerebellar atrophy, demyelination)
EIEE 52	SCN1B (ion channel subunit)	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, atypical absences, focal dyscognitive), Global developmental delay,Spasticity, Diffuse multifocal sharp waves and sharp waves-slow waves on EEG, Cortical atrophy on MRI
EIEE 53	SYNJ1 (Modulator of vescicular release)	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, tonic or focal seizures,), Hypo or hypertonia, Cortical visual impairment, Spasticity, Hypsarrhythmia on EEG, Cortical atrophy on MRI
EIEE 54	HNRNPU	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, atypical

	(Regulator of cellular cycle/signaling)	absences, tonic), Global developmental delay,Spasticity, Microcephaly, Autistic traits, Variable EEG abnormalities (Slow background , Diffuse multifocal sharp waves and sharp waves-slow wave, Paroxysmal fast activity).
EIEE 55	PIGP (Regulator of cellular cycle/signaling)	Early onset seizures, Profound intellectual disability, Microcephaly, Poor eye contact, Hypo/Hypertonia, Cortical visual impairment, Variable EEG abnormalities (Focal-spreading sharp waves, Multifocal epileptiform discharges, Slow background activity, and modified hypsarrhythmia),
EIEE 56	YWHAG (Regulator of cellular cycle/signaling)	Polymorphic seizures (myoclonic, absence, generalized tonic-clonic, febrile, and focal motor with eyelid fluttering or limb jerks), Intellectual disability, Ataxia, Psychiatric disorders, Variable EEG abnormalities (Dysrhythmic background, atypical spike waves, Sharp waves, polyspike waves, and Generalized spike waves).
EIEE 57	KCNT2 (ion channel subunit)	Polymorphic seizures (myoclonic, absence, generalized tonic-clonic, , and focal motor), Developmental regression, Variable EEG abnormalities (Disorganized background activity, Multifocal epileptogenic activity, Hypsarrhythmia), Hypomyelination and thin corpus callosuum on MRI
EIEE 58	NTRK2 (Regulator of cellular cycle/signaling)	Polymorphic seizures, Global developmental delay with intellectual disability, Optic atrophy Hypotonia, Spasticity
EIEE 59	GABBR2 (ion channel subunit)	West syndrome, Lennox-Gastaut syndrome
EIEE 60	CNPY3 (Regulator of cellular cycle/signaling)	Polymorphic seizures (prominentlt myoclonic), Developmental delay, Hypsarrhythmia on EEG, Cortical atrophy on MRI
EIEE 61	ADAM 22 (Synaptic secreted protein)	Early onset seiures, Developmental delay, Intellectual disability, Dysmorphisms, Microcephaly, Spasticity, Supratentorial atrophy on MRI
EIEE 62	SCN3A (ion channel subunit)	Polymorphic seizures (myoclonic, tonic, tonic-clonic), Microcephaly, Hypotonia, Spastic tetraparesis, Cortical blindness, Variable EEG abnormalities (Multifocal sharp waves and spikes, Intermittent slowing, Hypsarrhythmia), Variable MRI abnormalities (Polymicrogyria, Thin corpus callosum, White matter abnormalities)
EIEE 63	CPLX1 (Modulator of vescicular release)	Malignant migrating epilepsy or progressive myoclonus epilepsy, Iintellectual disability, Mild dysmorphic features, Generalized spikes on EEG, Cortical atrophy on MRI
EIEE 64	RHOBTB2 (Regulator of cellular cycle/signaling)	Early onset seizures, Intellectual disability, Poor motor development, Poor or absent speech, Hypotonia, Movement disorders, Nonspecific dysmorphic features.
EIEE 65	CYFIP2 (Structural protein)	Early onset seizures, Profound psychomotor developmental delay, Mild facial dysmorphism, Hypotonia, Spasticity, Pyramidal signs, Absent speech, Autistic traits, Variable EEG abnormalities (multifocal spikes, sharp waves, spike and slow wave complexes, suppression-burst patterns, and/or hypsarrhythmia), Cerebral atrophy or corpus callosuum abnormalities on MRI
EIEE 66	PACS2 (Regulator of cellular cycle/signaling)	Polymorphic seizures (prominently focal motor), Intellectual disability, Visual impairment, Spasticity, Microcephaly, Cerebellar abnormalities on MRI
EIEE 67	CUX2 (Regulator of cellular cycle/signaling)	Polymorphic seizures (focal, myoclonic, absence and atypical absence, tonic, atonic, and generalized tonic-clonic), Developmental regression , Movement disorders, Autistic features, Variable EEG (Generalized spike-wave or polyspike-wave patterns, Focal discharges, Multifocal discharges, Hypsarrhythmia, and focal slowing), Cerebellar or callosal abnormalities

Table 1. List of genes associated with early infantile epileptic encephalopathies and correlated phenotypes (https://www.ncbi.nlm.nih.gov/omim)

Mechanisms of epileptogenesis in monogenic epilepsies

Most of the reported genes associated with pediatric epilepsies encodes for ion channels subunits, membrane transporters, enzymes of the intermediate metabolism, regulators of neuronal cellular cycle and signaling, modulators of the release of synaptic vescicles, structural proteins and synaptic secreted proteins[3, 4]. Pathogenic variants in these genes result in dysfunctions in different stages of neuronal development and functioning, including synaptogenesis, pruning, neuronal migration and differentiation, neurotransmitter synthesis and release[3, 4].

a) Channellopathies

General aspects

Mutations of genes encoding for ion channels subunits are the most frequent cause of genetic epilepsies[3, 4]. Ion channels are pore-forming membrane proteins that are essential for the excitability of neurons including: a) the establishment of action potentials; b) the maintainance of the homeostasis by gating the ionic flow traversing the cell membrane; c) the management of the ionic flow across cells; d) the regulation of cell volume[6]. Alterations of these mechanisms are the basis of epileptogenic processes that are related to ion channels[6]. A recent analysis of several databases including OMIM (Online Mendelian Inheritance in Man), HGMD (Human Gene

Mutation Database), and EpilepsyGene) and recent publications in PubMed 977 identified 60 ion channel genes with a proved (28 genes) or a potential role (32 genes) in human epilepsies with more than 1600 pathogenic or likely reported pathogenic mutations[6]. Table 2 summarizes these ion channels and their main physiological functions.

ION CHANNELS	GENE (PROTEIN)	FUNCTIONS
SODIUM CHANNELS	SCN1A (NaV1.1), SCN1B (NaVb1), SCN2A (NaV1.2), SCN3A (NaV1.3), SCN8A (NaV1.6), SCN9A(NaV1.7)	Generation and propagation of action potentials
POTASSIUM CHANNELS		
Voltage gated	KCNA2 (KV1.2), KCNB1 (KV2.1), KCNC1 (KV3.1), KCND2 (KV4.2), KCND3 (KV4.3), KCNH2 (KV11.1), KCNH5 (KV10.2), KCNQ2 (KV7.2), KCNQ3 (KV7.3), KCNV2 (KV8.2)	Regulation of outward K currents and action potentials, modulation of neurotransmitter release
Calcium-activated	KCNMA1 (KCa1.1)	Regulation of neuronal firing properties and circuit excitability
Sodium-activated	KCNT1 (KCa4.1)	Regulation of delayed outward IK_{Na} currents and contribution to adaptation of firing rate
CALCIUM CHANNELS	CACNA1A (CaV2.1), CACNA1H (CaV3.2), CACNA2D2 (CaVa2d-2), CACNB4 (CaVb4),	React to membrane potential depolarization by opening and provide an elevation of Calcium ions to modulate many processes
CHLORIDE CHANNELS	CLCN2 (CLC-2), CLCN4 (CLC-4)	Maintenance of resting membrane potential and regulation of cell volume
C-AMINOBUTYRIC ACID TYPE A RECEPTOR	GABRA1 (GABAAa1), GABRA6 (GABAAa6), GABRB1(GABAAb1), GABRB2 (GABAAb2), GABRB3 (GABAAb3), GABRD (GABAAd), GABRG2 (GABAAc2)	Mediation of major inhibitory functions in neurotransmission
IONOTROPIC GLUTAMATE RECEPTORS	GRIN1 (GluN1), GRIN2A (GluN2A), GRIN2B (GluN2B), GRIN2D (GluN2D)	Excitatory synaptic transmission, plasticity, and excitotoxicity of the CNS
NICOTINIC ACETYLCHOLINE RECEPTORS	CHRNA2 (nAChRa2), CHRNA4 (nAChRa4), CHRNA7 (nAChRa7), CHRNB2 (nAChRb2)	Permeation of Na and K and modulation of neurotransmitter release
HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED CHANNELS	HCN1 (HCN1), HCN2 (HCN2)	Permeation of Na and K fluxes

Table 2 Ion channels that are implicated in genetic epilepsies

Illustrative disease: Dravet syndrome

Dravet syndrome (OMIM 607208) is the most frequent epileptic channellopathy and it represented the first example of epileptic/developmental encephalopathy that was associated with specific genetic basis[3]. The clinical phenotype includes prolonged generalized or unilateral clonic seizures triggered by fever, photo stimulation, or hot water, myoclonic seizures, atypical absences, and partial seizures[3]. Developmental milestones are usually normal before the onset of seizures, but are gradually impaired by recurrent epileptic episodes, resulting in mental delay, spasticity, or ataxia[3]. About 85% of patients with Dravet syndrome manifest sodium channel neuronal type 1a subunit (SCN1A) mutations[3]. A minority of patients carried mutations in other genes including PCDH19 or STXBP1 while various Dravet-like phenotypes were associated with CN2A, SCN8A, SCN9A, SCN1B, GABRA1, GABRG2, HCN1, CHD2, and KCNA2[4].

b) Disorders associated with mutations in genes encoding for membrane transporters

General aspects

Membrane transporters are proteins involved in the transport of molecules across blood-brain barrier or between cytosol and the internal parts of various organelles such as mitochondria or endoplasmic reticulum[4]. The major molecules involved in the transport are represented by glucose, aminoacids (i.e. glutamate, GABA),

creatine, vitamins (i.e. folate, thiamine) or trace elements (i.e. manganese, copper)[4].

The functional impairment of these proteins results in the activation of epileptogenic mechanisms that are based on the depletion of substrates for neuronal energy reactions (i.e. glucose in GLUT 1 deficiency syndrome) and cofactors for several biochemical processes including glycosylation of neuronal membrane proteins (i.e. manganese in SLC39A8 deficiency), and the biosynthesis of inhibitory or excitatory neurotransmitters (i.e. glutamate in SLC25A22 deficiency syndrome, folate in FOLR1 deficiency syndrome, thiamine in SLC22A1 deficiency syndrome[4]. Creatine in SLC6A8 deficiency, copper in Menkes disease)[4].

Common phenotypic features of this disorders often include a catastrophic and life-threatening neonatal epileptic encephalopathy associated with suppression burst at the EEG (i.e. in SLC25A22 deficiency syndrome) and a severe developmental impairment in the less severe cases (Glut 1 deficiency syndrome)[3, 4]. Movement disorders (i.e. paroxysmal kinesigenic dyskinesia in Glut 1 deficiency syndrome) or autistic spectrum disorder (i.e. FOLR1 deficiency syndrome) can also be observed[3, 4].

Illustrative disease; Glut 1 deficiency syndrome

Glut1 deficiency syndrome (OMIM 138140) is a valuable example of the possible phenotypic heterogeneity in epileptic transportopathies[7]. GLUT1 is a facilitative

glucose transporter with a prominent expression in brain, placenta, and erythrocytes[7]. GLUT1 deficiency syndrome is generally due to de novo SCL2A1 mutations or, in familial cases, due to mutations that are transmitted through an autosomal dominant mechanism[7].

GLUT1 deficiency syndrome includes a classical phenotype (early-onset epileptic encephalopathy, acquired microcephaly, developmental delay, hypotonia, spasticity, and movement disorders including dystonia and ataxia) and various nonclassical phenotypes (early-onset absences, paroxysmal exercise-induced dystonia with or without seizures, choreoathetosis, alternating hemiplegia, intermittent ataxia, language delay, expressive language difficulties, learning difficulties, different degree of cognitive delay, and migraine)[7].

Electroencephalogram can show various epileptic abnormalities[7]. It has reported a typical reduction of some abnormalities, such as slow waves, after a meal. Magnetic resonance is usually nondiagnostic[7]. Positron-emission tomography often demonstrates a decrease in cortical (prominently in the mesial temporal regions) and thalamic glucose uptake[7].

The main biochemical hallmark for GLUT1 deficiency syndrome is hypoglycorrhachia (especially if it is lower than the third percentile for the age)[7]. Cerebrospinal fluid-to-blood glucose ratio level lower than 0.35 is considered as strongly suggestive of GLUT1 deficiency (even if in milder phenotype, the ratio can be higher than 0.59)[7]. Clinicians should take into account that

hypoglycorrachia can be also observed in meningitis, status epilepticus, mitochondrial diseases, hypoglycemic states, subarachnoid hemorrhage, and meningeal carcinomatosis[7]. These disorders should be carefully excluded before performing second-level investigations for GLUT1 deficiency (test for uptake of 3-*O*-methylglucose into erythrocytes and SLC2A1 gene sequencing)[7]. The normal values of cells, proteins, and lactate, which are observed in GLUT1 deficiency, are useful in differential diagnosis of infectious, inflammatory, and mitochondrial diseases[7]. Ketogenic diet remains to be the gold standard for treatment of GLUT1 deficiency because it represents an alternative source of energy for the brain[7]. It includes high proportion of fats and a restriction of carbohydrates and it mimics the metabolic state of fasting with an increased production of ketones[7]. Ketogenic diet in patients with GLUT1 deficiency induces an optimal seizure control and it also results in a decrease in movement disorders (especially dystonia, paroxysmal exercise-induced dyskinesia, and ataxia)[7]. The evaluation of its effects on the cognitive outcome requires further studies[7].

Alternative promising treatments for GLUT1 deficiency in the future will be represented by alpha-lipoic acid (an antioxidant that improves cellular glucose uptake and transport) and triheptanoin (a triglyceride that strengthens the function of common ketones)[7].

c) Disorders associated with mutations in genes encoding for enzymes of the intermediate metabolism

General aspects

This group includes a wide number of diseases involving genes encoding for enzymes belonging to different metabolic pathways[3, 4, 7]. In this context epileptogenesis results from different mechanisms including: a) dysfunctions in the production of molecules that are involved in the synthesis of neurotransmitters (i.e. vitamin B6-dependant epilepsies due to mutations in ALDH7A1, PNPO, ALDH4A1 or PROSC); b) neurotoxic effects of intermediate compounds (i.e. urea cycle disorders or organic acidurias, some aminoacidopathies such as maple syrup urine disease); c) abnormalities in the production of energy substrates (i.e. mitochondrial disorders such as pyruvate dehydrogenase deficiency or defects of creatine metabolism); d) reduced availability of specific substrates (disorders of serine metabolism, disorders of molybdenum cofactor biosynthesis, biotinidase deficiency); e) abnormal storage of metabolites (lysosomal storage disorders such as Niemann Pick type C disease); f) disturbances in neuronal membrane permeability (i.e. holocarboxylase synthetase deficiency; g) misbalance in intracellular/extracellular ions (organic acidurias)[3, 4, 7].

An important quote of these disorders includes treatable conditions with effective available therapies that allow a satisfying seizure control : a) vitamin B6-dependent epilepsies; b) cerebral folate deficiency; c) congenital disorders of

serine metabolism; d) biotinidase deficiency; e) inborn errors of creatine metabolism; f) molybdenum cofactor deficiency[7]. These therapies don't result in similar benefits on symptoms other than seizures in these diseaeses (i.e. intellectual disability or movement disorders)[7].

Illustrative disease: ALDH7A1 deficiency

Pyridoxine dependent epilepsy due to ALDH7A1 mutations represented the first historical example of a treatable vitamin dependant epilepsy. ALDH7A1 encodes for alpha-aminoadipic semialdehyde dehydrogenase deficiency (antiquitin). Antiquitin is an enzyme involved in lysine catabolism[7]. Antiquitin deficient or absent activity results in the accumulation of precursors α-aminoadipic semialdehyde (α-AASA) and Δ1-1-piperideine-6-carboxylate (P6C)[7]. P6C induces a Knoevenagel condensation product with the active form of pyridoxine (pyridoxal-5′-phosphate [PLP])[7]. The above-mentioned chemical reaction removes PLP from several cellular processes (PLP is an essential cofactor for different enzymes involved in more than 140 neuronal intracellular process) and in a subsequent activation of different epileptogenic mechanisms[7]. The classical clinical presentation of ALDH7A1 deficiency encompasses an early-onset epileptic encephalopathy with variable seizure types and with its onset in the neonatal period or in the first months of life[7]. More recently, milder epileptic phenotypes with later onset have been reported[7]. Other clinical manifestations of patients with *ALDH7A1* deficiency include both neurological (abnormal fetal

movements, signs of hypoxic ischemic encephalopathy, dystonia, increased startle response, irritability, and intellectual disability) and non-neurological (respiratory distress, abdominal distension, bilious vomiting, hepatomegaly, hypothermia, shock, and acidosis) symptoms[7]. Electroencephalographic patterns vary from suppression burst or hypsarrhythmia to focal or multifocal epileptic discharges[7]. Possible structural brain abnormalities include hemispheric hypoplasia or atrophy, cerebellar or cortical dysplasia, intracerebral hemorrhage, or periventricular hyperintensity at magnetic resonance imaging (MRI)[7]. A therapeutic trial with an intravenous (100 mg) or an oral/enteral (30 mg/kg/day) administration of pyridoxine can be an important step also for the diagnosis[7]. Acute intravenous administration of 100 mg of pyridoxine should be followed by a long-term oral/enteral administration at the dosage of 15–30 mg/kg/day in responding patients[7]. Lysine-restricted diet or L-arginine supplementation could represent possible therapeutic alternatives[7].

d) ***Disorders associated with mutations in genes encoding for regulators of neuronal cellular cycle and signaling***

General aspects

This group of diseases are caused by mutations in genes encoding for proteins that are implicated in different phases of anchoring the synaptic machinery, neuronal cellular cycle, subcellular signaling pathways and, subsequently, in the regulation of neuronal excitability[4]. The phenotypes associated with mutations of genes involved in these processes include both catastrophic early

infantile onset epileptic encephalopathies associated with a severe developmental delay or movement disorders (i.e. ARX, CDKL5, PLCβ1, MAGI1, DOCK7, GNAO1, ARHGEF 9, ST3GalIII, WWOX) and less severe presentations (disorders of GATOR1 complex)[4].

Illustrative diseases: disorders of mTOR and GATOR1 pathways

The first studied subcellular cascade involved in focal epilepsies was represented by the mammalian target of rapamycin (mTOR) neuronal transduction signal pathway[8]. The mTOR pathway has a pivotal role in the synaptic protein synthesis and in the integrations of inputs resulting from NMDA and metabotropic glutamate receptors[8]. The mTOR pathway is also a modulator of the synaptic excitation/inhibitiom balance[8]. An abnormal activation of the mTOR occurs in tuberous sclerosis complex, which is a genetic multiple organ system disease, characterized by localized cellular overgrowth leading to benign tumor-like lesions[8]. Tuberous sclerosis is a developmental disorder resulting from loss of function of either hamartin or tuberin because of pathogenic mutations in TSC1 and TSC2 genes[8]. TSC1 and TSC2 act as negative regulators of mTORC1 (one of the two complexes forming mTOR pathway)[8]. Mutations in these genes induce an hyperactivation of the mTOR pathway, resulting in a downstream kinase signaling cascade that can consequently lead to alterations in excitation/inhibition balance, therefore, to abnormalities in numerous cell processes, including cell cycle progression, transcription, translation, and metabolic control[8]. Such events have been

thought to cause the clinical hallmarks of tuberous sclerosis such as: epileptic seizures, formation of dysplastic areas ("tubers"), cutaneous manifestations and benign tumours involving organs such as kidney or heart[8].

Recently, germline mutations have been found in genes encoding the proteins involved in the GATOR1 complex (DEPDC5, NPRL2, NPRL3), another repressor system of mTORC1[9]. These mutations are implicated in a wide and spectrum of focal epilepsy syndromes, with and without cortical structural abnormalities (mainly focal cortical dysplasia)[9]. Patients carrying mutations in DEPDC5, NPRL2 and NPRL3 have a similar epilepsy phenotype[9]. They present with focal epilepsy without predilection for a specific cortical area, even if nocturnal frontal lobe epilepsy is extremely frequent[9]. Age of seizure onset is variable[9]. Ictal electroencephalogram may evidence focal (frontal, temporal, more rarely parietal or occipital) epileptiform abnormalities that are relatively constant in the affected patients[9]. Brain MRI can be normal or may show focal cortical dysplasia, hemimegalencephaly, or polymicrogyria in a quote of patients[9]. Psychomotor development and cognition are usually normal even if intellectual disability or other neuropsychiatric manifestations can also be observed[9]. Drug-resistance rates may be higher than in other focal epilepsies[9].

e) Disorders associated with mutations in genes encoding for modulators of the release of synaptic vescicles

General aspects

The neurotransmitter release machinery includes various regulators of the synaptic vesicle formation, fusion, and recycling[3]. Mutations of many proteins involved in this multistep pathway cause epilepsy[3, 4]. The more studied proteins belonging to this group include: a) SV2A and Synapsins for synaptic vesicle formation; b) t- SNARE proteins (Syntaxin 1B and SNAP25b), SNARE-associated protein (STXBP1/MUNC18-1), and voltage-dependent P/Q-type calcium channel subunit a-1A (CACNA1A) for synaptic vesicle fusion; and c) Dynamin 1 for synaptic vesicle recycling[3, 4]. Given that the loss-of-function of these genes causes epilepsy, the reduced GABA release from inhibitory neurons or an imbalance between inhibitory and excitatory synaptic transmission may account for their pathogenic mechanism. their pathogenic mechanisms[4].

The most frequent genes involved in human diseases presenting with epilepsy are represented by STXBP1, DNM1, NECAP1 and TBC1D24[4].

Illustrative disease: STXBP1 encephalopathy

The syntaxin binding protein 1 (STXBP1, or Munc18) gene maps to 9q341, and includes 20 exons. Syntaxin binding protein 1 modulates the release of synaptic vesicles through specific interactions with syntaxin A (Stx1a) and

with the soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex[4]. An open conformation of syntaxin 1A that promotes the formation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex and subsequent vesicular release, and a closed conformation of syntaxin 1A that controls synaptic vesicular docking, are involved in these processes[4].

Over recent years, the phenotypic spectrum of patients with STXBP1 mutations has expanded from a severe neonatal or early infantile epileptic and developmental encephalopathy to a less severe Dravet-like pattern[4]. Epileptic spasms or tonic seizures are part of the clinical presentation in most patients at some point in their disease history[10]. Additional neurologic symptoms include intellectual disability, autistic features, dyskinesia, stereotypes, dystonia or parkinsonism, tremor, axial hypotonia and ataxia[10].

The main EEG features include is focal or multifocal spike and waves discharges, while burst-suppression or hypsarrhythmia are reported in about 30% of patients[10]. MRI can highlight cortical atrophy, delayed myelination, and thin corpus callosum even if no neuroradiological abnormalities are detected in 50% of patients[10].

f) ***Disorders associated with mutations in genes encoding for structural proteins***

<u>General aspects</u>

The genes causing this disorders encode for proteins involved in the modulation of neuronal structural integrity and in trans-synaptic adhesion[3, 4].

The prototype of structural proteins that are involved in early onset epilepsies is represented by SPTAN1[3]. Nonerythrocytic alpha-spectrin-1 (SPTAN 1) gene maps to 9q33-q34 and encodes for a filamentous cytoskeletal protein that regulates the stability of axonal structure[3]. Clinical presentation of patients with mutations in this gene mainly includes intractable seizures with hypsarrhythmia , mental retardation, spastic quadriplegia and progressive microcephaly[3].

Adhesion molecules are essentials for trans-synaptic communication and, subsequently, for synapse development and synaptic transmission and plasticity[11]. Recent studies have identified various synaptic adhesion molecules including: presynaptic Neurexins and postsynaptic Neuroligins, IL1RAPL1, TrkC, Slitrks, NGLs, LRRTMs, Dystroglycan, and SALMs[11]. These synaptic adhesion molecules have distinct but overlapping binding specificity that is further regulated by their isoforms and alternative splicing[11]. The linkage of these proteins to epilepsy still remains limited probably due to their functional redundancies[11]. Examples of human epilepsy mutations include compound heterozygous deletion of NRXN1, a microdeletion encompassing IL1RAPL1 , and mutations of CNTNAP2 (Caspr2)[11].

Illustrative disease: PCDH19-related epilepsy

Protocadherin 19 (PCDH19) gene maps to Xq22 and encodes for a transmembrane protein that controls calcium-dependent cell-cell adhesion[3]. PCDH19 may be involved in specific synaptic connections and transmissions and its impairment results in an altered neuronal excitability[3].

The role of PCDH in epilepsy was described for the first time within the so-called "epilepsy and mental retardation limited to females" or EFMR (OMIM 300088) and, then, in patients with SCN1A-negative Dravet syndrome[3].

EFMR is characterized by a seizure onset between 6 and 36 months, a combination of febrile and afebrile seizures and a variable psychomotor and cognitive impairment[3]. The typical prominent expression in females of EFMR, notwithstanding PCDH19 gene is on X chromosome, has been explained through two possible mechanisms: the existence of compensatory factors in males with mutated PCDH19 (such as Protocadherin 19Y gene) and the formation of tissue mosaicism with PCDH19-positive and PCDH19-negative cells and subsequent altered interactions between the two cellular populations[3].

g) Disorders associated with mutations in genes encoding for synaptic secreted proteins

General aspects

This group of disorders result from the deficient synthesis of proteins acting as extracellular synaptic organizers[11]. The most important proteins with this role include

C1q family proteins and SRPX2[11]. C1q complement regulates synapse elimination during development and in mouse models loss of C1q causes failure in pruning of excessive excitatory synapses in the retinogeniculate and neocortical neurons, leading to atypical absences[11]. SRPX2 pathogenic mutations were reported in patients with temporal seizures and speech impairment[11].

Illustrative disease: LGI1-related epilepsy

LGI1 mutations cause autosomal dominant lateral temporal lobe epilepsy[11]. The encoded protein LGI1 binds to an ADAM22 transmembrane protein that is anchored by a postsynaptic PSD-95 scaffold[11]. Loss of LGI1 or ADAM22 reduces AMPA-receptor mediated synaptic transmission and causes life threatening epilepsy in mouse models[11]. In the absence of LGI1, PSD-95 is unable to modulate AMPA receptor-mediated synaptic transmission with an increase of neuronal excitability[11]. In addition, LGI1 autoantibodies observed in patients with limbic encephalitis, which is characterized by seizures and amnesia, inhibit the LGI1-ADAM22 interaction, reducing the number of synaptic AMPA receptors[11].

Phenotypic heterogeneity in genetic epilepsies

Epilepsy phenotypes and severity, degree of developmental impairment, concurrent neurological and non neurological manifestations are extremely variable according to the functions of the different involved genes and their role in epileptogenic mechanisms[4]. Several studies also evidenced a remarkable heterogeneity in terms of different clinical conditions resulting from variants of the same genes (i.e.SCN2A

causes both familial benign neonatal infantile epilepsy and a severe epileptic encephalopathy; KCNQ2 was initially associated with familial benign neonatal seizures and, subsequently, with an early onset epileptic encephalopathy) or similar clinical syndromes caused by different genes (i.e. Dravet syndrome can be caused by pathogenic variants in SCN1A , PCDH 19, STXBP1 or GABRA1)[4]

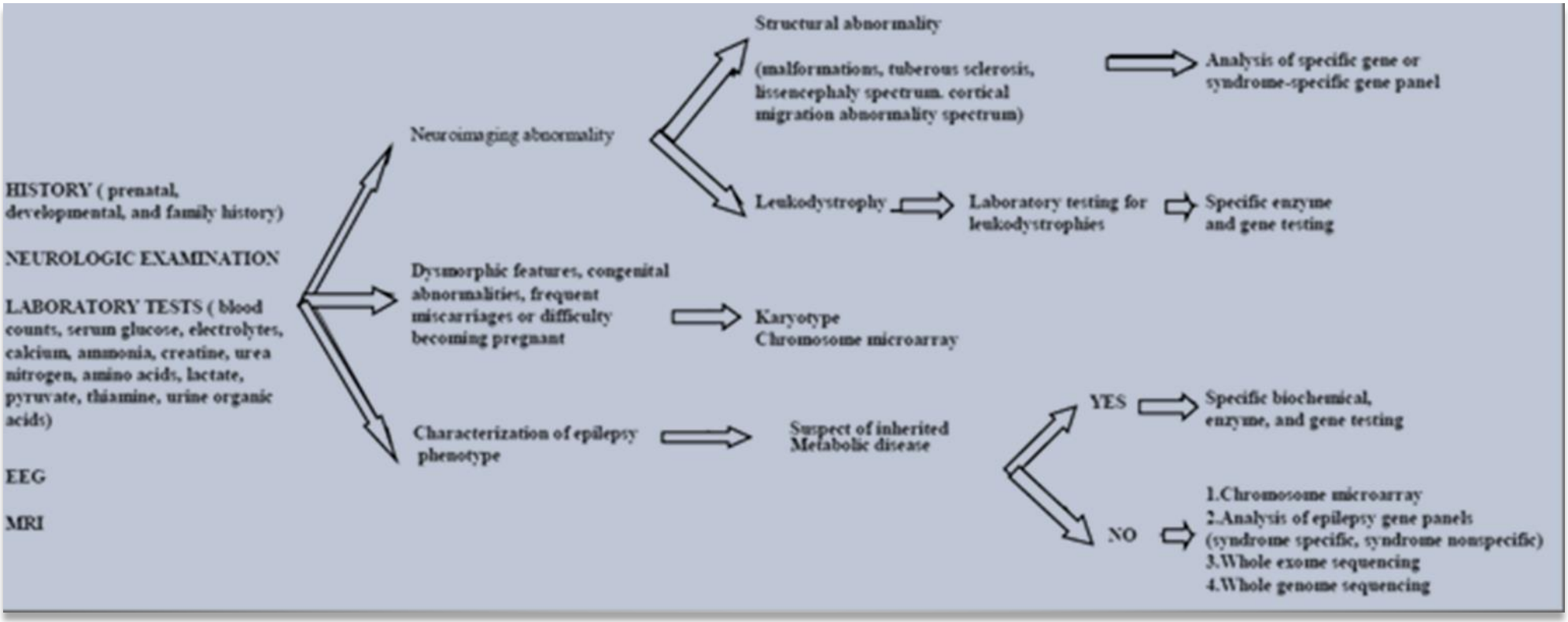


Fig. 1 A suggested *algorithm* for diagnostic work-up in genetic epilepsies

Molecular genetic work-up and the role of Next Generation Sequencing in genetic epilepsies

During pre-next generation sequencing era patients, who were suspected to have a single gene-related epilepsy, underwent a prolonged diagnostic odyssey including gene by gene Sanger sequencing and a complex biochemical and laboratory work-up as I previously discussed in three different reviews that were published between 2012 and 2015[3, 4, 5].

Next generation sequencing (NGS) includes different techniques that allow a simultaneous sequencing of exons belonging to a selected group of genes organized in panels (gene panels) or to the whole exome or genome[2]. Whole-exome sequencing (WES) involves the encoding part of the human genome (about 20,000 disease causing genes)[2]. WES analysis identifies the profile of the detected gene variants and subsequently a comparison with the polymorphic (non pathogenic) variants, distributed in the general population, is realized in order to identify the possible pathogenic variants[12]. The putative pathogenic mutations are subsequently characterized in terms of de novo occurrence (variant absent in the parents) and state of homozygosity (both gene copies suffering from the same mutation) or compound heterozygous (two different mutations in the same gene)[2, 12]. Whole genome sequencing (WGS) involves both the encoding and non encoding human genome[2, 12]. The three groups of NGS investigations (gene panels, WES and WGS) do not

identify non-coding regulatory sequences and deletions/duplications of exons that can be studied through array CGH and other cytogenetic techniques[2, 12].

Although every form of pediatric onset epilepsy could be diagnosed by NGS, in the literature it was observed a more useful applicability of these techniques for patients without gestaltic facial dysmorphisms or structural abnormalities at the neuroimaging[2, 12]. In this regard, a suggestion for a possible diagnostic algorithm including NGS is included in Figure 1. In Table 3, the objectives and the indications of all the molecular genetics diagnostic tests are summarized, with the current application in the study of epileptic children.

DIAGNOSTIC TESTS	DIAGNOSTIC AIM	INDICATIONS
Karyotype	Analysis of all chromosome for extended duplications/deletions	Patients with dysmorphism and/or multiorgan involvement
Array CGH	Identify single nucleotide polymorphisms (SNP arrays) or to determine chromosomal rearrangements submicroscopic (array-CGH) as copy number variants (CNVs).	Epilepsy with developmental delay, dysmorphism, Autism spectrum disorders
Single gene sequencing	Detects changes in the gene and if it causes amino acid alterations	Suspected single-gene defect (e.g. SCN1A-related Dravet syndrome)
Duplication/deletion of a single gene analysis	CNV of a single gene	Suspicious of a single gene defect when sequencing is inconclusive
Research of a specific mutation	Sequencing of a specific mutation	On parents to understand if an unknown mutation is pathological
Targeted-resequencing	Sequencing and duplication/deletion research of a gene panel for a specific disease (e.g. epilepsy)	Diseases with more genes involved
Fluorescent in situ hybridization (FISH)	Probes that analyse specific chromosome's portions	Confirmation of a duplication/deletion
Whole-exome and genome sequencing	Whole-exome and genome sequencing Sequencing of all DNA only for codifying regions (exons) or all regions (genome)	Suspected genetic aetiology with otherwise normal investigations

Table 3 Diagnostic objectives and indications for genetic tests in epileptic patients.

NGS approaches produced remarkable advantages in different fields including: a) the identification of an increasing number of new genes responsible for rare forms of monogenic epilepsies; b) an extension of the known phenotypes associated with previously discovered disease-causing genes c) an improvement in the potentialities of genetic counseling with an increase of molecular genetic diagnosis and with the demonstration that most of the pathogenic variants in pediatric onset epilepsies are de novo; d) an acceleration and optimization of diagnostic work-up and therapeutic choices; e) a reduction of economic costs[2, 12].

The possibility of analyzing concurrently a wider group of disease-causing genes and the faster gene-sequencing was counterbalanced by: a) the availability of a large amount of data that often complicate genotype-phenotype correlations; b) the frequent detection of variants of uncertain significance (VOUS); c) the frequent need for functional studies to assess the real pathogenic effect of the detected variants; d) a limited epidemiological impact (most of the known disease-causing genes associated with epilepsy accounts for a few dozen of the cases)[2, 12].

The interpretation of functional effects and pathogenicity of the detected variants can be supported by several additional bioinformatics tools[12]. The ExAC (http://exac.broadinstitute.org), the gnomAD (http://gnomad.broadinstitute.org) or the 1000 Genomes Project (http://www.internationalgenome.org) databases list variants and their alleles frequencies in the population[12]. The freely available ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), the Human Gene Mutation

Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php) or several locus-specific databases correlate genomic variants with reported clinical presentations[12].

A positive correlation can be assessed between the number of genes included in an NGS panel and its diagnostic yield[2, 12]. The number of sequenced genes should be continuously updated according to the progression of knowledge even if these frequent changes in the structure of the panels result in a concurrent increase of the identified variants and, subsequently, in an increasing complexity of both bioinformatic filtering process and genotype–phenotype interpretation[12].

AIM OF THE STUDY

In epileptic children recently published next generation sequencing studies evidenced a detection rate for pathogenic or likely pathogenic variants ranging between 18,3 and 40% according to different extensions of the specific gene panels that were built (Table 4) [13, 14, 15, 16, 17, 18, 19, 20, 21]. A recent Whole Genome Sequencing study in 197 subjects highlighted the role of several new genes in the pathogenesis of both epileptic and developmental encephalopathies with a clear prevalence of de novo point mutation if compared with other mechanisms of inheritance[22]. The present study aimed to characterize the principal epilepsy phenotypes and the main additional clinical features that could improve the selection of adequate candidates and the diagnostic power of targeted next generation sequencing techniques.

PATIENTS AND METHODS

Patients selection

We evaluated clinical, electroencephalographic and genetic data of all consecutive epileptic patients who were referred to the Infantile Neurology Unit of Sapienza-University of Rome from November 2015 to August 2018. All the patients underwent periodical neurological and developmental evaluations including neurocognitive testing, appropriated for their age.

All the selected epileptic patients were analyzed in terms of demographic features, seizures semiology, clinical evolution during the follow-up, neurological and non-neurological symptoms associated with epilepsy, EEG and MRI characteristics. Three

distinct phenotypical categories were selected for a targeted next generation sequencing study with the gene panels that is described in the following sections:

-Patients in which epilepsy was the primary cause of a progressive regression of motor, sensorial and cognitive functions (A group: patients with epileptic encephalopathies) [22, 23]:

-Patients in which epilepsy was part of a complex developmental impairment involving multiple functional areas (B group: patients with developmental encephalopathies with epilepsy)[22, 23];

-Patients with isolated idiopathic epilepsy without other signs of encephalopathy (C group)[23, 24, 25]. In this group subjects with a familial history for epilepsy and/or combination of febrile and non febrile seizures were included.

In a following stage, a critical revision of all the relevant clinical and molecular genetics differences among the members of each abovementioned groups was realized.

Written parental consent for all patients and approval from the Ethic Committee of our institution were obtained for the realization of this study.

Next Generation Sequencing Methods

Three distinct gene panels were realized over the years (including, respectively, 30, 95 and 148 genes associated with epilepsy) at the Neurogenetics Laboratory of Meyer

Children Hospital-University of Florence according to a previously published protocol (the whole list of the explored genes is shown in Table 5)[14].

DNA was obtained from peripheral blood leukocytes through a QiaSymphony SP robot (Qiagen, Hilden, Germany) according to the manufacturer‟s instructors. High-quality DNA was quantified through a Quantifluor Fluorometer (Promega, Madison, WI, USA)[14].

a) 30-genes panel analysis

The panel was designed through a custom target in solution enrichment NimbleGen SeqCap EZ Choice Library (Roche Inc., Madison, WI, USA) to target the genomic sequence of analyzed genes and the flanking regions at the 5′ and 3′ ends of each gene, accounting for a total of 109528 bp. 500ng of gDNA were nebulized and the libraries was built through a GS FLX Titanium Rapid Library Preparation Kit (Roche Inc., Madison, WI, USA)[14]. The libraries were multiplexed through different MID identifiers in order to obtain a sequencing involving up to 12 samples in a single run, and the pool was hybridized to SeqCap EZ Choice Library designed to capture the genes inserted in the panel[14]. Sequencing was realized according to the Roche FLX Titanium protocols and kits[14]. Briefly, captured sample libraries were subjected to emulsion-based clonal amplification[14]. DNA-carrying beads were enriched and used as template for sequencing by synthesis through the Titanium chemistry (XLR70 GS FLX Titanium sequencing kit - Roche Inc., Madison, WI, USA)[14]. GS FLX sequence reads were aligned to the NCBI37/hg19 reference genome through the GS Reference

Mapper v2.9 toolkit[14]. Variants were called through the same toolkit[14]. Exploiting the long reads generated by the GS FLX sequencer, we used the GS Reference Mapper to unravel potential structural rearrangements in the 30-genes panel[14].

95-genes panel analysis

The Haloplex panel was designed using the Agilent SureDesign. (https://earray.chem.agilent.com/suredesign/index.htm) to capture the 95 epilepsy genes. gDNA were purified and resuspended in water through the DNA Clean & Concentrator-5 columns (Zymo Research Corporation, Irvine, CA, USA) and the libraries prepared with the Haloplex target enrichment system (Agilent Technologies, Santa Clara, CA, USA) according the manufacturer protocol[14]. Probes were built to cover all coding exons and their flanking intronic sequences (10 base pairs padding)[14]. 225 ng of genomic DNA was used for restriction reactions, and hybridization with the Haloplex probe was prealized for 3 hours at 54°C[14]. Twelve libraries containing unique identifiers were quality controlled using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), pooled in equimolar concentration and sequenced on a MiSeq sequencer using a MiSeq Reagent Kit v3 and a 150 bp paired-end chemistry (Illumina, San Diego, CA, USA)[14]. Sequence reads were aligned to the NCBI37/hg19 reference genome using a pipeline based on BWA (Li and Durbin, 2009) and Picard (https://broadinstitute.github.io/picard/). Variants were called using the GATK toolkit (McKenna et al., 2010)[14].

148 genes panel analysis

The investigation was performed through a 150 bp Paired-End protocol through NexSeq (Illumina). The sequencing was preceded by a selective enrichment of the DNA regions of interest through a hybridization with specific probes (Nextera, Illumina). The result of the sequencing was considered as optimal if the following criteria were satisfied: a) >95% of covered target bases at 15X; b) >85% of covered target bases at 40X; c) mean cover > 100X.

Sequence reads were aligned to the NCBI37/hg19 reference genome using a pipeline based on BWA (V0.7.7-r441) and Picard (v1.109)[14]. Variants were called using the GATK toolkit (v3.1)[14]. Resulting variants were filtered through the elimination of possible artifacts of sequencing/alignment[14].

Variants annotation and filtering

For all the three panels, variants were annotated with gene name and classified according to their position and effect (frameshift, truncating, splicing, coding non synonymous, coding synonymous, intronic) using the ANNOVAR tool (v17 June15)[14].

Exonic non sinonimous and splice site variants (+/- nucleotides in encoding exons) were considered in the analysis when frequency of controls in the referring databases [the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/) , GnoMad (http://gnomad.broadinstitute.org/) and 1000 Genomes Project (http://www.1000genomes.org)] was below 0,1% for genes with an autosomal

dominant transmission and below 1% for genes with an autosomal recessive transmission[14]. Variants localized in intronic regions outside the 10 bp exon flanking boundaries and in the 5'- and 3'-UTR regions were excluded[14]. Variants reported in the ExAC database and/or in the 1000 Genomes Project and/or in the NHLBI Exome Sequencing Project (ESP6500 database, http://evs.gs.washington.edu/EVS), with a Minor Allele Frequency (MAF) > 0.01 (1%) were dropped out (with the exception of previously reported variants with a demonstrated pathogenicity)[14].

In silico prediction of mutations‟ pathogenicity were obtained using ANNOVAR and the dbNSFP database (v3.0a), which provides functional prediction scores on more than 20 different algorithms (https://sites.google.com/site/jpopgen/dbNSFP)[14]. To assess the effects of missense substitutions it was used both the dbNSFP ensemble rank scores MetaSVM and MetaLR[14].

The cDNA numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1[14].

NGS data analysis including both single nucleotide variant calling and exon copy-number variations analysis by CONVaDING tool was also performed[14].

Variants confirmation

Presumed causative variants were analyzed by Sanger sequencing to confirm the NGS results and investigated in the parents of probands to check their inheritance status[14]. The exons covering the coding regions flanking the variants were amplified by PCR[14]. PCR products were cycle sequenced on both strands using the BigDye

Terminator v 3.1 chemistry (Applied Biosystems, CA, USA) and run on a 3130XL genetic analyzer (Applied Biosystems, CA, USA)[14]. Relatedness within families was confirmed through the Powerplex Fusion Kit (Promega, Madison, USA) when *de novo* mutations occurred[14].

Criteria for Pathogenicity of Rare or Novel Variants

HGMD transcript was used for the nomenclature of the detected variants[14]. Rare or novel variants were classified as being ‟pathogenic‟, ‟likely pathogenic‟, ‟variant of uncertain significance (VOUS)‟, ‟likely benign‟ or ‟benign‟, according to the international guidelines of the ACMG Laboratory Practice Committee Working Group[14]. The interpretation of the detected variants was performed according to the phenotype of the analyzed patients[14]. Three main in silico prediction softwares (Polyphen-2, SIFT and Mutation Taster) supported the evaluation of presumed functional effects of the detected variants[14].

Referring databases of diseases-causing variants included HGMD, GnoMad and DGV (http://dgv.tcag.ca/dgv/app/home)[14].

Statistical analysis

The distribution of the main demographic and clinical features among the three groups was statistically analyzed through the software MEDCALC (version 18.5). Kolmogorov-Smirnov Z test was performed to assess if the variables had the normal distribution. Variables with a normal distribution were reported as mean ± standard deviation. The differences among the groups were evaluated through Kruskal-Wallis

test, ANOVA and χ^2 test. Results were considered as statistically significant when $p < 0.05$.

RESULTS

Demographic data and clinical phenotypes

In the analyzed temporal range \ with epilepsy was referred to our institution. 58 of these patients (28 males and 30 females), with a mean age of 9,06 ± 6,97 years, underwent targeted next generation sequencing gene panels for epilepsy (30 gene panel was performed in 7 patients, 95 gene panel in 39 patients and 148 gene panel in 12 patients). The A, B and C Groups included, respectively 9 (4 females and 5 males), 31 (16 females and 15 males) and 18 (9 males and 9 females) patients.

Table 6 summarized the main clinical features and the prevalent epilepsy phenotypes in the three-abovementioned groups.

In the group A an earlier onset of seizures and a higher quote of severe intellectual disability were observed. Although the detected differences were not statistically significant, seizure-types at onset were prominently represented by infantile spasms and myoclonic seizures in the A and B group while a higher relevance of focal and tonic clonic seizures was assessed in the C group. During the follow-up a remarkable increase of the quote of patients with atypical absences was reported in the B group.

Other significant differences among the three groups were detected about the presence of developmental delay, intellectual disability, movement disorders and abnormalities of cranial circumference.

The quote of subjects with developmental delay was mildly higher in the B group than in the A group (in which developmental impairment followed the onset of seizures). Movement disorders patterns were comparable in the A group and B group in terms of frequency with a clear prominence of dystonia while hyperkinetic movement disorders involved a minority of the cases.

The frequency of associated non neurological manifestations was variable in the B and C groups but none of them acquired a remarkable importance for diagnostic characterization.

Abnormalities of head circumference (mainly microcephaly) were prominent in the A group while facial dysmorphisms had a higher frequency, even if not statistically significant, in the B group.

The differences in EEG patterns were statistically significant at the onset but not during the follow-up. EEG mainly evidenced, both at the onset and during the follow-up, a prominence of multifocal spikes and waves discharges in the A and B groups while focal abnormalities represented the most common patterns in the C group. The quote of normal intercritical EEG at the onset was almost double in the C group if compared with the other groups.

Brain MRI abnormalities were relatively non specific in all the three groups with a prominence of corpus callosum abnormalities in the A group, cortical atrophy in the B group and variable degree of ventricular enlargement or asymmetry in the C group.

Most of the patients that was selected for NGS study had a remarkable drug-resistance even if some antiepileptic treatments evidenced a higher efficacy in specific contexts (i.e. ACTH for patients with West syndrome belonging to the A group and old generation drugs such as valproate and phenobarbital for the B and C groups).

Molecular genetics findings and genotype-phenotype correlations

Mean obtained coverage was: a) for 30 genes panel: 95% of covered bases at ≥10×; b) for 95 gene panel: 98% bases of covered at ≥30×; c) for 148 gene panel: 99,7% of covered bases at 15X and 99,8 of covered basis at 40X.

Pathogenic or likely pathogenic variants at the targeted next generation sequencing were assessed in 18/58 patients (3/7 patients through 30 gene panel, 7 /39 patients through 95 gene panel and 7/12 patients through 148 gene panel) with a detection rate of 31,03% in the whole sample (42,9% for 30 gene panel, 17,9% for 95 gene panel and 41,7% for 148 gene panel). Detection rate was higher for the B group (51,6%) than in the C group (11,1%) while in the A group the panels allowed the detection of variants of uncertain significance only. Most of the detected pathogenic/likely pathogenic variants were de novo (13/18) while 3 variants were inherited by epileptic parents by an autosomal dominant transmission. An autosomal recessive inheritance involved 2 patients. Among the 18 pathogenic or likely pathogenic variants, there were 9 frameshift mutations (50%), 7 (38,88%) missense mutations, 2 (11,11%)

nonsense mutations, and 1 (5,55%) deletion. Types of mutation were not correlated with clinical severity.

Genes with pathogenic/likely pathogenic mutations were represented by (Table 7): SCN1A (in 3 patients), IQSEC2 (in 2 patients), PRICKLE1, GABRB3, SLC2A1, MFF, SCN1B, KCTD7, CDKL5; FOXG1, SYNGAP1, ATP1A3, GRIN2A, PRRT2 and CACNA1A (one affected patients for each one of these genes).

Fifty five variants of uncertain significance with no apparent links with the reported clinical phenotypes were detected in 31/58 patients. Table 8 shows the detected VOUS and compares the phenotype of the patients that carried them with the expected phenotypes based on previously reported data about the involved genes .

No molecular alterations were reported in 14 /58 patients (3 /7 patients through 30 gene panel, 10/39 patients through 95 gene panel and 1/12 patients through 148 gene panel).

Clinical and genetic details of these patients are summarized in Table 7. The phenotypic characterizations in these cases underlined several atypical presentations. These atypical presentations included: 1) the association of an epilepsy phenotype consistent with the Dravet syndrome ones, with microcephaly, micrognathia and spherocytosis in Patient 2 (variant c.4814A>T in SCN1A); 2) a severe early onset epileptic encephalopathy in patient 6 (variant c.820G>A in PRICKLE 1)[26]; 3) an unusual pattern resembling epilepsia partialis continua with photosensitivity also at low frequencies in patient 11 (variant c.533C>T in KCTD7-Fig. 3)[27]; 4) a severe

developmental impairment in patient 18 (variant c.649dup in PRRT2); 5) a complex phenotype including a severe developmental delay before the onset of seizures, a pattern of seizures consistent with the Dravet syndrome ones (myoclonic seizures, atypical absences, febrile seizures), paroxysmal dyskinesia and a remarkable EEG photosensitivity at low frequencies in a 21 months'old male with the longer reported deletion in the literature involving chromosome 2q24.3 and the related sodium channel gene cluster (including SCN1A, SCN2A, SCN3A, SCN7A and SCN9A)[28].

Illustrative cases

Patient 3

a) Clinical Report

This 21 months-old boy was born after in vitro fertilization. A first generalized seizure appeared during a febrile rotavirus gastroenteritis at 5 months. He came to our attention after myoclonic jerks with staring appeared at age 8 months. On examination exhibited severe developmental delay and sub-continuous bursts of choreatic movements, exacerbated by pain or external stimuli that were still present at follow-up in the following months (the attached video at 21 months-see on-line supplementary material). EEG showed generalized sharp waves and a photoparoxysmal response. Brain MRI revealed fronto-temporal cortical atrophy and mild corpus callosum hypoplasia. Tonic and myoclonic seizures were observed in the following months. A clinical suspect of Dravet syndrome was formulated. Seizures were partially improved with a combination of levetiracetam, clobazam and

stiripentol after failure valproate and clonazepam had been of no benefit. 148 gene panel was performed using a SureSelect custom capture (Agilent). NGS data analysis including both single nucleotide variant calling and exon copy-number variations analysis by CONVaDING tool was performed. Copy-number analysis detected a heterozygous deletion including the SCN1A and SCN2A NGS targeted genes, both located within the 2q24 region. SCN1A MLPA analysis (P137-B2, MRC-Holland) confirmed the SCN1A gene deletion. Array-CGH analysis defined the boundaries of a 6.1 Mb microdeletion on 2q24.3q31.1, between the regions 164375953 and 170535670, and including the genes SCN1A, SCN2A, SCN3A, SCN7A, SCN9A, GRB14, SLC38A11, GALNT3, TTC21B, XIRP 2 and STK39 (Fig. 2).

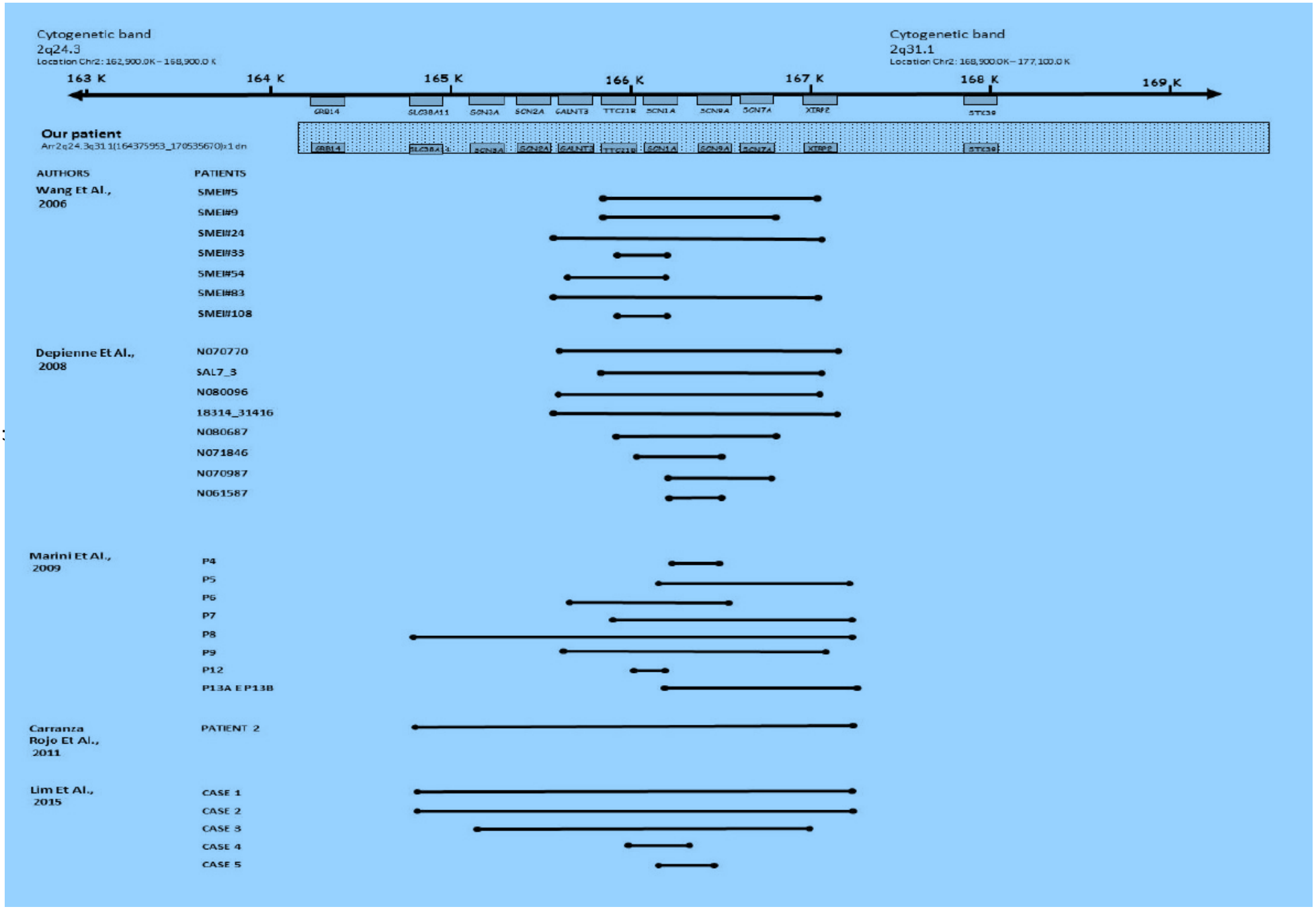
Discussion

Deletions involving the sodium channel gene cluster on chromosome 2q24.3, which includes SCN1A, SCN2A, SCN3A, SCN7A, and SCN9A, have been associated with variable phenotypes including Dravet syndrome, migrating partial seizures of infancy, and various occasional non specific dysmorphic features including ear abnormalities, microcephaly, micrognathia and brachysyndactyly[29]. None of the 72 previously published patients with 2q24.3 deletions exhibited movement disorders (Fig. 2). An hyperkinetic movement disorders with early onset was previously reported in 10 patients with Dravet syndrome carrying three different SCN1A missense point mutations (c.677C>T, c.4033C>T and c.1264G>T) and in about 7% of children with SCN2A encephalopathy[29]. The pathomechanisms causing these motor manifestations could reside in either loss or gain of function of Nav1.1 and

Nav1.2 channels that are both expressed in the basal ganglia[29]. In some patients with Dravet syndrome paroxysmal hyperkinetic movement disorders are precipitated by treatment with phenytoin or carbamazepine[29].

In the patient reported herein an epilepsy phenotype consistent with Dravet syndrome and a hyperkinetic movement disorder are associated with a deletion of the sodium channel gene cluster. Whether this unusual phenotype results from leading to haploinsufficiency of either SCN1A or SCN2A, or the combination of both, remains subject of speculation. However, there is no indication that any of the numerous reported patients with truncating mutations in SCN1A has ever manifested such a clinical phenotype[29]. Although dystonic posturing and a hypokinetic movement disorders, such as anterocollis and parkinsonian gait, have been described in adults with Dravet syndrome, signs of parkinsonism were not observed in our patient notwithstanding the deletion included the STK3 gene, which has been previously associated with this presentation in adults[30].

The molecular genetic basis underlying the reported phenotype was uncovered through an extended NGS analysis also including exon copy-number variations analysis. This approach, although not yet extensively included in the diagnostic process, allows a remarkable increase of the diagnostic yield.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GRB14
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SCN3A
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SCN7A
XIRP2
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Our patient
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AUTHORS
PATIENTS
Wang Et Al., 2006
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SMEI#9
SMEI#24
SMEI#33
SMEI#54
SMEI#83
SMEI#108
Depienne Et Al., 2008
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N080096
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N070987
N061587
Marini Et Al., 2009
P4
P5
P6
P7
P8
P9
P12
P13A E P13B
Carranza Rojo Et Al., 2011
PATIENT 2
Lim Et Al., 2015
CASE 1
CASE 2
CASE 3
CASE 4
CASE 5

Fig. 2 Topographic comparison between the deletions detected in our patients and those previously published in patients with available array CGH data. References of previously published patients:
- Wang J, Kurahashi H, Ishii A, et al Microchromosomal deletions involving SCN1A and adjacent genes in severe myoclonic epilepsy in infancy. Epilepsia 2006, 49:1528–1534.
- Depienne C., Trouillard O., Saint-Martin C. I. et al Spectrum of SCN1A gene mutations associated with Dravet syndrome: analysis of 333 patients, J Med Genet 2009;46:183–191.
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- Carranza Rojo D, Hamiwka L, McMahon JM , et al De novo SCN1A mutations in migrating partial seizures of infancy, Neurology 2011;77:380-3.
- Lim BC , Hwang H, Kim H , et al Epilepsy phenotype associated with a chromosome 2q24.3 deletion involving SCN1A:migrating partial seizures of infancy or atypical Dravet syndrome? Epilepsy Res. 2015; 109:34-9.

Patient 6

a) Clinical Report

This 25-month-old boy was born from non-consanguineous Indian parents after an uneventful pregnancy and labor. Family history evidenced a single relative in the maternal line with drug-responsive tonic seizures during childhood. The child's psychomotor development before the epilepsy onset was normal.

At the age of 10 months he manifested prolonged daily clusters of head-nodding attacks and myoclonic jerks. After the age of 13 months tonic and focal motor seizures also appeared and progressive developmental delay became apparent (at the age of 14 months Griffith's Mental Developmental Scales DQ was less than 50) with reduced alertness, poor social interactions and feeding. Despite ataxia, the child could still walk unsupported at 18 months of age. No notable dismorphic features or other non-neurological signs and symptoms were observed. Ictal EEG revealed generalized

delta activity associated with diffuse epileptiform discharges. Interictal EEGs showed multifocal spikes and sharp waves. Brain MRI and an extensive neurometabolic work-up were unremarkable.

Seizures were partially responsive to a combination of ACTH (2 cycles), valproate and clonazepam while other drugs (including phenobarbital, clobazam,pyridoxine and vigabatrin) were ineffective.

On the last examination, at 23 months of age, the boy showed mild ataxia, immature language, hyperactive behavior, and poor eye contact.

95 gene panel causing early infantile epilepsies revealed a novel homozygous missense mutation in the PRICKLE1 gene (NM_153026.2:c.820G>A, p.Ala274Thr). Both parents were heterozygous carriers of the mutation.

b) Discussion

The PRICKLE1 (Prickle Planar Cell Polarity Protein 1-MIM 608500) gene is involved in a calcium mediated regulation of planar neuronal polarity signaling during embryonic development as well as in neuronal morphogenesis, migration and networking[31].

PRICKLE1-related phenotypes not only include autosomal recessive progressive myoclonus epilepsy (PME)-ataxia syndrome (MIM 612437) and neural tube defects associated with heterozygous mutations but also agenesis of corpus callosum, polymicrogyria, and autistic spectrum disorder[26]. The broad heterogeneity of the phenotypic spectrum could be explained by a dosage effect involving the encoded

protein for patients carrying heterozygous variants and by the variable degrees of impairment that may occur in the cascade of signals modulated by PRICKLE 1 in the other cases[26].

PME has been reported in 23 subjects with homozygous variants and in 2 patients with heterozygous variants[26].

A positive neurocognitive outcome and a complete or partial responsiveness to valproate were reported in almost all cases[26]. In some patients a minor efficacy of antiepileptic treatment was observed even if no specific phenotypic feature was highlighted as a reliable predictor for an optimal responsiveness[26].

The patients so far reported presented a later onset of seizures (mean age higher than 4 years with the significant exception of a 7 month-old male carrying a de novo mutation and presenting with myoclonic seizures) and a less severe epilepsy than our patient[26].

Epilepsy with pleomorphic seizures and concomitant developmental arrest suggested the diagnosis of epileptic encephalopathy in our patient[26]. Seizures-related developmental and cognitive impairment have not been mentioned as part of the PRICKLE1-related phenotypes even though a systematic neurodevelopmental evaluation has not been performed in the previously reported patients[26]. Variants of PRICKLE 1 might contribute to epileptogenesis via various mechanisms such as: a) impairment of calcium mediated signaling in different brain regions, especially the cortex, thalamus and hippocampus; b) impairment of microtubule-associated vesicle

transport of neurotransmitters c) dysregulation of neurites' outgrowth and neuronal connectivity[26]. The mutation c.820G>A was indicated as pathogenic by different in silico prediction softwares (Mutation Taster, Polyphen 2 and SIFT) and the CADD score was of 31[26]. Three individuals heterozygous for this mutation, none homozygous, were present in the GnomAD database (http://gnomad.broadinstitute.org/)[26]. Mutation Taster and Interpro analysis predicted loss of the third LIM zinc binding domain of the protein[26]. As a consequence of the p.Ala274Thr transition, the substitution of an alanine residue with threonine changes the polarized protein distribution that is required for planar cell polarity signaling[26]. A similar effect was demonstrated in zebrafish for a mutation involving an adjacent residue (p.Thr275Met), which had been detected in a patient with neural tube defect and hydrocephalus but no epilepsy[26].

The role of PRICKLE 1 in different aspects of embryo neurodevelopment would suggest that cognitive and neurological functions can be impaired as a direct consequence of the defective protein, although severe epilepsy might have worsened the clinical picture[26]. Moreover, the alterations of neuronal signaling and networking cascades in which PRICKLE 1 is involved may result in dysfunctions of RE-1 silencing transcription factor or ubiquitin-specific peptidase 9 X-linked, which may as such contribute to the worsening of cognitive status[26].

Patient 11

a) Clinical Report

This 17 years old Italian girl had normal early developmental milestones. At the age of 10 months the patient was referred to a local hospital because of “jerky” movements involving the head and the upper limbs in the absence of EEG abnormalities (action myoclonus). She came to our attention at the age of 2 years, when she experienced her first seizures resembling the clinical pattern of an epilepsia partialis continua with sub-continuous jerks of her left upper limb distal extremity lasting more than 3 hours. EEG showed multifocal sharp waves, slow waves and spike and waves with a prominent involvement of the left hemisphere. Seizures were poorly responsive to benzodiazepines in the acute phases as well as to different maintenance treatments including phenobarbital and valproic acid. A progressive hemiparesis involving the left side of the body and an ataxic gait with a prominent trunk involvement were observed. Brain MRI was negative. Increased levels of GluR3 antibodies were detected in CSF after two distinct lumbar punctures. Ultrastructural features of a skin biopsy did not show any trace of CLN-type lysosomal storages.

A therapeutic trial with immunoglobulins and methylprednisolone resulted in a complete seizure freedom lasting for 4 months. After 4 months similar focal seizures re-appeared while the patient was under valproic acid and oral prednisone. Thereafter the patient completely lost all motor abilities and verbal functions and she became

wheelchair bound at the age of 28 months. At the age of 4 years staring episodes with eyelid myoclonia, often induced by intermittent light stimulation, were observed. In the following years, epileptic manifestations included both generalized (myoclonic seizures and generalized tonic-clonic seizures) and focal secondarily generalized seizures. EEGs were characterized by multifocal spikes and waves (mainly in the posterior regions- Fig. 3 A and B), photosensitivity also at very low frequencies (Fig. 1C), and an excess of slow activity (Fig. 3 B). Brain MRIs were negative at the age of 4, 7 and 10 years while a mild fronto-insular atrophy was observed at the age of 11 years (Fig.3 D). Epilepsy remained severely drug-resistant with recurring episode of status epilepticus over the years.

The homozygous mutation (c.533C>T) /p.(Ala178Val) in KCTD7 was demonstrated through 95 gene panel. The mutation was not included in the Human Gene Mutation Database (https://portal.biobase-international.com) and in the NCL database (http://www.ucl.ac.uk/ncl). In global/population databases this nucleotide variant was absent in the 1000 Genomes Project (www.1000genomes.org), showed a very low frequency in Exome Aggregation Consortium (http://exac.broadinstitute.org) (2.474e-05) and no homozygous in Gnomad (http://gnomad.broadinstitute.org/). The pathogenic role of the mutation was suggested by Mutation Taster and SIFT.

b) Discussion

Potassium channel tetramerization domain-containing protein 7 (KCTD7) gene encodes for a voltage-gated channel involved in the regulation of potassium fluxes in

the cell membrane excitability modulation and in the neuronal glutamine transporter SAT2 activity[27].

Clinical phenotypes associated with pathogenic mutations involving KCTD7 include epilepsy, action myoclonus, progressive ataxia and neurocognitive deterioration[27]. KCTD7-related progressive myoclonus epilepsy has been reported in 23 patients from 14 families with an autosomal recessive transmission[27]. Neuromotor and cognitive regression was observed in 17/23 patients including 6 subjects in which a complete seizure control was achieved with pharmacological treatment[27]. A clinical and pathological pattern consistent with a peculiar form of neuronal ceroid lipofuscinosis (CLN14) was reported in two previously reported patients[27].

Our patient is the first case of epilepsia partialis continua associated with a genetic channellopathy[27] . This case also expands the list of systemic monogenic disorders associated with this peculiar form of focal status epilepticus that includes genes encoding for signal transduction proteins (such as TBC1D24), cytoskeletal proteins (i.e. GFAP), mithocondrial proteins (i.e. ND3, POLG or ADCK3) or membrane transporters (i.e. ATP7A)[27]. The efficacy of methylprednisolone and immunoglobulins in the control of episodes of status epilepticus represented an interesting point of contact with other acquired etiologies of epilepsia partialis continua such as Rasmussen encephalitis[27]. This efficacy probably results from the interruption of the vicious circle including the activation of inflammatory cascade induced by epileptic seizures (overproduction of interleukins, complement proteins, prostaglandins, chemokines and adhesion molecules, infiltration of cell types

involved in innate and adaptive immunity, blood-brain barrier impairment) and the subsequent non transcriptional deleterious effects on ionic channels, potassium fluxes, glutamine transport, and glutamate synthesis and release[27].

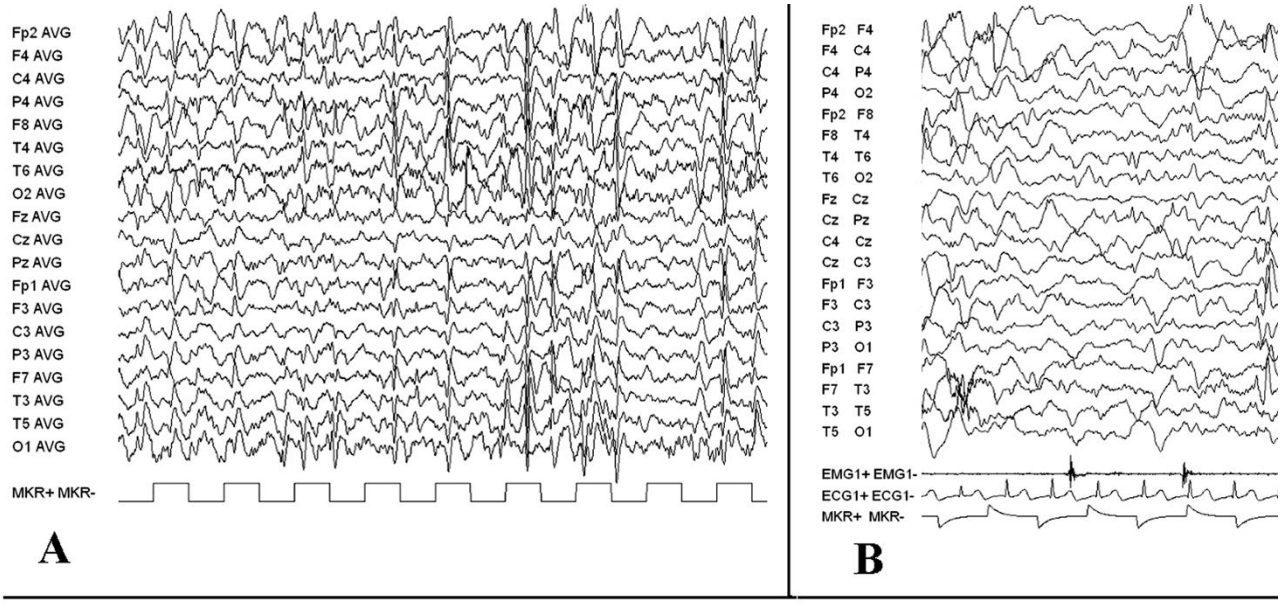
The disease natural history in our patient is consistent with the ILAE definition of "epileptic encephalopathy" as well as in the majority of previously published patients carrying mutations in KCTD7 gene[27]. The existence of six published patients with intellectual disability and motor symptoms but without seizures-induced neurodegeneration or drug-resistant epileps and the appearance of neurological regression before the onset of seizures in other patients suggest that KCTD7-related progressive myoclonus epilepsy could be also considered as a "developmental encephalopathy" in which cognitive regression represents a part of the phenotypic spectrum and not the consequence of epileptic manifestations[27]. Whether KCTD7-related progressive myoclonus epilepsy is to considered as a progressive epileptic encephalopathy is still a matter of discussion[27]. Some KCTD7 patients show disease progression for a few years after onset, followed by a clinical stabilization and large variation of their clinical conditions[27]. On the contrary, 9 out 19 patients whose head MRI record was reported, featured atrophic cortices and/or cerebellum, which are consistent with disease progression[27].

A single patient who developed myoclonic seizures, severe neurocognitive deterioration, optic atrophy leading to visual loss, and cortical frontal and cerebellar atrophy at the brain neuroimaging was reported[32]. These features (along with lysosomal storage consistent with ceroid-lipofuscin in fibroblasts and buffy coats at

electronic microscopy) allowed to include KCTD7-disease as a specific NCL form (CLN14)[27]. A second patient with similar features has been recently reported by our group[27]. Interestingly, the two reported CLN14 patients presented with an infantile onset and dramatically progressive course, as other NCL of infancy, a clinical feature which is not shared with the majority of KCTD7 patients reported so far[27].

The reasons of the existence of patients carrying KCTD7 mutations with and without lysosomal storage material has not a valuable explanation[27]. The mutation of our patient is located outside functional domain but upstream respect the other reported variants (p.Ala178Val)[27]. Staropoli et al demonstrated that the mutation c.550C>T, outside the BTB/POZ domain, impairs the interaction between KCTD7 and cullin 3 and, subsequently, endosomal and autofagosomal maturation processes resulting in an abnormal storage of ceroido lipofuscin-type material[27]. Our Patient with c.533C>T mutation did not show a similar storage despite she carried a mutation in a site close to the one involved in the mutation of the patient with CLN14 reported by Staropoli et al.[27] Some discrepancies result from the comparative analyses of the ultrastructural findings from skin biopsies as reported in literature[27]. Negative findings were described from ten patients[27]. Lysosomal storage seems to be a common ultrastructural marker shared among several progressive myoclonus epilepsies regardless the mutated gene[27]. Such findings may reflect the effort of the cells to discharge potentially toxic cytoplasmic aggregates, whose formation might be due to negative effects of the mutated genes on cell trafficking[27]. Furthermore, the presence of engulfed lysosomal might also be related to the disrupted autophagy, as described

in a knock-in mouse model of juvenile NCL as well as to impaired ubiquitin-proteasomic system, as suggested by the molecular interactions between pKCTD7 and Cullin-3[27]. Functional experiments showed that the different variants (p.F232fs, p.R94W,p.R184C, p.N273I and p.Y276C) have differential impact on KCTD7 and SAT2 function which regulates neuronal membrane potential and glutamine transport for glutamate synthesis[27]. However only in vitro experiments may elucidate the effect of p. Gly58Arg and p.Ala178Val on KCTD7 protein interactions.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F3 C3
C3 P3
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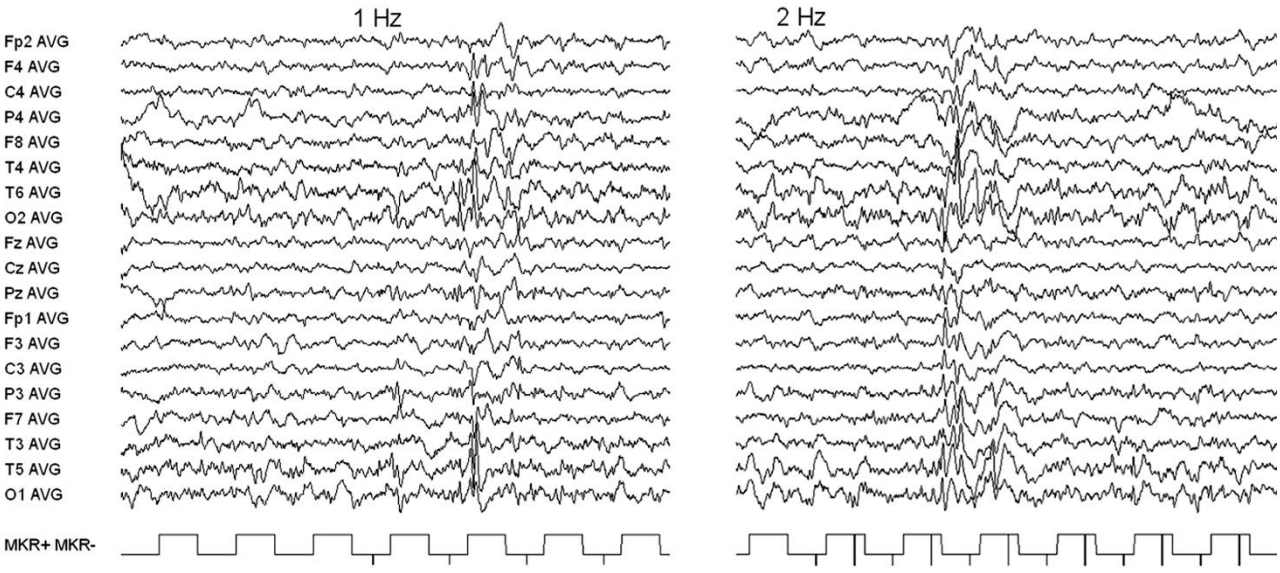
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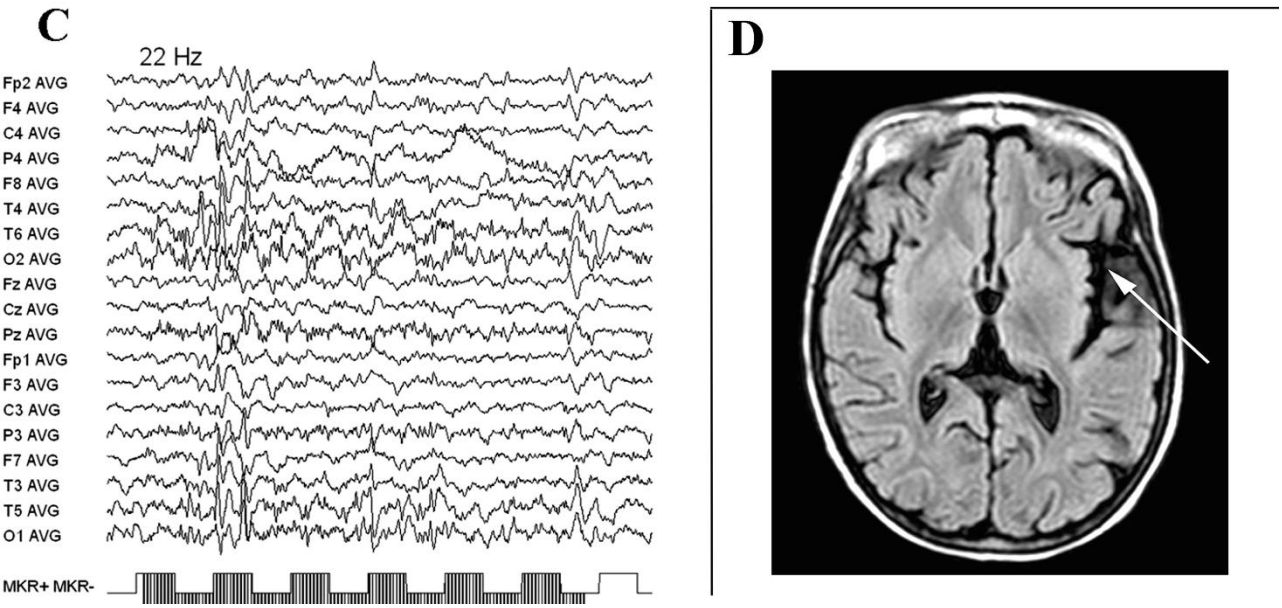
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Figure 3. Patient 11: A. Awake EEG: multifocal high-voltage sharp waves, slow waves, and atypical spike and wave complexes with slight prevalence in the temporo-occipital regions, tending to spreading. B. Polysomnography: multifocal epileptic abnormalities similar to those recorded in wakefulness; on the right deltoid muscle (EMG-1), two "jerky" movements not time-related with the EEG abnormalities. C. Spikes, poly-spikes, and spike and wave complexes in the temporo-occipital areas, spreading to the anterior regions, elicited by Intermittent Light Stimulation at low, medium, and high frequency stimulus rate. D. Brain MRI: mild localized atrophy in the left fronto-insular region (arrow). (EEG setting: filters 1.6 - 70 Hz; marker amplitude in A and C = 200 µV; in B = 250 µV).

Patients with negative results at NGS panels who underwent whole exome sequencing

10/ 46 patients, in which targeted gene panels gave no useful results for the diagnosis, underwent whole exome sequencing. Four out of ten patients received a molecular diagnosis: 1) a 4 years old male with a pathogenic variant of the gene *unc80 (*compound heterozygosis for c.1513C>T/p.Arg505Stop in exon 10 and c.3899de1C/p.Ala1300fs in exon 24*); 2)* a 13 years old boy with a de novo pathogenic variant of *Grin1*(c.1643G>A; p.R548Q); 3) a 18 year male with a de novo variant on the gene KCND3 (c.901T>C;p.Ser301Pro); 4) a 7 years old male with a de novo variant on ADSL gene (compound heterozygosis for the variants c.65C>T; p.Ala22Val and c.340T>C;p.Tyr114His). The first two patients presented with a developmental encephalopathy with epilepsy, profound intellectual disability and facial dysmorphisms. The third patient presented with a drug-responsive generalized epilepsy and a dystonia-parkinsonism. The fourth patient presented with a presenting

with an infantile epileptic and developmental encephalopathy with microcephaly and spastic tetraparesis.

DISCUSSION

The demonstration that a careful phenotypic evaluation remains essential for an adequate molecular diagnosis in epileptic subjects, also during the next generation sequencing era, represents the main indication for real world clinical practice from this study. In our sample three distinct targeted gene panels resulted in a higher detection rate for pathogenic/likely pathogenic variants in patients with developmental encephalopathies including epilepsy (B group) that accounted for half of the diagnosed cases. The clinical profile of this group, that emerged from our study, included a prominent onset of seizures after the second year of life, prevalence of plurifocal spikes and waves discharges at the EEG and the association of epilepsy with other clinical signs including movement disorders or microcephaly.

We used a restricted definition of "developmental encephalopathy including epilepsy" (B group) for that clinical presentations in which epilepsy and developmental delay occurred together as a consequence of the underlying etiology[23]. These disorders were distinguished from "epileptic encephalopathies" (A group) in which a neurocognitive deterioration resulted from epileptic activity itself[24]. Although we did not encounter any difficulty in establishing if developmental impairment preceded or followed the onset of epilepsy, this differentiation could not always be easy to realize[24]. For this reason, ILAE recently suggested the inclusion of

both the conditions in the single term “epileptic and developmental encephalopathies”[23]. The present study supports an approach based on the distinction of the two concepts of “epileptic encephalopathies” and “developmental encephalopathies”. Physicians should always systematically investigate the developmental and intellectual status before the onset of seizures because a pre-existing developmental delay or intellectual disability implicate that it is not realistic an expectation of clinical reversibility after an aggressive antiepileptic therapy[23]. Despite the differences in the available data, we tried to analyze, according to this criterium, the distribution of epileptic encephalopathies, developmental encephalopathies and idiopathic epilepsy without encephalopathy in the previously published patients who underwent targeted next generation sequencing (Table 4). The results of this analysis confirmed that a minority of patients who obtain a molecular diagnosis have an isolated idiopathic epilepsy (6,39% versus 35,6% of patients with developmental encephalopathy and 58,13% with epileptic encephalopathy) while in most of cases a developmental impairment or other signs of encephalopathy were reported (Table 4)[13-21]. Unlike the data of the literature, no patients with an ascertained epileptic encephalopathy obtained a molecular diagnosis in our cohort (10 variants of uncertain significance in 6 patients and no significant variant in 3 subjects)[13-21]. These data could have variable explanations including: a) the small number of patients in the A group (that is secondary to the strict selection of patients that was followed in this study, excluding subjects in which developmental impairment was not due to epilepsy); b) the frequent misuse of the term “epileptic

encephalopathy" in the literature (that is often erroneously used also for patients in which epilepsy is not the direct cause of developmental arrest/regression)[25]; 3) the frequent involvement of the mutated genes in pathological alterations of neurological development other than epileptogenesis[23].

The detection of pathogenic/likely pathogenic variants of SCN1A was the most frequent molecular diagnosis in our cohort, as in the other published series, while a higher quote of molecular diagnosis of IQSEC2 encephalopahy was performed (11,11% versus 0,86%)[13-21]. This result could probably suggest a possible underestimation of IQSEC 2 encephalopathy in subjects with severe intellectual disability because of the extreme variability of phenotypes associated with pathogenic/likely pathogenic variants of this gene and the different degrees of clinical severity that was recently highlighted between males and females[33].

In most of the diagnosed patients phenotypes were consistent with the ones that were previously reported in the literature with significant exception in less than one third of the cases[13-21, 34, 35, 36]. These results highlighted that targeted gene panels allow a remarkable increase of the molecular diagnosis of diseases with overlapping clinical presentation and also in atypical cases[13-21].

A high number of VOUS was detected (in 53% of analyzed patients) with no doubtful cases in which: a) consistent phenotypic data could add useful information in order to switch the interpretation of their potential role towards a possible pathogenic effect and/or b) the evaluation of functional effects of the detected

variants, through in silico prediction softwares, could result in significant structural/functional impairment of the encoded protein[37]. These results mirror the actual state of knowledges and it is not excluded that further clinical observations of different phenotypes associated with the involved genes and future in vitro functional studies on mutant proteins could change the role attributed to the VOUS that were detected in the present study[37].

The most important aim beyond the molecular characterization of children with genetically determined epilepsies is represented by the possibility to build tailored therapies[19]. To date, this goal has been achieved for few cases including ketogenic diet for SCL2A1, retigabine for KCNQ2, memantine for GRIN2A or GRIN2B, and quinidine for KCNT1[20]. In other cases targeted next generation sequencing allowed a more adequate antiepileptic treatment with traditional drugs resulting in the optimization of seizure control[20]. In our cohort the molecular diagnosis addresses therapeutic choices with positive results in almost all cases (Table 7): ketogenic diet induced a complete seizure remission in Patient 8 (mutation in SLC2A1); memantine was not used in Patient 17 (mutation in GRIN2A) because seizures freedom had already been obtained with valproate; a partial improvement in seizure control was observed in 8 patients and a complete seizure control in 5 patients.

The strength of this study is represented by the rigorous phenotypic criteria for the selection of candidates for targeted next generation gene sequencing for epilepsy.

The main limitations include the small dimension of the analyzed cohort and the restricted dimension of the used gene panels (if we consider that the number of genes that were associated with epilepsy in the literature range between 4500 and 5000)[20]. Other limitations are typical about the next generation sequencing methods: a) the high number of the detected variants of uncertain significance requiring detailed and prolonged interpretations of the correlated data and subsequent functional studies; b) the necessity of an efficient updating of the gene panels that should be always consistent with the continuous expansion of the knowledge about genetic basis of epilepsy (even if it implicates an increased complexity in bio-informatic analysis and genotype-phenotype correlation)[13-21].

CONCLUSIONS

De novo monogenic variants and, in a minority of the cases, large deletion of genes involved in epileptogenesis represent a relevant underlying etiology for epileptic and developmental encephalopathies and for epilepsies with a probable genetic etiology such as the ones in which a strong familial history or a combination of febrile and non febrile seizures are observed. Our study highlighted that the diagnostic yield is higher in developmental encephalopathy with epilepsy and in subjects with an onset of seizures in the first 3 years of life. This result could suggest an eventual modification of actual ILAE definitions towards the use of the terms “epileptic encephalopathies” and “developmental encephalopathies” as distinct concepts.

The early use of targeted panel testing for epilepsy results in economic advantages and cuts the prolonged diagnostic pathways of the past decades. Careful clinical phenotyping improves the detection rate of pathogenic variant and eases pharmacological planning. The achievement of these aims requires a close collaboration between the geneticists and epileptologists to ensure the proper management of genetic investigations in patients with epilepsy. This interaction is crucial for both paediatric and adults patients towards the aim of a personalized (precision) medicine.

PAPER	EXTENSION OF THE NGS GENE PANEL	NUMBER OF PATIENTS	AGE RANGE OF AT SEIZURES ONSET	DETECTION RATE FOR PATHOGENIC/ LIKELY PATHOGENIC VARIANTS	GENES WITH PATHOGENIC/ LIKELY PATHOGENIC VARIANTS (number of patients)	EPILEPSY PHENOTYPE OF PATIENTS WITH PATHOGENIC/ LIKELY PATHOGENIC VARIANTS	NUMBER OF PATIENTS WITH EPILEPTIC ENCEPHALOPATHIES CARRYING PATHOGENIC / LIKELY PATHOGENIC VARIANTS	NUMBER OF PATIENTS WITH DEVELOPMENTAL ENCEPHALOPATHY CARRYING PATHOGENIC / LIKELY PATHOGENIC VARIANTS	NUMBER OF PATIENTS WITH ISOLATED IDIOPATHIC EPILEPSY CARRYING PATHOGENIC / LIKELY PATHOGENIC VARIANTS
Parrini et al 2016	30 genes and 95 genes	349	1 day-12 years	19,3% for 30 gene panel; 18,3% for 95 gene panel;	*SCN2A (9);*	Neonatal onset EE in 8 (2 patients with Ohtahara syndrome); Childhood onset EE in 1 patient presenting with MMPSI and spasms	9	0	0
					SCN1A (8);	Dravet syndrome in 6 patients ; 2 patients with non specified drugresistant epilepsy;	0	6	2
					KCNQ2 (6);	Neonatal onset EE in 4 patients ; EiEE in 2 patients;	6	0	0
					STXBP1 (6);	Neonatal onset EE in 4 patients ; EIEE in 2 patients	6	0	0
					SCN8A (5);	EIEE in 4 patients ; Infantile onset focal epilepsy	4	1	0
					CDKL5 (4);	EIEE in 4 patients (2 patients with West syndrome)	4	0	0
					MECP2 (4);	Rett syndrome in 4 patients	0	4	0
					KCNT1, UBE3A, SPTAN1, SYNGAP1, HCN1, GABRB3 (2);	MMPSI in 1 patient with KCNT1 mutations;	8	4	0
						Angelman syndrome in patient with UBE3A mutations;			
						EIEE in 1 patient and focal epilepsy in 1 patient with SPTAN1 mutations;			
						West syndrome in 1 patient and generalized epilepsy in 1 patient with SYNGAP1 mutations;			

						Dravet-like syndrome in 1 patient and neonatal EE in 1 patient with HCN1 mutations,			
						EIEE in 1 patient and West syndrome in 1 patient with GABRB3 mutations;			
					KCNB1, IQSEQ2, GABRG2, GABRA1, ARX, PCDH19, SLC25A22, MEF2C, CNTNAP2, PNPO, DEPDC5, PDHA1, PIGA, GNAO1, KCNA1, ATP1A2 and KCNA2 (1 patient)	Neonatal EE in 1 patient with KCNA1 mutations;	11	3	3
						Generalized epilepsy in 1 patient with KCNA2 mutations;			
						West syndrome in 1 patient with KCNB1 mutations;			
						Infantile EE in 1 patient with GABRA1 mutations;			
						Generalized epilepsy in 1 patient with GABRG2 mutations:			
						Childhood onset EE in 1 patient with IQSEC2 mutations;			
						Focal epilepsy (febrile and afebrile seizures) in 1 patient with PCDH19 mutations;			
						Ohtahara syndrome in 1 patient with SLC25A22 mutations;			
						Infantile EE in 1 patient with MEF2C mutations;			
						Focal epilepsy in 1 patient with *CNTNAP2 mutations;*			
						Infantile EE in 1 patient with ARX mutations;			
						Neonatal EE in 1 patient with PNPO mutations;			
						Focal epilepsy in 1 patient with DEPDC5 mutations;			
						Infantile EE in patient with PDHA1 mutations;			
						Infantile EE in patient with PIGA mutations;			
						Focal epilepsy in 1 patient with GNAO1			

						mutations;			
						EIEE in 1 patient with ATP1A2 mutations			
Gokben et al 2016	16 genes	30	7 months-17 years	40%	*SCN1A (3)*	Dravet syndrome in 2 patients and MMPSI in 1 patient	7	5	0
					KCNQ2 (2)	Early onset EE in 1 patient; Ohtahara syndrome in 1 patient			
					SCN2A (1)	Ohtahara syndrome			
					PCDH19 (1 patient)	Epilepsy and mental retardation limited to females			
					CDKL5 (1 patient)	Rett syndrome			
					STXBP1 (1 patient)	Early onset EE			
					FOXG1 (1 patient)	Rett syndrome			
					CNTNAP 2 (1 patient)	Early onset EE			
					MBD5 (1 patient)	Early onset EE			
Ortega Moreno et al 2017	83 and 106 genes	87	2 days-3years	19,5%	*KCNQ2 (4),*	Early onset EE	16	1	0
					CDKL5 (3),	Unclassified EE			
					POLG (2);	Unclassified EE			
					SCN1A (1 patient), PCDH19 (1 patient), STXBP1 (1 patient), SLC2A1 (1 patient), ARX (1 patient), ALG13 (1 patient), SYNGAP1 (1 patient), GRIN1 (1 patient), CHD2 (1 patient);	1 patient with Dravet syndrome (SCN1A mutation);			
						1 patient with Lennox Gastaut syndrome syndrome (ALG13 mutation),			
						1 patient with early onset EE (STXBP1 mutation);			
						Unclassified EE in 5 patients with mutations in PCDH 19, SLC2A1, ARX, SYNGAP 1 and GRIN1			
					GABRB2 (11)	NA			
					NUS1(8)				
					SCN1A (6)				
					SCN8A (5)				
					NTRK2 (5)				
					RAB11A (4)				
					SCN2A (3)				
					KCN1T (3)				
					IQSEC2 (2)				
					CACNA1A (2)				

					GNAO 1 (2) *GABRG2, KIIA2022, KCNQ2, HIVEP2, ANKRD11, ATP1A3, DNM1, FGF12, HECW2, DDX3X, MEF2C, NAA10, , ARID 18, COL4A1, , PPP2R1A, KCNA2, MED 13L, SNAP25, NF1, SYNGAP 1, WWOX, SZT2, NAGA, TBC12D24, SLC9A6, DNMT3A, PCDH 19, UBE3A (1 patient for each gene)*				
Bevilacqua et al 2017	70 and 377 genes	305	NA	31%	*ADGRV1 (44), COL18A1 (26), KMT2D (23), PCNT (21), RELN (19), not specified the other genes carrying pathogenic abnormalities*	NA	NA	NA	NA
Rim et al 2018	172 genes	74	Mean age: 7.5 ± 7.8 months	37,8%	*STXBP1 (3)* *CDKL5 (2)* *KCNQ2 (2)* *SCN1A (2)* *SYNGAP 1 (2)* *GNAO1 (2)* *KCNT1 (2)* *BRAT 1 (2)* *WWOX (2)* *ZEB (1 patient)* *CH2 (1 patient)* *PRICKLE 2 (1 patient)* *COL4A1 (1 patient)* *DNM1 (1 patient)* *SCN8A (1 patient)* *MECP2 (1 patient)* *SLC9A6 (1 patient)* *Pathogenic copy number variants (3)*	Infantile spasms in 16 patients : BRAT1 (1), CDKL5 (2), COL4A1 (1), DNM1 (1), GNAO1 (2), KCNQ2 (2), MECP2 (1), STXBP1 (3), WWOX (1), CNV (2). Dravet syndrome in 2 patients with SCN1A mutations; MMPSI in 1 patient (CH2 mutation); Doose syndrome in 1 patient (KCNT1 mutation); Not specified epilepsy syndrome in 8 patients : KCNT1 (1), PRICKLE2 (1), SLC9A6 (1), SCN8A (1), SYNGAP1 (2), ZEB2 (1), CNV (1)	17	11	0

Ko et al 2018	172 genes	278	3-18 months	37,1%	*SCN1A (11 patient) ;*	Dravet syndrome in 11 patients with SCN1A mutations;	76	11	6
					CDKL5 (9),	Early onset EE in 5 patients , West syndrome in 2 patients ; Ohtahara syndrome in 2 patients			
					CHD2 (8),	Unspecified generalized epilepsy in 5 patients ; Doose syndrome in 2 patients ; Lennox Gastaut syndrome in 1 patient;			
					KCNQ2 (7),	Ohtahara syndrome in 4 patients ; Eearly onset EE in 3 patients			
					STXBP1 (7),	Focal epilepsy in 3 , patients; Ohtahara syndrome in 2 patients , Early onset EE in 2 patients			
					SCN2A (5),	Ohtahara syndrome in 3 patients , Lennox-Gastaut syndrome in 1 patient, Focal epilepsy in 1 patient			
					SCN8A (5),	Early onset EE in 1 patient ; West syndrome in 5 patients			
					SYNGAP1 (5),	Lennox Gastaut in 4 patients , Doose syndrome in 1 patient			

					KCNT1 (3), PCDH 19 (3); BRAT 1 (3); ALDH7A1 (2); DNM1 (2); EEF1A2 (2); KCNB1 (2); UBE3A (2); ZEB (2); ARX (1 patient); CACNA1A (1 patient); CACNB4 (1 patient); CASK (1 patient); GNAO 1(1 patient) ; GRIN2A (1 patient) ; HCN1(1 patient) ; IQSEC2 (1 patient) patient) ; KANSL 1(1 patient) ; KCNA1 (1 patient);PRODH(1 patient) ;SCN1B (1 patient);SCN3A (1 patient); SYN1 (1 patient); WWOX(1 patient)	Non specified the relationship mutated genes-phenotypes			
Peng et al 2018	308 and 540 genes	141	NA	32,6%	**** *SCN1A (21), TSC2 (7), SCN8A (5), CDKL5(5), TSC1(4), KCNMA1(4), STXBP1(3), KMT2D(2), MECP2(3), KCNQ2(3), HCN1(2), ITPR1(2), CHD2 (2), KCNT1(2), GABRG2(1patient), SCN2A(1patient), SCN9A(1patient), TRPM6(1patient); ALDH7A1(1patient) ,PP1(1patient), PNPO(1), SLC35A2(1) PCDH19(3), SPTAN1(1patient) ARX(1patient),*	**** Dravet syndrome (31 patients), West syndrome (19 patients), epilepsy combined with global developmental delay (14 patients), epilepsy with focal seizures (10 patients), Malignant migrating partial seizures of infancy (3 patients), progressive myoclonic epilepsy (3 patients), early onset epileptic encephalopathies (2 patients), Ohtahara syndrome (2 patients), early infantile EE (1 patient), and epilepsy with generalized tonic clonic seizures (1	**** 30	**** 45	**** 11

					FOXG1 (1patient), MED17(1patient)), DOLK(1patient),GNAO1(1patient), GNB1(1patient), TBC1D24(1patient), DNM1(1patient), SLC6A1(1patient),	patient)			
Liu et al 2018	153 genes	173	1 day-14 years	23,3%	*SCN1A (16), TSC2 (5), STXBP1 (2), SCN8A (2), TSC1(1), MECP2 (1), CHD2 (1), PCDH19 (1), GABRA1 (1), GABRB3 (1), SLC2A1 (1), SLC9A6 (1), IQSEC2 (1), KCNQ2 (1), SCN2A (1), CACNA1A (1), KCNT1 (1), SYNGAP1 (1), ATP1A2 (1), CDKL5 (1), ADSL (1), VRK2 (1)*	23 patients with Dravet syndrome, 10 patients with Ohtahara syndrome, 2 patients with Ohtahara syndrome evolving to West syndrome; 10 patients with West syndrome; 2 patients with West syndrome evolving to Lennox-Gastaut Syndrome; 5 patients with Lennox-Gastaut syndrome; 4 patients with Doose syndrome; 2 patients with epilepsy of infancy with migrating focal seizures; 2 patients with epileptic encephalopathy with continuous spike and wave during sleep, 1 patient each with temporal lobe epilepsy, early myoclonic encephalopathy, Landau-Kleffner syndrome	12	31	0

NA: not available; EE: epileptic encephalopathy

****** The quote of with pathogenic/likely pathogenic variants at targeted NGS panels and the correlated clinical features were not specified. The authors realized a whole analysis of a wider sample(273) that also included who underwent whole exome sequencing. The results reported in these column consider the whole sample.**

Table 4 Molecular genetic and epilepsy phenotype in the main published targeted NGS studies in the literature

AARS	CDKL5	EPM2A	GOSR2	KCNB1	MEF2C	PIGN	SCARB2	SLC25A22	STXBP1
ADRA2B	CHD2	FARS2	GRIN1	KCNC1	MMF	PIGQ	SCN1A	SLC2A1	SYN1
ALDH7A1	CHRNA2	FGF12	GRIN2A	KCNH1	MTOR	PIGT	SCN1B	SLC35A2	SYNGAP1
ALG13	CHRNA4	FOLR1	GRIN2B	KCNJ10	NECAP1	PLCB1	SCN2A	SLC35A3	SYNJ1
AP3B2	CHRNB2	FOXG1	GRIN2D	KCNK18	NHLRC1	PLPBP	SCN8A	SLC6A1	SZT2
ARHGEF9	CPA6	FOXP1	HACE1	KCNMA1	NPRL2	PNKD	SCN10A	SLC6A5	TBC1D24
ARV1	CSNK1G1	FOXP2	HCN1	KCNQ2	NPRL3	PNKP	SERPINI1	SLC6A8	TCF4
ARX	CSTB	FRRS1L	HNRNPU	KCNQ3	NRXN1	PNPO	SHANK3	SLC9A6	TPP1
ATP1A2	DDX3X	GABRA1	HUWE1	KCNT1	PC	POLG	SIK1	SMC1A	UBA5
ATP1A3	DENND5A	GABRB3	IQSEC2	KCTD7	PCDH19	PRICKLE1	SLC12A5	SON	UBE3A
ATRX	DEPDC5	GABRG2	ITPA	LGI1	PDHA1	PRRT2	SLC13A5	SPATA5	WDR45
BRAT1	DLAT	GLRA1	ITPR1	LIAS	PDHB	PURA	SLC19A3	SPTAN1	WWOX
CACNA1A	DNM1	GLRB	KANSL1	MBD5	PDP1	QARS	SLC1A2	ST3GAL3	
CACNB4	DOCK7	GNAO1	KCNA1	MDH2	PIGA	RELN	SLC1A3	ST3GAL5	
CAD	EEF1A2	GNB1	KCNA2	MECP2	PIGG	ROGDI	SLC25A12	STX1B	

Table 5 List of 148 genes included in the targeted gene panels for epilepsy that were performed in our cohort

CLINICAL FEATURES	GROUP A	GROUP B	GROUP C	P (test)
MEAN AGE AT THE ONSET OF SEIZURES	5,88±4,56 months	24,35±33,31 months	48,72± 47,42 months	**0,0004 ((kruskal-wallis test)**
SEIZURES TYPES AT THE ONSET	**IS** 30%**; M** 30%; **T** 10%; **F** 10%; **FS** 10 %;	**M** 17,6%; **C** 17,6%; **T** 14,7%; **F** 14,7%; **AA** 11,8%; **A**11,8%; **FS** 8,8%; **TC**2,9%	**FS** 22,22 %; **TC**22,22 %; **T** 16,7%; **F**11.2%; **M** 11,1 %; **C** 5,6%; **TA** 5,6 %; **A** 5.6 %;	>0,05 (χ^2 test)
PROMINENT EEG PATTERNS AT THE ONSET	**p**33,3 %, **f** 22,2%, **i**22,2; **n** 11,1 %; **sba:** 11,1%.	**p** 45,2%, **f** 32,3%, **n**16,1%, **dfa** 6,5%	**f** 50%, **n** 33,3 %, **p** 16,7%.	**0,02 (χ2 test)**
PROMINENT SEIZURES TYPES DURING FOLLOW-UP	**C** 19%,**M** 14,3%,**T**14,3% **AA**14,3%,**F** 14,3%, **A=** 10,5% **IS** 9,5%,**TC** 4,8%	**AA** 24,1%,**C** 16,7%,**M** 14,8%,**T** 13% ,**TC** 13%,**A** 11,1%, **F** 3,8%	**TC**20 %,**AA**16,7%,**TA=**13,3% , **M** 13,3 %,**C**13,3 %,**T** 10% ,**A**6,7 %, **F** 6,6 %	>0,05 (χ2 test)
PROMINENT EEG PATTERNS DURING FOLLOW-UP	**p** 55,6%, **f** 22,2 %, **n** 11,1%, **dfo** 11,1%	**p** 41,9%, **f** 32,3%, **n** 19,4%, **dfo** 6,4%	**f** 44,4%, **n**33,3 %, **p**22,2%,	>0,05 (χ2 test)
DEVELOPMENTAL DELAY	66,7%	74%	16,7%	**0,002 (χ2 test)**
INTELLECTUAL DISABILITY	**S** 88,8 %; **MO** 12,1 % ;	**S** 35,5%; **MO** 29% ; **MI** 22,6%; **B** 12,9	0 %;	**0,0002 (χ2 test)**
MOVEMENT DISORDERS	**N** 44,4%; **D** 44,4%; **HMD 11,1** %;	**N** 41,7%; **D** 22,5%; **At** 9,6%; **HMD** 9,6%;	0 %;	**0,0018 (χ2 test)**
FACIAL DYSMORPHISMS	11,1%	25%	0%	>0,05 **(χ2 test)**
ABNORMALITIES OF CRANIAL CIRCUMFERENCE	44,4%	25%	0%	**0,0039 (χ2 test)**
NON NEUROLOGICAL MANIFESTATIONS	**N** 100 %	**H** 16,1%; **G** 6,45%; **Ca** 3,22%; **Sk**3,22%	**H** 5,5%;**E** 5,5%; **AD** 5,5 %; **Sk** 5,5%	>0.05 (χ2 test)
BRAIN MRI ABNORMALITIES	**N** 44,4 %; **CCA** 22,2 %; **CA** 11,1 %; **VE** 11,1 %; **PVL** 11,1 %;	**N** 38,8%; **CA** 28,95%; **VE** 12,9%; **PVL** 9,6%; **CCA** 6,45%; **BGA** 3,22%	**N** 77,7%; **VE** 11,1%; **CA** 5,5%; **CBA** 5,5%; **PVL** 5,5 %; **CCA** 5,5%; **BGA** 5,5 %	>0.05 (χ2 test)
MORE EFFECTIVE ANTIEPILEPTIC TREATMENTS	**ACTH** 33,3**%; VA** 11,1%; **PTH** 11,1% **LTG**11,1 %; **CBZ** 11,1%; **TPM** 11,1%; **CLB** 11,1%; **PB** 11,1%; Z**NS**11.1%; **FBM** 11,1%	**VA** 29%; **LTG** 9,3%; **CBZ** 6,45%; **TPM** 6,45%; **CLB** 6,45%; **PB** 6,45%; **LEV** 3,22%; **CLN** 3,22%; **KD** 3,22%; **LAC** 3,22%; P**YR** 3,22%; E**TS** 3,22%	**VA**22,2 %; **PB** 16,6%; **PTH** 11,1 **TPM** 11,1 %; **LEV** 11,1 %; **CLB** 5,5%;	Not applicable

LEGEND: M=myoclonic; T=tonic; C=clonic; F=focal; AA=atypicalabsences; A=atonic; FS= febrileseizures; TC=tonicclonic; IS=infantile spasms; TA=typicalabsences; p= plurifocalspike and waves discharge; f=focal abnormalities; i=hypsarrhytmia; n= normal; dfa=disorganized for age; sba=slow background activity; S=severe; MO=moderate; MI=mild; B= cognitive borderline; D=dystonia; At=ataxia; HMD=hyperkineticmovementdisorders; N=none; H=hematologicalabnormalities; G=gastrointestinal abnormalities; Ca=cardiological abnormalities; Sk= skin abnormalities; E= endocrinological abnormalities; AD=autoimmune disorders; VE=ventricular enlargement; PVL=Periventricular leukomalacia; CA=cortical atrophy; CCA=corpus callosuum abnormalities; MCD= malformations of cortical development; BGA= basal ganglia abnormalities; CBA=cerebellar atrophy;VA= valproic acid; CBZ= carbamazepine: PTH= phenytoin; TPM= topiramate; CLB=clobazam;LTG= lamotrigine; LEV= levetiracetam; CLN=clonazepam; KD=ketogenicdiet; LAC= lacosamide; PYR=pyridoxine; ETS=ethosuximide; ZNS=zonisamide: FBM=felbamate.

Table 6 Main clinical features and epilepsy phenotype of the reported cohort

PATIENT/ SEX	AGE	AGE AT ONSET OF SEIZURES	AGE AT THE MOLECULAR DIAGNOSIS	PROMINENT SEIZURE TYPES	OTHER CLINICAL FEATURES	DISEASE-CAUSING GENES , PATHOGENIC VARIANTS AND TYPE OF VARIANTS	REFERENCE FOR THE DETECTED VARIANTS	EEG	BRAIN MRI	RESPONSE TO TREATMENT
1 F	10 Y	3 D	9 Y	T	Moderate intellectual disability Ataxia	**SCN1 A** c.4907G>A p.(Arg1636Gln) De novo Likely pathogenic variant	NPR	p	Normal	Partial seizure control with valproate and clonazepam
2M	18 Y	4 m	10 Y	C, M, TC	Moderate intellectual disability Microcephaly Micrognathia Spherocytosis	**SCN1 A** c.4814A>T p.(Asn1605Ile) De novo Likely pathogenic variant	NPR	p	Normal	Partial seizure control with valproate and topiramate
3M	2Y	5 m	16 m	M, AA	Developmental delay Paroxysmal dyskinesia Dystonic postures	**SCN1A and SCN2A** 6,1 Mb microdeletion on 2q24.3q31.1 between the regions 164375953 and 170535670 De novo likely pathogenic variant	NPR	P	Normal	Partial seizure control with levetiracetam, clobazam and stiripentole
4	8 Y	2Y	7y	T, A, M	Severe intellectual disability Spastic dystonic tetraparesis Micrognathia Recurrent dermatitis	**IQSEC2** c.854del p.(Pro285Leufs*21) De novo likely pathogenic variant	NPR	p	Normal	Partial seizure control with ketogenic diet
5	8Y	12 m	6Y	M	Severe intellectual disability Rett-like phenotype	**IQSEC2** c.4110_41111del p.(Tyr1371Glnfs*15) De novo likely pathogenic variant	NPR	f	Corpus callosuum hypoplasia	Complete seizure control with valproate
6	3Y	10 m	18m	M, T, F	Severe developmental regression	**PRICKLE 1** c.820G>A p.(Ala274Thr)	14	p	Normal	Partial seizure control with ACTH valproate

					Autism spectrum disorder Ataxia	De novo likely pathogenic variant				
7	8Y	9m	12y	FS, AA	None	**GABRB3** C.146A>G p.(Asp49Gly) De novo likely pathogenic variant	NPR	n	Normal	No treatment
8	12Y	2Y	11Y	F, AA	Mild intellectual disability Ataxia	**SLC2A1** c.470dup p.(Thr158Hisfs*79) De novo likely pathogenic variant	NPR	p	Periventricular leukomalakia	Complete Seizure control with ketogenic diet
9	12Y	12m	11Y	M	Leigh syndrome	**MFF** c.892C>T p.(Arg298*) Pathogenic variant Autosomal recessive inheritance (both parents were carriers)	18	p	Cortical atrophy Bilateral basal ganglia lesions	Severe Drug-resistance
10	5Y	12 m	5Y	FS, C	None	**SCN1B** c.6del p.(Arg3Glyfs*5) Likely pathogenic variant Autosomal dominant inheritance (inherited by the epileptic mother)	NPR	n	Ventricular asimmetry	No treatment
11	18Y	2Y	16Y	M, AA	Developmental Regression Spastic-dystonic Tetraparesis	**KCTD7** c.533C>T p.(Ala178Val) Pathogenic variant Autosomal recessive inheritance (both parents were carriers)	15	p	Cortical atrophy	Severe Drug-resistance
12	16 Y	1m	13Y	C, T, AA	Severe intellectual disability Spastic-dystonic tetraparesis Rett-syndrome phenotype	**CDKL5** c.587C>T p.(Ser196Leu) De novo likely pathogenic variant	19	p	Normal	Partial seizure-control with valproate and lacosamide
13	2Y	6m	15m	C	Rett-syndrome	**FOXG1**	20	f	Corpus	Complete seizure

					phenotype	c.946del p.(Leu316Cysfs*10) De novo likely pathogenic variant			callosuum hypoplasia	control with phenobarbital
14	4Y	3Y	3Y	AA	Developmental Delay Dystonia	**SYNGAP1** c.3706C>T p.(Gln1236*) De novo likely pathogenic variant	NPR	p	Normal	Complete seizure control with valproate
15	6Y	3Y	6Y	AA, M	Moderate intellectual disability	**CACNA1A** c.4446del p.(Tyr1483Thrfs*27) De novo likely pathogenic variant	NPR	f	Normal	Partial seizure control With valproate
16	10 Y	1 m	8Y	T, C	Severe intellectual disability Dystonia	**ATP1A3** c.2324C>G p.(Pro775Arg) De novo likely pathogenic variant	NPR	p	Normal	Complete seizure control with topiramate
17	7Y	4Y	7Y	AA	None	**GRIN2A** c.1784dup p.(His595Glnfs*21) Likely pathogenic variant Autosomal dominant inheritance (inherited by the epileptic mother)	NPR	p	Normal	Complete seizure control with valproate
18	12Y	5m	11Y	T, M, AA	Moderate intellectual disability Spastic diplegia	**PRRT2** c.649dup p.(Arg217Profs*8) Likely pathogenic variant Autosomal dominant inheritance (inherited by the epileptic father)	16	p	Normal	Partial seizure control with lamotrigine and clobazam

LEGEND: Y= years; m=months; M=myoclonic; T=tonic; C=clonic; F=focal; AA=atypical absences; A=atonic ; FS= febrile seizures; TC=tonic clonic; NPR= not previously reported p= plurifocal spike and waves discharge; f=focal abnormalities; i=ipsaritmia; n= normal

Table 7 Clinical and molecular phenotype of the patients with pathogenic/likely pathogenic variant in our cohort.

PATIENT	GENE	VOUS	MUTATION STATUS	EXPECTED PHENOTYPE	OBSERVED PHENOTYPE	NOTES
A	SPTAN1	c.5881G>A p.(Gly1961Arg)	heterozygosis	Infantile spasms with hypsarrhythmia, Other Generalized seizures, Developmental delay, Intellectual disability, Spastic quadriplegia, Progressive microcephaly, Hypomyelination and diffuse brain atrophy on MRI	Focal epilepsy, mild intellectual disability, allucination	Phenotype not compatible with the detected gene including the VOUS
B	CACNA1H CACNA1H SLC6A8	c.1367C>G p.(Ala456Gly) c.2561G>A p.(Arg854Gln) c.1649C>G p.(Thr550Ser)	heterozygosis heterozygosis heterozygosis	Absence epilepsy, Febrile seizures; Myoclonic-atonic seizures, Temporal lobe seizures, generalized tonic-clonic seizures, Hyperaldosteronism (rare) Febrile seizures, generalized tonic-clonic seizures, Myoclonic seizures, language delay, movement disorders, intellectual disability	Myoclonic seizures with photosensitivity, selective mutism,	Phenotype not compatible with the detected genes including the VOUS
C	GRIN2B	c.3983G>A p.(Gly1328Asp)	heterozygosis	West syndrome	Myoclonic seizures, Absence seizures, microcephaly, developmental delay, language delay	Phenotype not compatible with the detected gene including the VOUS
D	SPTAN1	c.4045C>T p.(Arg1349Trp)	heterozygosis	Infantile spasms with hypsarrhythmia, Other Generalized seizures, Developmental delay, Intellectual disability, Spastic quadriplegia, Progressive microcephaly, Hypomyelination and diffuse brain atrophy on MRI	Dravet syndrome (Febrile or afebrile seizures, Generalized or unilateral clonic seizures, Myoclonic seizures, Atypical absences, Partial seizures, Photosensitivity, Developmental delay or regression, Ataxia); Genetic epilepsy with seizures plus (GEFS+)	Pathogenic variant on SCN1A (Patient in Table 7)
E	SCN10A	c.3087+2T>C	heterozygosis	FIRES, Infantile spasms, Lennox-Gastaut syndrome, Episodic pain disorder, Autism spectrum disorders	Early onset absences, ataxia, intellectual disability	Pathogenic variant on SLC2A1 (Patient 8 in Table 7)
F	SCN1B PLCB1	c.574G>A p.(Ala192Thr) c.2474A>G p.(Lys825Arg)	heterozygosis heterozygosis	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, atypical absences, focal dyscognitive), Global developmental delay, Spasticity, Diffuse multifocal sharp waves and sharp waves-slow waves on EEG, Cortical atrophy on MRI Tonic seizures, Infantile spasms, Developmental delay, Suppression burst or Hypsarrhythmia on EEG	Focal seizures, autism spectrum disorder, intellectual disability, Motor stereotypes, Microcephaly, Developmental delay with no language	Phenotype not compatible with the detected genes including the VOUS
G	GRIN2A	c.3118G>A p.Glu1040Lys	heterozygosis	Focal Epilepsy and Speech Disorder (epilepsy-aphasia spectrum disorder) with or without intellectual disability,	Generalized seizures, Rett-like phenotype	Phenotype not compatible with the detected gene including the VOUS
H	DNM1	c.1315G>A p.(Val439Ile)	heterozygosis	Polymorphic seizures (Infantile spasms Myoclonic, atonic, tonic or focal seizures, Atypical absences), Variable EEG abnormalities (Hypsarrhythmia, Slow background Diffuse multifocal sharp waves and sharp waves-slow waves, Paroxysmal fast activity), Possible diffuse brain atrophy on MRI	Generalized seizures, autism spectrum disorders,	Phenotype not compatible with the detected gene including the VOUS
I	ST3GAL	c.145G>A	heterozygosis	West syndrome	West syndrome	Disorder

		p.(Ala49Thr)				associated with an autosomal recessive transmission. Gene coverage for the NGS: 100%. Negative MLPA analysis of the gene. Diagnosis excluded.
L	GABRG2	c.1244C>G p.(Ala415Gly)	heterozygosis	Absence epilepsy, febrile seizures, generalized epilepsy with febrile seizures plus,	Polymorphic seizures, Intellectual disability, Dystonia-Parkinsonism	Pathogenic variant on KCND3 at Clinical exome sequencing (including disease-causing genes only)
	DENND5A	c.675A>T p.(Gln225His)	heterozygosis	Polymorphic seizures (Myoclonic, tonic-clonic,), Hypo or hypertonia, Spasticity, Global developmental delay, Diffuse multifocal sharp waves and sharp waves-slow waves on EEG, Basal ganglia calcifications or corpus callosuum abnormalities on MRI		
	DENND5A	c.544G>A p.(Asp182Asn)	heterozygosis			
M	SYN1	c.1781C>T p.(Pro594Leu)	heterozygosis	X-linked epilepsy with various learning disabilities and language disorders	Focal seizures, febrile seizures, normal intellectual disability	Pathogenic variant on SCN1B (Patient 10 IN Table 7)
	MTHFR	c.1167-2A	heterozygosis	Focal epilepsy, generalized seizures, gait disorder (from motor central or peripheral origin), cognitive decline, psychotic symptoms and thrombotic events		
N	CASR	c.2915C>T p.(Thr972Met)	heterozygosis	Generalized seizures in 1 family, Disorders of calcium metabolism and renal re-uptake	Myoclonic seizures, borderline cognitive functioning	Phenotype not compatible with the detected gene including the VOUS
O	IQSEC2	c.11G>A p.(Gly4Glu)	heterozygosis	Severe drug-resistent epileptic encephalopathies in males, Rett-like phenotype in females	Focal seizures, developmental delay	Phenotype not compatible with the detected genes including the VOUS
	MBD5	c.2254A>G p.(Ile752Val)	heterozygosis	Tonic-clonic seizures, absence seizures, focal dyscognitive seizures, focal seizures, and tonic seizures associated with multiple EEG abnormalities, consistent with epileptic encephalopathy. Milder phenotypes with short stature, macrocephaly, mild intellectual disability, seizures, and sleep and behavioral problems		
P	ARHGEF9	c.1300G>C p.(Gly434Arg)	hemizygosis	Focal seizures, Status epilepticus during sleep, Developmental delay, Focal epileptic abnormalities or spike and waves during sleep on EEG, Frontal hypoplasia or Polymicrogyria on MRI ,	Febrile seizures, Myoclonic-atonic seizures, Intellectual disability	Phenotype not compatible with the detected genes including the VOUS
	CACNA1A	c.6104G>A p.(Arg2035His)	heterozygosis	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, tonic or focal seizures,), Hypo or hypertonia, Ataxia, Hyperkinetivc movement disorders, Abnormal eye movement, Variable EEG abnormalities (Slow background Diffuse multifocal sharp waves and sharp waves-slow waves),		
Q	SHANK3	c.3679dup p.(Ala1227Glyfs*69)	heterozygosis	Severe cognitive deficits, including language and speech disorder and autism spectrum disorder, minor dysmorphic features, polymorphic seizures, psychosis,	Absence-like seizures, focal seizures, Rett-like phenotypes, ataxia, intellectual disability	Phenotype not compatible with the detected genes
	TCF4	c.1349T>C	heterozygosis	Severe epileptic encephalopathy		

		p.(Met450Thr)		with mental retardation and intermittent hyperventilation, characteristic facial gestalt (Pitt Hopkins syndrome),		including the VOUS
R	POLG1	c.3650C>T p.(Ala1217Val)	heterozygosis	Severe epileptic encephalopathy, Ataxia, peripheral neuropathy, ophtalmoplegia, movement disorders	Polymorphic seizures, lacking developmental milestones, Spastic tetraparesis	Phenotype not compatible with the detected genes including the VOUS
	CACNA1A	c.2128T p.(Leu710Val)	heterozygosis	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, tonic or focal seizures,), Hypo or hypertonia, Ataxia, Hyperkinetivc movement disorders, Abnormal eye movement, Variable EEG abnormalities (Slow background Diffuse multifocal sharp waves and sharp waves-slow waves)		
	PLCB1	c.3643C>T p.(Pro1215Ser)	heterozygosis	Tonic seizures, Infantile spasms, Developmental delay, Suppression burst or Hypsarrhythmia on EEG		
S	SLC13A5	c.1412T>C p.(Leu471Ser)	heterozygosis	Polymorphic seizures (Myoclonic, Focal or Tonic seizures), Profound developmental delay, Multifocal epileptic Discharges on EEG	Neonatal onset tonic and myoclonic seizures, developmental delay, intellectual disability	Phenotype not compatible with the detected gene including the VOUS
T	SYNGAP1	c.3959C>A p.(Pro1320His)	heterozygosis	Polymorphic seizures (focal seizures, myoclonic seizures, infantile spasms, myoclonic-atonic seizures), Intellectual disability, Autistic spectrum disorders,	Focal seizures, obsessive compulsive disorder	Phenotype not compatible with the detected gene including the VOUS
U	MBD5	c.3194C>G p.(Pro1065Arg)	heterozygosis	Tonic-clonic seizures, absence seizures, focal dyscognitive seizures, focal seizures, and tonic seizures associated with multiple EEG abnormalities, consistent with epileptic encephalopathy. Milder phenotypes with short stature, macrocephaly, mild intellectual disability, seizures, and sleep and behavioral problems	Early onset absences, autism spectrum disorder	Phenotype not compatible with the detected gene including the VOUS
V	NPRL3	c.1642G>A p.(Arg547His)	heterozygosis	Focal epilepsy, Focal cortical dysplasia,	Myoclonic seizures,	Phenotype not compatible with the detected gene including the VOUS
X	GABRD	c.649A>T p.(Thr217Ser)	heterozygosis	Myoclonic seizures, generalized seizures with febrile seizures plus, absence seizures	Tonic seizures, Myoclonic seizures, hypotonia, profound intellectual disability, developmental delay	Phenotype not compatible with the detected genes including the VOUS
	CDKL5	c.295T>C p.(Thr217Ser)	heterozygosis	Infantile spasms, Intellectual disability, Severe motor impairment, Hypotonia, Poor eye contact Rett-like phenotype (secondary deceleration of head growth, sleep disturbances, hand apraxia, and stereotypies)		
Y	SCN8A	c.95G>A p.(Ser32Asn)	heterozygosis	Polymorphic seizures (Infantile spasms, Migrating partial seizures in infancy, Focal, tonic, clonic, myoclonic and absence Seizures), Developmental delay, Dystonia, Hypotonia, Non specific EEG abnormalities (Background slowing Focal or multifocal epileptic discharges, Electrical status epilepticus),	Myoclonic seizures,lacking motor development, profound developmental delay, microsomy, hearing and visual imèpairment, cerebellar atrophy	Phenotype not compatible with the detected genes including the VOUS

				Non specific MRI abnormalities (Possible brain or cerebellar Atrophy, Possible callosal		
	CNTNAP2	c.1028A>G p.(Asn343Ser)	heterozygosis	Polymorphic seizures, Pitt-Hopkins-like phenotype, focal cortical dysplasia		
	ST3GAL	c.782G>a p.(Arg261Gln)	heterozygosis	West syndrome		
	DEPDC5	c.2576C>T p.(Thr859Met)	heterozygosis	Focal epilepsy, focal cortical dysplasia,		
Z	SCN1A	c.1552G>A p.(Asp518Asn)	heterozygosis	Dravet syndrome (Febrile or afebrile seizures, Generalized or unilateral clonic seizures, Myoclonic seizures, Atypical absences, Partial seizures, Photosensitivity, Developmental delay or regression, Ataxia); Genetic epilepsy with seizures plus (GEFS+)	Focal seizures, severe intellectual disability, Self-injurious behavior	Phenotype not compatible with the detected genes including the VOUS
	NECAP1	c.436C>T p.(Arg146Cys)	heterozygosis	Multifocal clonic or tonic seizures, Global developmental Delay, Non specific EEG and MRI abnormalities (Multifocal dischargesSlowed background activity, Possible diffuse brain Atrophy)		
A1	RELN	c.7643C>T p.(Ser2548Leu)	heterozygosis	Polymorphic seizures, Developmental delay, Intellectual disability, Lissencephaly, temporal lobe epilepsy	Generalized seizures, normal intellectual development	Phenotype not compatible with the detected genes including the VOUS
	GRIN2B	c.3076G>A p.(Gly1026Ser)	heterozygosis	West syndrome		
B1	NRXN1	c.385G>A p.(Val129Ile)	heterozygosis	Pitt-Hopkins-like syndrome, polymorphic seizures, intellectual disability, autistic spectrum disorders,	Severe infantile omnset epileptic encephalopathy, developmental delay, spastic diplegia, lacking language development	Phenotype not compatible with the detected gene including the VOUS
C1	POLG1	c.3152G>C p.(Gly1051Ala)	heterozygosis	Severe epileptic encephalopathy, Ataxia, peripheral neuropathy, ophtalmoplegia, liver impairment, movement disorders	Tonic seizures, developmental and language delay	Phenotype not compatible with the detected gene including the VOUS
D1	ARX	c.1462A>G p.(Met488Val)	heterozygosis	Infantile spasms, Myoclonic epilepsy, Tonic spasms and other Seizures, Intellectual disability, Generalized spasticity, Dyskinetic movements, Generalized dystonia, Ambigous genitalia, Suppression burst or Hypsarrhythmia on EEG	Focal seizures, Myoclonic seizures, Normal developmental milestones	Phenotype not compatible with the detected genes including the VOUS
	HNRNPU	c.2375A>G p.(Asn792Ser)	heterozygosis	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, atypical absences, tonic), Global developmental delay,Spasticity, Microcephaly, Autistic traits, Variable EEG abnormalities (Slow background , Diffuse multifocal sharp waves and sharp waves-slow wave, Paroxysmal fast activity).		
	ADGRV1	c.17902G>A p.(Glu5968Lys)	heterozygosis	Febrile seizures, myoclonic seizures, reflex seizures, audiogenic seizures hearing loss, retinite pigmentosa		
E1	CACNA1A	c.2924G>T p.(Arg975Leu)	heterozygosis	Polymorphic seizures (Myoclonic, atonic, tonic-clonic, tonic or focal seizures,), Hypo or hypertonia, Ataxia, Hyperkinetivc movement disorders, Abnormal eye movement, Variable EEG	Focal seizures, language delay	Phenotype not compatible with the detected genes

				abnormalities (Slow background Diffuse multifocal sharp waves and sharp waves-slow waves),		including the VOUS
	WWOX	c.1184G>A p.(Arg395Gln)	heterozygosis	Polymorphic seizures (Epileptic spasms, Tonic, clonic, or myoclonic seizures), Hypotonia, Developmental delay, Retinopathy, Hypokinesia, Microcephaly, Variable EEG abnormalities (Slow background activity, Focal or plurifocal epileptic discharges, Hypsarrhythmia), Variable MRI abnormalities (Delayed myelination Brain atrophy, Corpus callosum, hypoplasia, Hippocampal dysplasia)		
F1	MTHFR	c.1408_1409delinsCT p.(Glu470Leu)	heterozygosis	Focal epilepsy, generalized seizures, gait disorder (from motor central or peripheral origin), cognitive decline, psychotic symptoms and thrombotic events	Polymorphic seizures (myoclonic seizures, tonic Microcephaly, Spastic tetraparesis,	Pathogenic variant on ADSL gene at exome sequencing

Table 8 List and discussion of the VOUS detected in the present study

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“Il bambino non sa che esistono i draghi perché è scritto nelle favole. Il bambino sa già che esistono i draghi, ma legge nelle favole che i draghi possono essere sconfitti.”

GILBERT KEITH CHESTERTON