SAPIENZA UNIVERSITY OF ROME

**SCHOOL OF BIOLOGY AND MOLECULAR MEDICINE**

PhD THESIS

# Targeted Resequencing as a diagnostic tool in patients with epilepsy

Human Biology and Medical Genetics PhD course

Medical Genetics curriculum

XXXII Cycle

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A.A. 2018-2019

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## Abstract

Epilepsy is one of the most common neurological disorder, affecting 5–8/1.000 individuals worldwide. Approximately 20–30 % of epilepsy cases are caused by acquired conditions such as stroke, tumor or head injury, but the remaining 70–80 % of cases are believed to be due to one or more genetic factors. In the last decade, advances in genomic technologies have led to a rapid increase in understanding of epilepsy genetics and to date, to the best of our knowledge, about 1000 genes have been associated with epilepsy. The aim of this study is to determine the contribution of some currently known disease-causing genes in a cohort of Italian patients affected by syndromic or non-syndromic forms of epilepsy. We designed a genes panel for Targeted Resequencing (TRS) containing 85 relevant epilepsy genes responsible for the most common epilepsy phenotypes known so far. A cohort of 49 patients (23 male and 26 female) with a clinical diagnosis of epilepsy, including both sporadic and familial cases, has been enrolled for the study and analyzed by TRS. This approach allowed us to identify variants in 25/49 (51%) patients analyzed. In detail, disease-causing mutations (classified as pathogenic or likely pathogenic following the American College of Medical genetics guidelines), has been identified in 10/25 (40%) affecting the genes *ARX*, *GAMT*, *KCNQ2*, *MECP2*, *SCN1A*, *POLG*, *SPTAN1*, *STXBP1* and *TCF4*, while variants of uncertain clinical significance (VUS) has been identified in the remaining 15/25 patients (60%) affecting the genes *ATP1A2*, *CACNB4*, *CLN3*, *CLN6*, *CNTN4*, *CACNA1H*, *CNTNAP2*, *GRIN2A*, *GRIN2B*, *KCNMA1*, *LIAS*, *POLG*, *PNKP*, *PRICKLE2*, *SCN1A*, *SCN2A*, *SPTAN1*, *SCN9A*, *TSC1*. Next Generation Sequencing technologies have revolutionized our approach to genetic epilepsies both from research than clinical perspective. The identification of novel mutations in known epilepsy associated genes is useful to increase our knowledge about the molecular mechanisms of the disease. More importantly, our study highlight once again the utility of next generation sequencing in establishing an etiological basis in clinically and genetically heterogeneous conditions such as epilepsy. Knowing the genetic basis of the disease can be valuable not only for diagnosis but also for guiding treatment and, above all, estimating recurrence risk.

# 1. INTRODUCTION

## 1.1 Epilepsy: definitions and epidemiology

Epilepsy is one of the most common neurological disorders characterized by recurrent unprovoked seizures due to neuronal hyperexcitability and abnormal synchronization.

The term "seizure" describes a paroxysmal alteration of neurologic function caused by the excessive, hypersynchronous discharge of neurons in the brain, while the term "epileptic seizure" is used to distinguish a seizure caused by abnormal neuronal firing from a nonepileptic event, such as a psychogenic seizure. "Epilepsy syndrome" refers to a group of clinical characteristics that consistently occur together, with similar seizure type(s), age of onset, EEG findings, triggering factors, genetics, natural history, prognosis, and response to antiepileptic drugs (AEDs) (Stafstrom and Carmant, 2015). Approximately 3% of the general population is affected by epilepsy (Hauser et al., 1993).

The WHO's 2010 Global Burden of Disease study classify epilepsy as the second most heavy neurologic disorder worldwide in terms of disability (Cross, 2011).

Incidence and prevalence studies are critical to provide measures of frequency and therefore the burden of disease, and allow for proper planning of services. Prevalence is an estimate of the number of people with epilepsy in a given population at a specified time (point prevalence), or during a defined time interval (period prevalence). In most countries worldwide, the prevalence of epilepsy ranges from 4 to 10/1000. The incidence, instead, is the number of new cases per year. The incidence of epilepsy ranges from 40 to 70/100.000 in most developed countries and is nearly double in developing countries (Fiest et al., 2017).

In particular 2–5% of the general population will suffer an epileptic seizure, while a third of those patients will eventually develop epilepsy according to its historical definition (at least two unprovoked seizures 24 hours apart). It was recently proposed that a diagnosis of epilepsy could be performed after the first seizure, when the risk of seizure recurrence is at least 60%. This complex relationship between seizure and epilepsy explains some of the challenges encountered in their epidemiology (Behr et al., 2016).

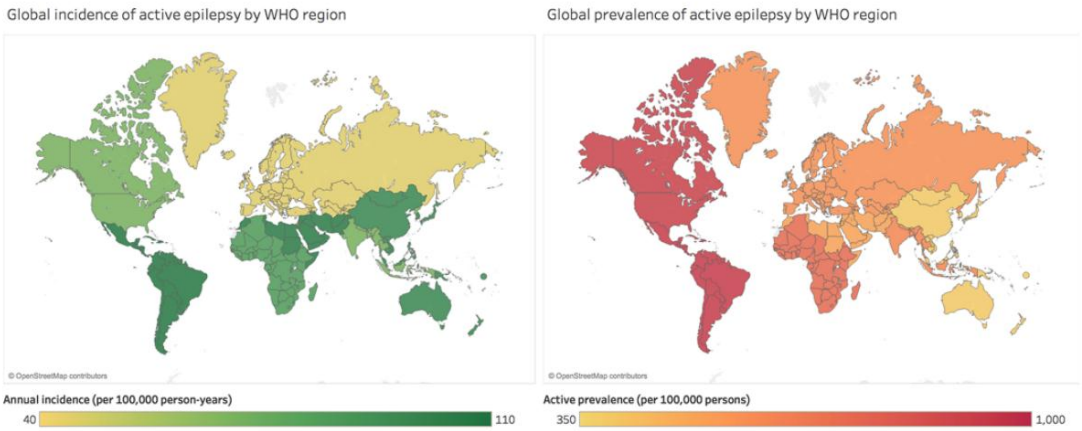


**Fig.1** Estimate of the epilepsy incidence (left) and prevalence (right).

## 1.2 Classification of seizure and epilepsy

The classification of seizures, epilepsies, and epilepsy syndromes creates a framework for clinicians, researchers, and patients and their families. This classification has evolved over the years, and in 2017 the International League Against Epilepsy (ILAE) published a classification of seizures, epilepsies and epilepsy syndromes. Understanding this classification is important in the diagnosis, prognosis and treatment (Pack, 2019).

***Seizure*** classification is based on the propagation and localization of the neurological event and, taking into account this, seizures can be divided in focal and generalized. Focal seizures originate within a neuronal network limited to one hemisphere, whereas generalized seizures originate at some point within the brain and rapidly engage neuronal networks located on both the hemispheres. If the onset of the seizure is missed or is unclear, the seizure is classified as unknown etiology (Pack, 2019).

The second level of classification is based on the ***epilepsy type*** which is clinically determined (characteristic EEG findings provide supportive evidence). Also in this case, as for the seizure classification, the epilepsies are distinguished in generalized and focal. The new classification system additionally recognizes two new categories: combined generalized and focal epilepsy (examples of combined generalized and focal epilepsy include Dravet syndrome and Lennox-Gastaut syndrome), and unknown epilepsy.

Finally, the group ***'epilepsy syndromes'*** has been recently added to the current classification system and is defined as "a cluster of features incorporating seizure types, EEG, and imaging features that tend to occur together." Factors that contribute to epilepsy syndrome include age of onset, remission, triggers, diurnal variation, intellectual and psychiatric dysfunction, EEG findings, imaging studies, family history, and genetics (Pack, 2019).

In 2017, the ILAE released a new classification of seizure types, largely based upon the existing classification formulated in 1981. The differences include new focal seizure types, classifying focal seizures by the first clinical manifestation, a few new generalized seizure types, ability to classify some seizures when onset is unknown, and renaming of certain terms to improve clarity of meaning.

At the level of epilepsy syndrome, the 2017 ILAE document introduced the concept of "development and epileptic encephalopathies", an important distinction for geneticists.

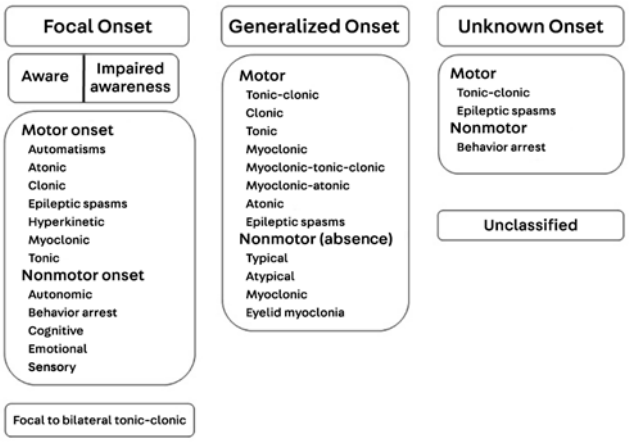


**Fig.2** Expanded version of International League Against Epilepsy seizure type classification (2017).

## 1.3 Etiology of epilepsy

The etiology of epilepsy is emphasized in the new classification system; in particular, six etiological categories (structural, genetic, infectious, metabolic, immune, unknown) have been defined, each reflecting underlying brain dysfunction (Shorvon et al., 2011).

About 1% of all people develop recurrent unprovoked seizures for no obvious reason and without any other neurological abnormalities. These are named 'idiopathic epilepsies', and they are assumed to be mainly genetic in origin (Steinlein, 2004).

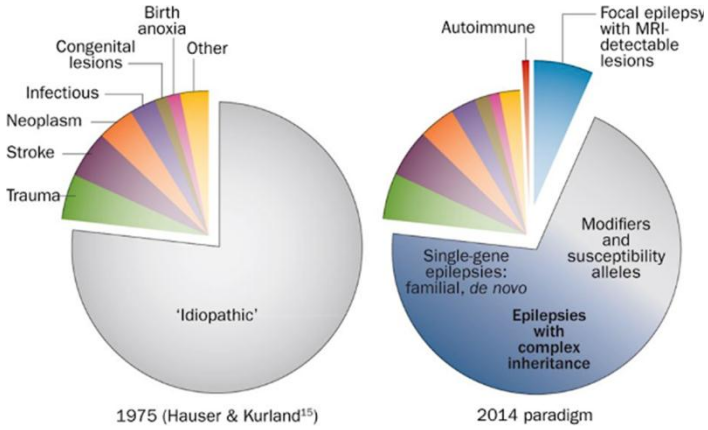


**Fig. 3** Advances in understanding the causes of epilepsy.

In particular, about 20–30% of epilepsy cases are due to acquired conditions such as stroke, tumor or head injury, but the remaining 70–80% of cases are believed to be due to one or more genetic factors (Myers and Mefford, 2015).

Genetic etiologies are determined if there is a genetic mutation in one or more genes known to be associated or responsible for a clinical condition in which the epilepsy is the main symptom of the disorder. Although some epilepsies are inherited, many occur secondary to a *de novo* mutation in the affected individual. In some cases, the genetic mutation is not identified, but the clinical presentation, EEG findings, and family history suggest a genetic etiology (Pack, 2019).

## 1.4 Genetic basis of epilepsy

A genetic basis for epilepsy has been hypothesized for decades, but the first evidence of a genetic component emerged from epidemiological studies that reported an increased risk of epilepsy in relatives of affected individuals. Studies of twins showed that monozygotic twins have a higher concordance rate for both genetic generalized epilepsy (GGE) and focal epilepsy than dizygotic twins, supporting the hypothesis that epilepsy has a genetic basis (Myers and Mefford, 2015). In particular, twin studies suggested that the heritability of epilepsy is ~25%-70%. While genetic linkage analyses have identified several susceptibility loci for epilepsy, recent advances in genomic technology have made it feasible to identify single nucleotide variants, and copy number variants (CNVs) associated with epilepsies (Chen et al., 2017).

To date, extensive research has identified different genetic component of epilepsy including genetic aberrations now known to cause or contribute to the condition. In particular, ring chromosome 20 syndrome is a rare but well-known cause of epilepsy. Atkins et al. (1972) first described this condition in a 7 year old boy with behavioral issues, mental retardation and grand mal seizures. Other examples of chromosomal aberrations associated with epilepsy include Klinefelter Syndrome (47,XXY) (Elia et al., 1995; Tatum et al., 1998) and Pallister-Killian Syndrome (OMIM #601803) (12p tetrasomy) (Pallister et al., 1977; Peltomaki et al., 1987).

Copy number variations (CNVs) have also been associated with epilepsy and other neurological disorders (Mullen et al., 2013; Mefford, 2015; Borlot et al., 2017). Briefly, CNVs are classified as deletions or duplications of DNA, larger than 1 kb in size, which can be recognized as either a normal variation of the genome or to be pathogenic based on the location and number of genes encompassed by the rearrangement (Mefford, 2014). Examples of CNVs associated with epilepsy include deletions at Xp22.31, 1q21.1, 15q11.2, 15q13.3, and 16p13.11 chromosome regions respectively as well as duplications involving the 1p36.33 and 22q11.2 chromosome regions which have been all previously identified as risk factors for genetic generalized epilepsy (Mefford, 2014; Addis et al., 2016).

Other factors, such as uniparental disomy (UPD) or genetic imprinting, have also been associated with epilepsy as reported, for example, for the Angelman syndrome (OMIM #105830) involving the chromosome region 15q11q13 (Lalande et al., 1999; Valente et al., 2005).

In addition, epigenetic factors, including DNA methylation (Kobow and Blumcke, 2012; Wang et al., 2016), histone modification, transcriptional regulation (Hwang et al., 2013; Jagirdar et al., 2015),

and microRNAs involvement (Henshall, 2014; Raoof et al., 2017), have been implicated in epilepsy. They are able to regulate the neuro-inflammatory responses, neuronal cell growth and other relevant cellular process (Roopra et al., 2012; Boison, 2016; Kobow and Blumcke, 2017).

Mutations of mitochondrial DNA (mtDNA) have also been identified in patients affected by epilepsy and/or other neurological diseases (Wallace et al., 1994). The first example was described by Shoffner et al., in 1990, when they reported a mutation in tRNALys as cause of myoclonic epilepsy with ragged red fibers (MERFF; OMIM #545000). Subsequently, Tatuch et al. (1992) reported a mutation in ATPase6 that cause Leigh syndrome (OMIM #256000) if present in a high percentage of cells. Mosaic mutations in well-known epilepsy genes, such as *SCN1A* and *SLC6A1*, have also been identified to cause the epilepsy phenotype (Shi et al., 2012; Halvorsen et al., 2016). In a recent study by Stosser and collaborators (2017), they found a 3.5% overall frequency of mosaicism in 893 affected patients across 9 different nuclear genes (*CDKL5*, *GABRA1*, *GABRG2*, *GRIN2B*, *KCNQ2*, *MECP2*, *PCDH19*, *SCN1A*, and *SCN2A*). Mosaicism is thought to be an underreported cause of genetic disorders, due to detection challenges, although there are numerous studies aimed at improving this using Next Generation Sequencing (NGS) technology (Stosser et al., 2017). Furthermore, mosaicism is not limited to point mutations regarding mtDNA or nuclear DNA, but it can be also observed at chromosomal level (i.e. aneuploidies and/or CNVs) (Gajecka, 2016).

To date, approximately 977 genes have been associated with epilepsy grouped into the following categories: i) 84 genes causing epilepsy as a core symptom; ii) 73 neurological genes associated with brain gross development and epilepsy; iii) 536 epilepsy-associated genes where epilepsy is a symptom of another neurological disorder; iv) 284 potential-epilepsy genes (Wang et al., 2017).

Some of these genes are associated, not only with epilepsy as the only symptom, but also with different phenotypes including well-characterized syndromes with variable clinical manifestations.

## 1.4.1 Genetics of Epilepsy Syndromes

Although the definition of epilepsy suggests that it is a single disorder, it is more accurate to describe epilepsy as a group of disorders clinically and etiologically heterogeneous.

A crucial issue underpinning gene discovery in epilepsy is that each gene shows phenotypic pleiotropy, and that each epilepsy syndrome shows genetic heterogeneity. Phenotypic heterogeneity or pleiotropy, in which mutations in a single gene cause different phenotypes, is increasingly recognised in epilepsy and across many neurological disorders. Many factors contribute to phenotypic heterogeneity, including the following: type of mutations during development, gene expression, epigenetic factors and modifier genes (McTague et al., 2016).

Channelopaties are such an example. *SCN1A* mutations are associated with Dravet syndrome (OMIM #607208) but also with milder phenotypes such as genetic epilepsy with febrile seizures plus (GEFS+; OMIM 604233, 604403, 609800, 611277, 612279, 613060, 613828 613863, 616172, 618482). *KCNQ3* mutations are associated with benign neonatal seizure while *KCNQ2* can be associated with early-onset epileptic encephalopathy or benign neonatal seizure, highlighting phenotypic variations resulting from mutations in the same gene (El Achkar et al., 2015).

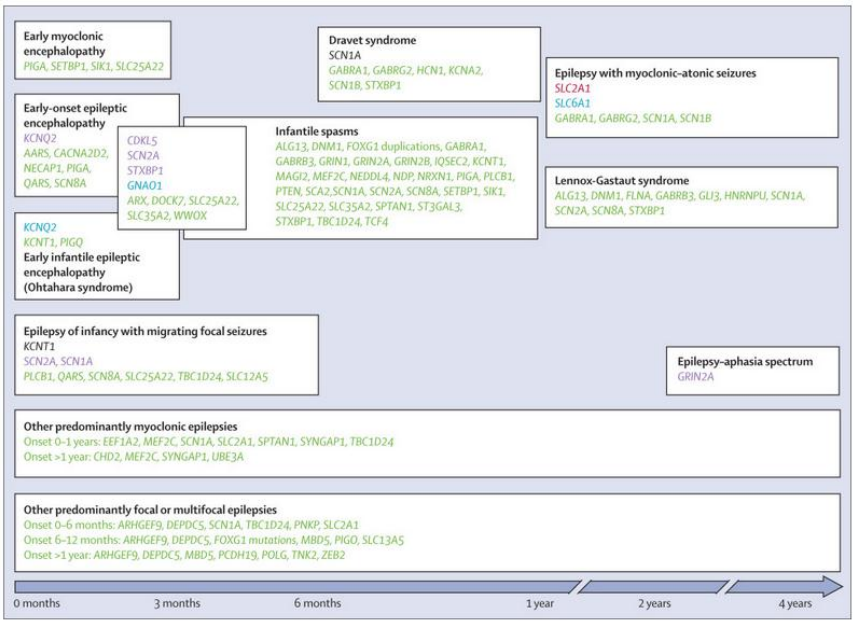


**Fig.4** Clinical heterogeneity of epilepsy genes.

According to the ILAE, epilepsy syndromes are classified based on the electroclinical features and age of epilepsy onset. It is therefore clinically relevant to understand the diverse genetic

mechanisms of each of these syndromes as they might shed a light on diagnosis, treatment and prognosis.

**I) Early-onset epileptic encephalopathies (EOEEs)**

Epileptic encephalopathies are disorders caused by recurrent clinical seizures or prominent interictal epileptiform discharges and usually seen during the early infantile period. They are associated with impaired cognitive, sensory, and motor development. The most common epileptic encephalopathies are Ohtahara syndrome (OS), early myoclonic encephalopathy, epilepsy of infancy with migrating focal seizures (EIMFS), West syndrome, and Dravet syndrome (Mastrangelo et al., 2012). Based on current literature, single gene variants explain at least 20-30% of epileptic encephalopathies (Olson et al., 2017).

Ohtahara syndrome is a rare form of epilepsy, characterized by intractable seizures within the first few weeks to months of neonatal period. It is usually associated with poor developmental outcome. Infants may develop acute generalized or lateralized tonic spasms that can occur either singly or in clusters and are independent of the sleep cycle. The seizure frequency may be very high, ranging from 10 to 300 spasms in 10 to 20 clusters per day. To date, various genes, which have essential roles in lower brain's neuronal and interneuronal functions, have been reported to be associated with Ohtahara syndrome. For instance, syntaxin binding protein 1 (*STXBP1*) regulates synaptic vesicle release; aristaless-related homeobox (*ARX*) acts as a regulator of proliferation and differentiation of neuronal progenitors; solute carrier family 25 member 22 (*SLC25A22*) encodes a mitochondrial glutamate transporter; and potassium voltage-gated channel, KQT-like subfamily, member 2 (*KCNQ2*) plays a key role in a cell's ability to generate and transmit electrical signals. (Beal et al., 2012).

Dravet syndrome is another genetic form of early-onset epileptic encephalopathies. It affects between 1 of 20 000 and 1 of 40000 live births. A positive family history is usually present in 25% to 71% of patients, while mutations in *SCN1A* are identified in 70-80% of cases. To date, more than 500 mutations of *SCN1A* have been associated with Dravet syndrome. However, other genes have been found as cause Dravet or Dravet-like clinical syndrome, including *PCDH19, SCN1B, STXBP1, GABRA1, CHD2, HCN1* and *GABRG2* (Chopra and Isom, 2014).

EIMFS is an even more heterogeneous syndrome. While *KCNT1* mutations account for about one third of reported cases of EIMFS, the other cases are caused by mutations in a number of other genes (*SCN1A, SCN8A, SLC25A22, SCN2A*) (Barcia et al. 2012; Ohba et al., 2014).

Infantile spasms are another form of epileptic disorder that occur during the first year of life. Genetic analysis of children with unexplained infantile spasms have demonstrated mutations in diverse genes including *ARX, CDKL5, ALG13* as well as *de novo* mutations in autosomal genes, including *PDZ, MAGI2, STXB1, SCN1A, SCN2A, GABRB3* and *DMN1* (Mastrangelo et al., 2012).

| Genes | Associated epilepsy syndromes | Gene localization and functions | Clinical findings | EEG findings | Estimated percentage in associated epilepsy syndromes |
|---|---|---|---|---|---|
| *STXBP1* | Ohtahara syndrome<br>West syndrome<br>Nonsyndromic intellectual disability with or without epilepsy | 9q34.1, 20 exons<br>Controls synaptic vesiculer docking | Early-onset seizures<br>Typically frequent epileptic spasms<br>Severe developmental retardation | Suppression-burst pattern | 10%-33% |
| *ARX* | X-linked infantile spasms<br>X-linked lissencephaly with ambiguous genitalia<br>Ohtahara syndrome<br>X-linked myoclonic epilepsy<br>Nonsyndromic intellectual disability | Xp21.3, 5 exons<br>Modulates cerebral development and patterning through the regulation of differentiation, proliferation, and tangential migration of neuronal precursors and cortical interneurons | Developmental delay<br>Affected males<br>Severe movement disorder<br>Ambiguous genitalia<br>Intractable seizures | Suppression burst pattern<br>Slow spike and wave | ~5% |
| *SLC25A22* | Ohtahara syndrome<br>Early myoclonic encephalopathy | 11p15.5, 9 exons<br>Encoding a mitochondrial glutamate carrier Motor coordination, eye movement | Epileptic encephalopathy beginning in the first days of life<br>Hypotonia<br>Microcephaly<br>Abnormal electroretinogram | Suppression burst pattern | |
| *KCNQ2* | Neonatal epileptic encephalopathy<br>Ohtahara syndrome<br>Benign familial neonatal seizures | 20q13.3, 17 exons<br>Encodes the voltage gated potassium channel Kv7.2<br>Cell's ability to generate and transmit electrical signals | Intellectual disability<br>Tonic seizures<br>Good response to sodium channel blockers | Suppression burst pattern | 5%-10% |
| *CDKL5* | West syndrome<br>Rett-like phenotype | Xp22, 20 exons<br>Essential for normal brain development and function | Affected females<br>Severe mental retardation<br>Hypotonia<br>Autistic features<br>Hand stereotypies<br>Hypermotor-tonic-spasm sequence | Multifocal spikes, sharp waves<br>Hypsarrhythmia<br>Low-voltage fast activity in the frontocentral regions | 8%-28% in females<br>5.4% in boys |
| *SCN1A* | Dravet syndrome<br>Generalized epilepsy with febrile seizure plus spectrum | 2q24.3, 26 exons<br>Encodes the voltage-gated sodium channel NaV1.1<br>Neuronal excitability | Generalized or unilateral clonic or tonic–clonic seizures usually triggered by fever<br>Learning difficulty<br>Global developmental delay | | 80% |
| *PCDH19* | Dravet syndrome<br>Epilepsy limited to females with mental retardation | Xq22.1, 6 exons<br>Calcium-dependent cell-cell adhesion | Affected females<br>Onset of seizures between ages 6 and 36 mo<br>Febrile and afebril seizures<br>Psychomotor and cognitive impairment | | 5% |

**Fig.5** List of genes associated to EOEEs.

### II) Benign familial neonatal/infantile seizures

Benign familial neonatal (BFNC), neonatal infantile, and infantile seizures (BFIS) are genetically distinct syndromes, although it has been proposed patients to be grouped into a single group broad referred to as benign familial infantile epilepsy (BFIE). Benign familial neonatal seizures are characterized by convulsions occurring shortly after birth and continuing only in the first month of life (Pandolfo, 2011). Genetic causes include channelopathy causing mutations (e.g., *KCNQ2*, *KCNQ3*, and *SCN2A*), as well as *PRRT2* present in the 16p11.2 chromosome region which is also associated with choreoatheosis syndrome (Méneret et al., 2013).

### III) Progressive myoclonus epilepsies (PMEs)

The progressive myoclonus epilepsies (PMEs) comprise a group of rare and heterogeneous disorders defined by the combination of myoclonus, epileptic seizures, and progressive neurologic deterioration. The gene defects for the most common forms of PME (Unverricht–Lundborg disease, Lafora disease, several forms of neuronal ceroid lipofuscinoses, myoclonus epilepsy with ragged-red fibers (MERRF), and type 1 and 2 sialidoses) have been identified (Kälviäinen, 2015). The most common subtype, Unverricht-Lundborg disease (ULD), can be caused by mutations in *CSTB, SCARB2, PRICKLE1*, and *GOSR2* (Lalioti et al., 1997; Corbett et al., 2011). While, Lafora disease can be caused by *EPM2A* or *EPM2B* (Ferlazzo et al., 2014). Neuronal ceroid lipofuscinosis (NCL) can be caused by numerous mutations: *CLN1* in classic infantile-onset form, *CLN2*, *CLN5*, *CLN6*, *CLN7*, and *CLN8* in late infantile-onset forms, *CLN3* in classic juvenile-onset form, *CLN4* and *CLN6* in adult-onset forms, and *CLN10* in congenital NCL. A recently identified PME mutation in *KCNC1*, encoding a subunit of a voltage gated potassium channel, was found in 11 individuals with phenotype resembling classic ULD (Muona et al., 2014).

### IV) Genetic generalized epilepsies (GGE)

The genetic generalized epilepsies (GGE), characterized by generalized seizures that involve both sides of the brain, include juvenile myoclonic epilepsy (JME), childhood absence epilepsy (CAE), juvenile absent epilepsy (JAE) and epilepsy with generalized tonic-clonic seizure alone

(IGE-TCS) (Guerrini et al., 2019). The GGEs tend to start in childhood or adolescence and are usually associated with normal development and intellect.

| Gene | Locus | Protein | Phenotype |
|---|---|---|---|
| *GABRG2* | 5q31–33 | $GABA_A$ receptor $\gamma_2$-subunit | CAE and FS |
| *GABRA1* | 5q34–35 | $GABA_A$ receptor $\alpha_1$-subunit | JME, CAE |
| *CLCN2* | 3q26 | ClC-2 voltage-gated $Cl^-$ channel | CAE, JAE, JME, EMA |
| *EFHC1* | 6p11–12 | Myoclonin1 | JME |
| *CACNA1H* | 16p13.3 | T-type $Ca^{2+}$ channel $\alpha_{1H}$-subunit | CAE |
| *CACNB4* | 2q22–23 | $Ca^{2+}$ channel $\beta_4$-subunit | JME, JAE |
| *CACNA1A* | 19p13 | P/Q-type $Ca^{2+}$ channel $\alpha_{1A}$-subunit | CAE with ataxia |
| *RORB* | 9q21.13 | Transcriptional factors | Eyelid myoclonia with Ab |
| ***List of loci from genome-wide linkage analyses of small multiplex families*** | | | |
| 1p, 2q36, 3q26, 5q12-q14, 5q34, 6p21, 8q24, 8p, 9q32–33; 10q25–26, 10p11, 11q13, 13q22–31, 14q23, 15q14, 18q21, 19q13 | | | IGE, CAE, JME |
| ***List of CNVs, risk factors of IGE*** | | | |
| Microdeletions: 15q13.3, 15q11.2, 16p13.11 | | | IGE, JME |
| Duplication: 1q21.3 | | | Early onset CAE |

*CAE*, childhood absence epilepsy; *EMA*, myoclonic–astatic epilepsy; *FS*, febrile seizures; *IGE*, idiopathic generalized epilepsy; *JAE*, juvenile absence epilepsy; *JME*, juvenile myoclonic epilepsy.

**Fig.6** List of genes associated to GGE

An important clinical example is mutations in *SLC2A1* gene that cause glucose transporter 1 deficiency, and can present early-onset absence epilepsy or other generalized epilepsies such as typical childhood absence or juvenile myoclonic epilepsy. The treatment of choice is ketogenic diet. Copy number variations (CNV) have been frequently found in this type of epilepsies. These include 1q21.1, 15q11.2, 15q13.3, 15q11-q13, 16p11.2, 16p13.11, and 1q21.1 (Mefford et al., 2010; Helbig I et al. 2009). Other identified genes are likely susceptibility genes for generalized epilepsies and include *CACNA1H*, *CACNB4*, and *CLCN2*, in addition to *CACNA1A*, which is associated with both focal and generalized seizures (Lu¨ JJ et al., 2005; Chioza et al., 2001).

## V) Genetic epilepsy with febrile seizures plus (GEFS+)

Genetic epilepsy with febrile seizures plus (GEFS+) is a familial epilepsy syndrome in which affected individuals within a family show a variety of epilepsy phenotypes, including varying from simple febrile seizures and febrile seizures plus to severe epileptic encephalopathy. The GEFS+ is associated with mutations mainly in *SCN1A, SCN1B, GABRG2* and *GABA*. Other genes have been implicated in GEFS+, including *STX1B, SCN9A, GABRD* and *FGF13* (Bonanni et al., 2004).

## VI) Focal epilepsy

| Familial Focal Epilepsy Syndrome | Genes |
|---|---|
| Autosomal dominant sleep-related hypermotor epilepsy (ADSHE) | *CHRNA4, CHRNA2, CHRNB2, DEPDC5, KCNT1, NPRL2, NPRL3* |
| Autosomal dominant epilepsy with auditory features (ADEAF) | *LGI1, RELN* |
| Autosomal dominant rolandic epilepsy with speech dyspraxia | *GRIN2A* |
| Benign familial neonatal epilepsy | *KCNQ2, KCNQ3* |
| Benign familial neonatal-infantile epilepsy | *KCNQ2, SCN2A* |
| Benign familial infantile epilepsy | *KCNQ2, KCNQ3, PRRT2,* SCN2A, SCN8A* |
| Familial focal epilepsy with variable foci (FFEVF) | *DEPDC5, NPRL2, NPRL3* |
| Familial mesial temporal lobe epilepsy (FMTLE) | *DEPDC5* |
| Familial posterior quadrant epilepsies | Unknown |
| Partial epilepsy with pericentral spikes | Unknown |

**Fig.7** List of Familial Focal Epilepsy Syndrome.

Focal seizures originate in one hemisphere of the brain. Examples of focal epilepsy syndromes are autosomal dominant sleep-related hypermotor Epilepsy (ADSHE), temporal lobe epilepsy (TLE), and autosomal dominant epilepsy with auditory features. (Myers and Mefford, 2015). ADSHE (previously known as "autosomal dominant nocturnal frontal lobe epilepsy") is characterized by seizures beginning in the first 2 decades of life. A severe form of ADSHE includes drug-resistant, and intellectual disability and displays autosomal dominant inheritance, with a penetrance of ~70% (Perucca, 2018).

The first epilepsy gene, with autosomal dominant inheritance, identified in a SHE family has been *CHRNA4*. Subsequently, two other genes were implicated in SHE, *CHRNB2* and *CHRNA2*. In addition, mutations in *CRH*, which encodes corticotropin-releasing hormone, have been implicated in families with autosomal dominant SHE. The underlying molecular mechanism in these cases is unclear, although corticotropin-releasing hormone is known to have a potentially pro-convulsive properties. The sodium-gated potassium channel, *KCNT1*, has also been associated with severe familial autosomal dominant SHE, as well as sporadic cases with *de novo* mutations. (McTague et al., 2018).

To date, the use of next generation sequencing technologies in research and diagnostic laboratories has given rise to the rapid identification of genes associated with epilepsy syndromes.

# 2. NEXT GENERATION SEQUENCING TECHNOLOGY

Next generation sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology which revolutionised genomic research. NGS has the potential to find causal mutations, including *de novo*, new and familial mutations associated with both epilepsy and epilepsy-related phenotypes, providing high performance in very few times (Dunn et al., 2018).

Conventional DNA sequencing developed by Sanger in 1977 (Sanger et al., 1977), led to many genetic discoveries and has been widely used for over 30 years in research and diagnostic laboratories. Although considered a major technological breakthrough, and still finding utility today for variant verification, the technique has limitations, in particular when examining large regions of the genome. More recently, NGS technology has begun to replace Sanger sequencing due its ability to quickly sequence large numbers of genes, the whole exome (protein-coding regions) or entire genome at once.

The applications of NGS include targeted gene panels, whole exome sequencing (WES) and whole genome sequencing (WGS). Custom gene panel testing allows to screen multiple clinically relevant genes and for more flexibility in phenotype–genotype correlations than required when testing individual genes (Poduri et al., 2014). WES focuses on the protein coding regions in the genome, comprising approximately 1–2% of the genome, attributable to ~85% of disease related mutations. In contrast, WGS provides information on the entire genome (both coding and non-coding regions), providing additional information on mutations in regulatory regions. The ~99% of the genome contains untranslated regions which may have a regulatory role (e.g., non-coding RNAs or transcription binding sites) along with potential protein coding sites yet to be annotated as genes. The impact of variants found in non-coding regions are not currently well understood, however it is feasible that a single or a combination of variants could have a significant impact on the pathology conditions such as epilepsy. This is most evident for non-coding variants that may influence expression levels or mRNA splicing, affecting protein abundance or isoforms (Dunn et al., 2018).

In particular:

i. **Targeted resequencing:** a set number of genes at a higher sequencing depth and lower cost when compared to whole exome and whole genome sequencing, however the number and specificity of genes included in the panel may influence the success of diagnosis.

ii. **Whole exome sequencing** is associated with a high sequencing depth of the protein coding regions at a lower cost compared to whole genome sequencing.

iii. **Whole genome sequencing** can theoretically provide the coverage of the full genome, compared to whole exome sequencing and gene panels, but it shows a lower sequencing depth and a higher cost per sample.

One key analytical difference between the three NGS methods is the number of variants identified. Approximately 3–4 million variants per individual are commonly identified through WGS and approximately 30,000–40,000 variants that differ to the reference genome per person are obtained by WES. Although the increased content generated from WGS allows for a better chance at finding pathogenic variants, it also increases the number of incidental findings (Dunn et al., 2018).

## 2.1 Workflow of next generation sequencing

The NGS workflow consists of multiple steps including: library preparation, sequencing and bioinformatics analysis.

### Library preparation

In all NGS approaches, DNA is fragmented prior to sequencing. This is performed in several ways and is dependent upon the specific kit used for library preparation and sequencing platform. DNA can be sheared using high frequency soundwaves (sonication), via enzymatic digestion or transposase. The key differences between the different assays is summarized in figure 8 which includes the current commonly utilized fragmentation and hybridisation techniques.

| Company | Assay name | DNA fragmentation technique | Fragment type and hybridisation method |
|---|---|---|---|
| ThermoFisher | AmpliSeq (Ion Torrent) | Primers bind to the genomic DNA creating known amplified target regions. | Primers amplify targeted regions resulting in overlapping amplicon panels |
| Agilent | HaloPlex | Transposase Digestion | Circular DNA probes align to capture regions of genomic DNA |
| Agilent | SureSelect | Sonication | Randomly sized DNA fragments are created and synthetic oligonucleotides then bind to regions of interest in solution |
| Illumina | Nextera | Restriction Enzyme Digestion | Evenly spaced, gapped probes bind to DNA. Paired-end sequencing is then used to fill the resultant gaps |
| Pacific Biosciences | Pacific Biosciences | Random shearing of DNA or amplification of specific sequences | Template fragments are ligated to hairpin adapters at each end, resulting in circular DNA with a constant ssDNA strand |
| Oxford Nanopore Technolgies | Oxford Nanopore Technologies | Optional fragmentation via Covaris g-TUBE™ using centrifugal force (NOTE: DNA shearing is not recommended when longer reads are required). | Template fragments are ligated to hairpin adapters at each end, resulting in circular DNA with a constant ssDNA strand |
| NanoString Technologies | nCounter Analysis System | Restriction enzyme digestion | Barcoded probes for targeted genes of interest bind to the DNA whilst another probe anchors the sequence target for sequencing |

**Fig.8** Common assays for library preparation.

An important step of the library preparation is amplification. Amplification is needed so that the ensuing sequencing reactions produce sufficient signal for detection by the instruments optical system.

## Sequencing

NGS technologies can perform massively parallel sequencing of millions of DNA strands. This phenomenal sequencing capability of NGS, have made this method important both for research and clinical diagnostic applications (Ballester et al., 2016). The characteristics of the DNA sequencing reaction are different for each platform, emphasizing the range of innovation in chemistry, molecular biology and engineering required to produce sequence (Mardis, 2014). In particular, the NGS sequencing technologies are based on two different sequencing systems: optical imaging and non-optical imaging. The optical imaging-based NGS technologies use sensitive optical imaging to detect and identify the nucleotides being incorporated during the sequencing. This is the most common NGS technology used by commercially available platforms. In figure 9 are summarized the principal sequencing platforms.

| Company | Platform | Amplification | Sequencing | Read length | Throughput/ time per run | Dominant error type | Overall error rate |
|---|---|---|---|---|---|---|---|
| Roche/454 Life Sciences | GS FLX Titanium XL+ | Emulsion PCR | Pyrosequencing | Up to 1 kb | 700 Mb/23 h | Indel | 0.5% |
| | GS FLX Titanium XLR70 | | | Up to 600 bp | 450 Mb/10 h | | |
| | GS Junior | | | ~400 bp | 35 Mb/10 h | | |
| Illumina | HiSeq 2000 | Bridge PCR | Sequencing-by-synthesis with reversible terminator | 36–100 bp | 105–600 Gb/2–11 days | Substitution | 0.2% |
| | Genome Analyzer IIx | | | 35–150 bp | 10–95 Gb/2–14 days | | |
| | MiSeq | | | 36–250 bp | 540 Mb–8.5 Gb/4–39 h | | |
| Life Technologies/ Applied Biosystems | 5500xl SOLiD™ system | Emulsion PCR | Sequencing by ligation | 35–75 bp | 10–15 Gb/day | Substitution | 0.1% |
| | SOLiD™ 4 system | | | 25–50 bp | 25–100 Gb/3.5–16 days | | |
| Life Technologies/Ion Torrent | Ion Proton™ sequencer (Proton I chip) | Emulsion PCR | Ion semiconductor sequencing | Up to 200 bp | Up to 10 Gb/2–4 h | Indel | 1% |
| | Ion PGM™ sequencer (318 chip) | | | 35–200 bp | 300 Mb–1 Gb/0.9–4.5 h | | |

**Fig.9** Overview of major next-generation sequencing platforms.

## Bioinformatics analysis

The detection of Single Nucleotide Variants (SNVs) and small insertions and deletions (indels) from raw NGS data consists of the following major steps:

- **Sequence generation**: Sequence generation (signal processing and base calling) is the process that converts sensor (optical and non-optical) data from the sequencing platform and identifies the sequence of nucleotides for each of the short fragments of DNA in the sample prepared for analysis. For each nucleotide sequenced in these short fragments (ie, raw reads), a corresponding Phred-like quality score is assigned, which is sequencing

platform specific. The read sequences along with the Phred-like quality scores are stored in a FASTQ file, which is a de facto standard for representing biological sequence information. After read quality control, alignment of the sequence reads to the human reference genome is performed.

- **Sequence alignment** is the process of alignment of each short DNA sequence (each typically <250 bp) with a reference genome (eg, the human reference genome used in clinical laboratories). This computationally process assigns a Phred-scale mapping quality score to each of the short sequence reads, determining the quality of the alignment process. This step also can be used to calculate the proportion of mapped reads and depth (coverage) of sequencing for one or more regions of interest. The sequence alignment data are usually stored in a de facto standard binary alignment map (BAM, binary alignment map) file format, which is a binary version of the sequence alignment/map format. Sequence alignments (BAM file formats) usually undergo quality control and alignment recalibration steps.

- **Variant calling:** is the process of accurately identifying the differences or variations between the sample and the reference genome sequence. Variant calling is based on numerous algorithmic strategies in order to create a list of sequence variants such as single nucleotide variants (SNVs), small insertions and deletions (indels), copy number alterations, and large structural alterations (insertions, inversions, and translocations). Sequence variants identified are written onto standard format files called VCF (Variant Call Format). The accuracy of variant calling is highly dependent on the quality of called bases and aligned reads. Therefore, variant calling processing, such as local realignment around expected indels and base quality score recalibration, is routinely used to ensure accurate and efficient variant calling. Typically 15,000 to 20,000 variants are discovered per exome, by contrast, about 3 million human SNV per genome are discovered using whole-genome sequencing (Ng et al., 2009; Koboldt et al., 2010).

- **Variant Filtering**: variant filtering is the process by which variants representing false-positive artifacts of the NGS method are flagged or filtered from the original VCF file on the basis of several sequence alignment and variant calling associated metadata (eg,

mapping quality, base-calling quality, strand bias, and others). This is usually a post-variant calling step, although some variant callers incorporate this step as part of the variant calling process. This automated process may be used as a hard filter to allow annotation and review of only the assumed true variants.

- **Variant Annotation:** variant annotation performs queries against multiple sequence and variant databases in order to characterize each called variant such as variant location, predicted cDNA and amino acid sequence change (HGVS nomenclature), minor allele frequencies in human populations, and prevalence in different variant databases (eg, Catalogue of Somatic Mutations in Cancer, The Cancer Genome Atlas, Single-Nucleotide Polymorphism (SNP) Database, and ClinVar). This information is used to further prioritize or filter variants for classification and interpretation.

- **Variant Prioritization:** variant prioritization is central to every Mendelian disease discovery and diagnosis effort. It is the process of determining which variants identified in the course of genetic testing are the most likely to damage gene function and underlie the disease phenotype (Karen Eilbeck et al., 2017). To perform an accurate and detailed clinical classification of each variants, the American College of Medical Geneticists guidelines (Richards et al., 2014) are follow. These recommendations suggest to assign one of the following six categories: 1) pathogenic: i.e. disease causing, sequence variation has previously been reported and is a recognized cause of the disorder; 2) Likely pathogenic: i.e. probably disease causing, sequence variation has not previously been reported and it is of a type expected to cause the disorder, usually in a known disease gene (for example, a nonsense mutation in a gene for which other mutations of this type, but at a different residue, have been reported); 3) Benign: sequence variation has previously been reported and is a recognized neutral variant; 4) Likely benign: sequence variation has not previously been reported and is probably not causative of disease; 5) Variant of unknown clinical significance (VUS): the variant is not described as pathogenetic in the literature or public database (such as ClinVar, dbSNP etc.) and/or involves a gene that could be functionally-related with the patient's clinical phenotype but animal models and/or functional validation studies have not yet been performed.

## 2.2 Next generation sequencing and epilepsy

The advent of next-generation (massively parallel) sequencing technology has revolutionized gene discovery in many disorders, including epilepsy. The most significant recent advance in understanding the genetics of epilepsy has come from exome sequencing in the EEs. The discovery in 2001 that *de novo* mutations in *SCN1A* gene cause Dravet syndrome set the stage for a better comprehension for this class of disorders (Claes et al., 2001). Targeted resequencing and the exome sequencing has proven to be an essential tool to confirm the importance of *de novo* mutations, facilitate rapid gene discovery and highlight the genetic heterogeneity of epilepsy.

In 2012, whole-genome sequencing in a family with a severely affected child revealed a *de novo* *SCN8A* mutation in the proband (Veeramah et al., 2012). Subsequent studies have confirmed the importance of this gene in the etiology of EE, with more than 25 cases reported. In a slightly larger study, using exome sequencing in 39 patients with fever-associated epilepsies similar to Dravet syndrome, Nava and colleagues identified two *de novo* mutations in *HCN1*. Subsequent sequencing of *HCN1* in 157 affected individuals has identified causative mutations in this gene (Nava et al., 2014). In particular, *HCN1* belongs to a family of hyperpolarization-activated, cyclic-nucleotide-gated channels that regulate neuronal excitability. Previous studies suggested that rare variants in *HCN1* play a significant role in the onset of GGE, while *HCN*2 and *HCN4* variations can predispose to the disease (Tang et al., 2008). Another study of 13 patients with Dravet syndrome revealed the importance of mutations in *GABRA1* and *STXBP1* (Carvill et al., 2014), two genes previously implicated in other EEs, in addition to the Dravet-related gene *SCN1A*. *GABRA1* encodes the $\alpha$1 subunit of the GABAA receptor, a multi subunit chloride channel that serves as the receptor for the GABA inhibitory neurotransmitter. *STXBP1* encodes a syntaxin-binding protein that is critical for presynaptic vesicle docking and fusion. Another gene identified by exome sequencing as causative for epilepsy is *KCNB1*, a voltage-gated potassium channel, in which new pathogenic variants were detected in three affected families (Torkamani et al., 2014).

A growing class of genes implicated in epilepsy and related disorders are those that encode for proteins involved in chromatin remodeling and transcriptional regulation.

Through targeted sequencing of candidate genes, have been identified 5 of 500 patients with EE who had a *de novo* mutation in *CHD2* (Carvill et al., 2013). To date, more than 20 patients with mutations in *CHD2* have been identified, with the majority of mutations arising as *de novo* events (Thomas et al., 2015; Suls et al., 2013). In addition, a massive study on 580 patients with GGE

(photosensitive epilepsy or photoparoxysmal response as determined by EEG ) sequenced by TRS and WES showed that variants in *CHD2* are a risk factor for photosensitivity in the GGEs, in particular disruptive variants (Galizia et al., 2015).

Another gene that causes a specific neural phenotype is the transcription factor myocyte enhancer factor 2C (*MEF2C*). Although *MEF2C* has important regulatory roles in other tissue types such as cardiac and skeletal muscle, it is a critical gene in neural progenitor cell differentiation and maturation, and the causative gene in 5q14 deletion syndrome. In fact, its haploinsufficiency can cause a range of features, including hyperkinesis, variable epilepsy, ID and autism, as well as atypical Rett syndrome (Zweier et al., 2012; Lambert et al., 2012).

The figure 10 show the timeline of gene discovery.

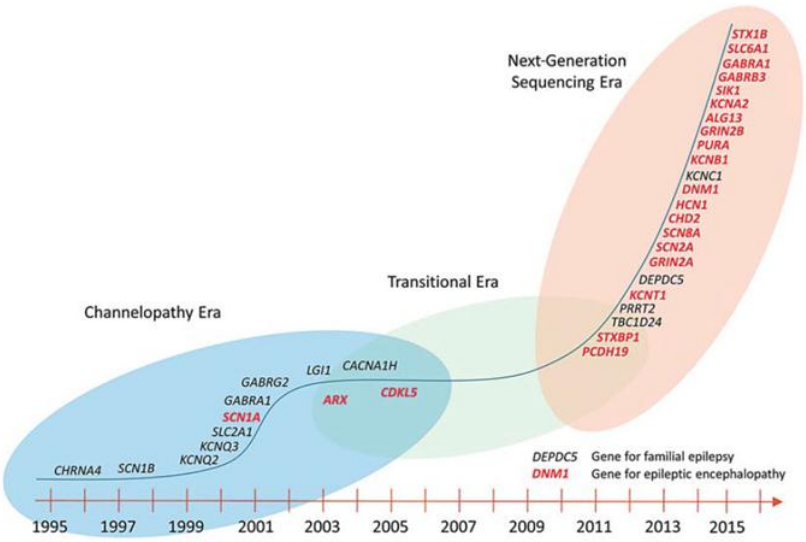


**Fig.10** Timeline of gene discovery in human epilepsies.

# 3. AIMS OF THE STUDY

Epilepsies have a highly heterogeneous background with a strong genetic contribution. The variety of unspecific and overlapping syndromic and non syndromic phenotypes often hampers a clear clinical diagnosis and prevents straight forward genetic testing. Knowing the genetic basis of a patient's epilepsy can be valuable not only for diagnosis but also for guiding treatment and estimating recurrence risk.

The aim of my project is to use TRS on a clinically well-selected cohort of patients affected by epilepsy and neurodevelopmental disorders in order to:

i) identify novel variants in candidate genes and some currently known disease-causing genes useful to expand our knowledge about the molecular mechanisms of the disease;
ii) characterize, both from molecular than from clinical point of view, novel syndromic and non-syndromic forms of the disease useful for diagnosis purpose, counseling and management of affected patients and their families.

# 4. MATERIALS AND METHODS

## 4.1 Cohort of the study

A total of 49 (23 male and 26 female) Caucasian patients were recruited at the Medical Genetic Unit of IRCCS Casa Sollievo della Sofferenza (San Giovanni Rotondo, Foggia, Italy) for genetic study by targeted resequencing.

All participants had a clinical diagnosis of concise epilepsy phenotype, often with additional symptoms such as myoclonus or intellectual disability (with or without another medical condition).

The patients were selected according to the following criteria:

• Cases with similar clinical characteristics in at least one aspect between clinical, cognitive phenotype, behavioral, pregnancy type, major abnormalities.

• Negatives to genetic tests including karyotype and high resolution SNP-array (CytoScan HD and CytoSca XON arrays).

From a clinical point of view, the patients enrolled for the study were subdivided into two groups:

- Patients affected by a syndromic form of epilepsy (table 1);
- Patients affected by a non-syndromic form of epilepsy (table 2).

| **Clinical diagnoses of syndromic epilepsy** | | **n** | **%** |
|---|---|---|---|
| Early-onset epileptic encephalopathy (EOEE) | | 2 | 4.08% |
| Rett Syndrome (RS) | | 4 | 8.16% |
| Dravet Syndrome (DS) | | 1 | 2.04% |
| Lennox-Gastaut Syndrome (LGS) | | 1 | 2.04% |
| Myoclonic epilepsy | | 1 | 2.04% |
| Mowat-Wilson Syndrome | | 1 | 2.04% |
| Unclassified epileptic syndromes | Epileptic encephalopathies (EEs) | 23 | 46.93% |
| | Generalized epilepsies | 3 | 6.12% |
| | Focal epilepsies | 2 | 4.08% |

**Tab. 1.** n: number of patients; %: percentage of patients

| **Clinical diagnoses of non syndromic epilepsy** | **n** | **%** |
|---|---|---|
| Epilepsy | 11 | 22.44% |

**Tab 2.** n: number of patients; %:percentage of patients

Clinical data was provided by the referring physician and included medical history (i.e. anamnesis, personal and family histories, physical and dysmorphological examination) and basic complementary tests, including electroencephalogram test, brain magnetic resonance imaging (MRI).

Written informed consent for genetic testing was obtained from all individuals enrolled for the study.

## 4.2 Targeted Resequencing (TRS)

### 4.2.1 Gene selection and panel design

A list of known and candidate genes associated with epilepsy was compiled on the basis of current literature (PubMed), clinical procedures suggested and adopted by the LICE (Lega Italiana Contro l'Epilessia) and in-house data. We selected 85 genes reported to cause different genetic forms of epilepsy and NDD. In particular we subdivided the genes into six categories: epilepsy genes (71 genes), neurodevelopment-associated epilepsy genes (13 genes), epileptic encephalopathy genes (16 genes) and (table 3).

| Phenotype | Genes |
|---|---|
| Epilepsy genes (71 genes) | ADSL ALDH7A1 ARHGEF9 ATP1A2 ATP6AP2 CACNA1H CACNB4 CDKL5 CHD2 CHRNA2 CHRNA4 CHRNA7 CHRNB2 CLN3 CLN5 CLN6 CLN8 CPA6 CSTB DNAJC5 EFHC1 EPM2A FOLR1 FOXG1 GAMT GATM GABRA1 GABRD GABRG2 GOSR2 GRIN2A GRIN2B KCNJ10 KCNMA1 KCNQ2 KCNQ3 KCTD7 LGI1 LIAS MAGI2 MBD5 MECP2 MEF2C MFSD8 NRXN1 NHLRC1 PCDH19 PLCB1 PNPO PRICKLE1 PRRT2 PRICKLE2 POLG PPT1 SCARB2 SCN1A SCN1B SCN2A SCN8A SCN9A SLC25A22 SLC2A1 SPTAN1 ST3GAL3 STXBP1 SLC9A6 TCF4 TPP1 TBC1D24 UBE3A ZEB2 |
| Neurodevelopment-associated epilepsy genes (13 genes) | ARX CNTN4 CNTNAP2 CRBN IER3IP1 KANSL1 PNKP SRPX2 SYN1 TMLHE TSC1 TSC2 TSEN2 |
| Epileptic encephalopathy genes (16 genes) | ARX CDKL5 CHD2 GABRA1 GRIN2B KCNQ2 PCDH19 PLCB1 SCN1B SCN2A SCN8A SLC25A22 SPTAN1 ST3GAL3 STXBP1 TBC1D24 |

**Tab 3**. Epilepsy genes: mutations in these genes cause pure or relatively pure epilepsies, or syndromes with epilepsy as the core symptom; Neurodevelopment-associated epilepsy genes: mutations in these genes produce gross neurodevelopmental malformations and epilepsy, which may vary in severity; Epileptic encephalopathy: are severe brain disorders in which the epileptic electrical discharges may contribute to progressive psychomotor dysfunction.

From a functional point of view, the majority of selected genes encodes for proteins involved in membrane protein (i.e ion channel), enzyme, synaptic formation/remodeling/maintenance, neurotransmission (or DNA methylation/chromatin remodeling). Table 4 summarized the selected genes to be included in the gene panel as well as their function, associated disorders, chromosomal position and sequencing details.

| Gene | Inheritanc | Description | Cellular Function | Phenotype | Chr. Location | Coverage (%) | Regions |
|---|---|---|---|---|---|---|---|
| *ADSL* | AR | adenylosuccinate lyase | Enzyme | adenylosuccinase lyase deficiency (ADSLD) | 22q13.1 | 100.00% | 15 |
| *ALDH7A1* | AR | aldehyde dehydrogenase 7 family, member A1 | Enzyme | Pyridoxine-dependent epilepsy (EPD) | 5q23.2 | 100.00% | 19 |
| *ARHGEF9* | XLR | RHO guanine nucleotide exchange factor 9 | Adaptor protein | Early infantile epileptic encephalopathy (EIEE) | Xq11.1 | 100.00% | 12 |
| *ARX* | XLR | aristaless related homeobox | Nucleic acid binding | Early infantile epileptic encephalopathy (EIEE) | Xq21.3 | 100.00% | 5 |
| *ATP1A2* | AD | ATPase Na+/K+ transporting subunit alpha 2 | Membrane protein | Enzymatic deficiency | 1q23.2 | 98.81% | 24 |
| *ATP6AP2* | XLR | ATPase H+ transporting accessory protein 2 | Membrane protein | Seizures, generalized tonic-clonic | Xp11.4 | 99.19% | 9 |
| *CACNA1H* | UN | calcium channel, voltage-dependent, T type, alpha-1H subunit | Membrane protein | Childhood absence epilepsy (CAE) | 16p13.3 | 99.91% | 34 |
| *CACNB4* | AD | calcium channel, voltage-dependent, beta-4 subunit | Membrane protein | Juvenile myoclonic epilepsy (JME)/Epilepsy, idiopathic generalized | 2q23.3 | 99.62% | 18 |
| *CDKL5* | XLD | cyclin-dependent kinase-like 5 | Enzyme | Early infantile epileptic encephalopathy (EIEE) | Xp22.13 | 100.00% | 20 |
| *CHD2* | AD | chromodomain helicase DNA-binding protein 2 | Nucleic acid binding | Childhood-onset epileptic encephalopathy (COEE) | 15q26.1 | 99.83% | 39 |
| *CHRNA2* | AD | cholinergic receptor,neuronal nicotinic, alpha polypeptide 2 | Neuronal receptors | Nocturnal frontal lobe epilepsy (NFLE) | 8p21.2 | 100.00% | 6 |
| *CHRNA4* | AD | cholinergic receptor, neuronal nicotinic, alpha polypeptide 4 | Acetylcholine receptor | Nocturnal frontal lobe epilepsy (NFLE) | 20q13.33 | 99.32% | 8 |
| *CHRNA7* | AR | cholinergic receptor nicotinic alpha 7 subunit | Acetylcholine receptor | Epilepsy, juvenile myoclonic/Epilepsy, idiopathic generalized | 15q13.3 | 89.53% | 15 |
| *CHRNB2* | UN | cholinergic receptor, neuronal nicotinic, beta polypeptide 2 | cholinergic receptor, neuronal nicotinic, beta polypept | Nocturnal frontal lobe epilepsy (NFLE) | 1q21.3 | 100.00% | 6 |
| *CLN3* | AR | CLN3 lysosomal/endosomal transmembrane protein | lysosomal function | Ceroid lipofuscinosis, neuronal | 16p12.1 | 100.00% | 16 |
| *CLN5* | AR | CLN5 intracellular trafficking protein | lysosomal function | Ceroid lipofuscinosis, neuronal | 13q22.3 | 100.00% | 4 |
| *CLN6* | AR | CLN6 transmembrane ER protein | lysosomal function | Ceroid lipofuscinosis, neuronal | 15q23 | 100.00% | 8 |
| *CLN8* | AR | CLN8 transmembrane ER and ERGIC protein | recycle between the ER and ER-Golgi | Ceroid lipofuscinosis, neuronal | 8p23.3 | 100.00% | 2 |
| *CNTN4* | AD | contactin 4 | cell adhesion molecule | 3p- syndrome/Seizures (rare) | 3p26.3-p26.2 | 100.00% | 24 |
| *CNTNAP2* | AR | contactin associated protein like 2 | Cell adhesion molecule | Cortical dysplasia-focal epilepsy syndrome | 7q35-q36 | 99.83% | 25 |
| *CPA6* | AR, AD | carboxypeptidase A6 | Enzyme | Familial febrile seizures (FFS)/Familial temporal lobe epilepsy (FTLE) | 8q13.2 | 100.00% | 11 |
| *CRBN* | AR | cereblon | Cytoplasm localized with a calcium channel membran | Nonsyndromic cognitive disability | 3p26.2 | 100.00% | 11 |
| *CSTB* | AR | cystatin B | Enzyme modulator | Progressive myoclonic epilepsy (PME) | 21q22.3 | 100.00% | 3 |
| *CTSD* | AR | cathepsin D | Enzyme | Ceroid lipofuscinosis, neuronal | 11p15.5 | 100.00% | 9 |
| *DNAJC5* | AD | DnaJ heat shock protein family (Hsp40) member C5 | membrane trafficking and protein folding | Ceroid lipofuscinosis, neuronal | 20q13.33 | 99.88% | 5 |
| *EFHC1* | AD | EF-hand domain (C-terminal)-containing protein 1 | Signal transduction/molecule | Juvenile absence epilepsy (JAE) | 6p12.2 | 99.40% | 13 |
| *EPM2A* | AR | EPM2A gene, encodes laforin | Enzyme | Progressive myoclonic epilepsy (PME) | 6q24.3 | 100.00% | 10 |
| *FOLR1* | AR | folate receptor alpha | Receptor protein | Neurodegeneration due to cerebral folate transport deficiency | 11q13.4 | 100.00% | 4 |
| *FOXG1* | AD | forkhead box G1 | Repressor protein | Rett syndrome | 14q12 | 99.80% | 1 |
| *GABRA1* | AD | gamma-aminobutyric acid receptor, alpha-1 | GABA-A receptor | Early infantile epileptic encephalopathy (EIEE)/Childhood absence epilepsy (CAE)/Juvenile myoclonic epilepsy (JME) | 5q34 | 100.00% | 10 |
| *GABRD* | AD | gamma-aminobutyric acid receptor, delta | GABA-A receptor | Generalized epilepsy with febrile seizures plus (GEFS + ) | 1p36.33 | 100.00% | 9 |
| *GABRG2* | AD | gamma-aminobutyric acid receptor, gamma-2 | GABA-A receptor | Familial febrile seizures (FFS)/Generalized epilepsy with febrile seizures plus (GEFS + )/Childhood absence epilepsy (CAE) | 5q34 | 98.09% | 12 |
| *GAMT* | AR | guanidinoacetate N-methyltransferase | Enzyme | Cerebral creatine deficiency syndrome | 19p13.3 | 100.00% | 6 |
| *GATM* | AR | glycine amidinotransferase | Enzyme | Cerebral creatine deficiency syndrome | 15q21.1 | 100.00% | 9 |
| *GOSR2* | AR | golgi snap receptor complex member 2 | Membrane trafficking | Progressive myoclonic epilepsy (PME) | 17q21.32 | 100.00% | 9 |
| *GRIN2A* | AD | glutamate receptor, ionotropic, N-methyl-D-aspartate, subunit 2A | NMDA receptor | Focal epilepsy, with speech disorder and with or without mental retardation | 16p13.2 | 99.04% | 12 |
| *GRIN2B* | AD | glutamate receptor, ionotropic, N-methyl-D-aspartate, subunit 2B | NMDA receptor | Early infantile epileptic encephalopathy (EIEE) | 15p13.1 | 99.86% | 12 |
| *IER3IP1* | AR | immediate early response 3 interacting protein 1 | Cell differentiation and apoptosis | Microcephaly, epilepsy, and diabetes syndrome | 18q21.1 | 100.00% | 3 |
| *KANSL1* | AD | KAT8 regulatory NSL complex subunit 1 | Nuclear protein | Koolen-De Vries syndrome | 17q21.31 | 100.00% | 14 |
| *KCNJ10* | AR | potassium inwardly rectifying channel subfamily J member 10 | Potassium channel | SESAME syndrome | 1q23.2 | 100.00% | 1 |
| *KCNMA1* | AR, AD | potassium channel, calcium-activated, large conductance, subfamily M, alpha member | Potassium channel | Generalized epilepsy and paroxysmal dyskinesia (GEPD) | 10q22.3 | 100.00% | 41 |
| *KCNQ2* | AD | potassium channel, voltage-gated, KQT-like subfamily, member 2 | Potassium channel | Benign familial neonatal seizures (BFNS)/Early infantile epileptic encephalopathy (EIEE) | 2oq13.33 | 100.00% | 19 |
| *KCNQ3* | AD | potassium channel, voltage-gated, KQT-like subfamily, member 3 | Potassium channel | Benign familial neonatal seizures (BFNS) | 8q24.22 | 99.78% | 18 |
| *KCTD7* | AR | potassium channel tetramerization domain-containing protein 7 | Potassium channel | Epilepsy, progressive myoclonic, with or without intracellular inclusions | 7q11.21 | 98.91% | 16 |
| *LGI1* | AD | leucine-rich gene, glioma-inactivated,1 | Neuronal growth regulation and cell survival | Familial temporal lobe epilepsy (FTLE) | 10q23.33 | 100.00% | 8 |
| *LIAS* | AR | lipoic acid synthetase | Enzyme | Hyperglycinemia, lactic acidosis, and seizures | 4p14 | 99.41% | 11 |
| *MAGI2* | AR | membrane associated guanylate kinase, WW and PDZ domain containing 2 | Enzyme | Nephrotic syndrome | 7q21.11 | 98.57% | 24 |
| *MBD5* | AD | methyl-CpG binding domain protein 5 | Enzyme | Kleefstra syndrome | 2q23.1 | 99.60% | 10 |
| *MECP2* | XLR | methyl-CpG binding protein 2 | Enzyme | Encephalopathy, neonatal severe | Xq28 | 95.76% | 5 |
| *MEF2C* | AD | myocyte enhancer factor 2C | Transcription factors | Mental retardation, stereotypic movements, epilepsy, and/or cerebral malformations | 5q14.3 | 100.00% | 11 |
| *MFSD8* | AR | major facilitator superfamily domain containing 8 | Membrane protein | Neuronal ceroid lipofuscinosis | 4q28.2 | 100.00% | 12 |
| *NHLRC1* | AR | NHL repeat-containing 1 gene | Enzyme | Progressive myoclonic epilepsy (PME) | 6p22.3 | 100.00% | 1 |
| *NRXN1* | AR | neurexin 1 | Membrane protein | Pitt-Hopkins-like syndrome | 2p16.3 | 97.44% | 38 |
| *PCDH19* | XL | protocadherin 19 | Cell adhesion molecule | Early infantile epileptic encephalopathy (EIEE) | Xq22.1 | 100.00% | 6 |
| *PLCB1* | AR | phospholipase C, beta-1 | Enzyme | Early infantile epileptic encephalopathy (EIEE) | 20p12.3 | 99.90% | 36 |
| *PNKP* | AR | polynucleotide kinase 3'-phosphatase | Enzyme | Microcephaly, seizures, and developmental delay | 19q13.33 | 100.00% | 16 |
| *PNPO* | AR | pyridoxamine 5-prime-phosphate oxidase | Enzyme | Pyridoxamine 5'-phosphate oxidase deficiency | 17q21.32 | 100.00% | 7 |
| *POLG* | AR | DNA polymerase gamma, catalytic subunit | Enzyme | Mitochondrial disease | 15q26.1 | 99.67% | 22 |
| *PPT1* | AR | palmitoyl-protein thioesterase 1 | Enzyme | Neuronal ceroid lipofuscinosis | 1p34.2 | 98.42% | 11 |

| Gene | Inheritanc | Description | Cellular Function | Phenotype | Chr. Location | Coverage (%) | Regions |
|---|---|---|---|---|---|---|---|
| *PRICKLE1* | AR | prickle planar cell polarity protein 1 | nuclear receptor | Progressive myoclonic epilepsy (PME) | 12q12 | 100.00% | 7 |
| *PRICKLE2* | AR | prickle planar cell polarity protein 2 | Unclassified | Progressive myoclonic epilepsy (PME) | 3p14.1 | 100.00% | 7 |
| *PRRT2* | AD | proline-rich transmembrane protein 2 | transmembrane protein | Benign familial infantile seizures (BFIS) | 16p11.2 | 100.00% | 2 |
| *SCARB2* | AR | scavenger receptor class B, member 2 | Receptor | Progressive myoclonic epilepsy (PME) | 4q21.1 | 100.00% | 13 |
| *SCN1A* | AD | sodium channel, neuronal type I, alpha subunit | Sodium channel | Dravet syndrome (DS) | 2q24.3 | 99.10% | 26 |
| *SCN1B* | AR | sodium channel, voltage-gated, type I, beta subunit | Sodium channel | Generalized epilepsy with febrile seizures plus (GEFS + ) | 19q13.11 | 99.68% | 5 |
| *SCN2A* | AD | sodium channel, voltage-gated, type II, alpha subunit | Sodium channel | Benign familial infantile seizures (BFIS)/Early infantile epileptic encephalopathy (EIEE) | 2q24.3 | 99.48% | 27 |
| *SCN8A* | AD | sodium channel, voltage-gated, type VIII, alpha subunit | Sodium channel | Benign familial infantile seizures (BFIS)/Early infantile epileptic encephalopathy (EIEE) | 12q13.3 | 100.00% | 28 |
| *SCN9A* | AD | sodium channel, voltage-gated, type IX, alpha subunit | Sodium channel | Familial febrile seizures (FFS) | 2q24.3 | 98.87% | 27 |
| *SLC25A22* | AR | solute carrier family 25 (mitochondrial carrier, glutamate), member 22 | Transporter | Early infantile epileptic encephalopathy (EIEE) | 11p15.5 | 100.00% | 9 |
| *SLC2A1* | AD | solute carrier family 2 (facilitated glucose transporter), member 1 | Transporter | Idiopathic generalized epilepsy (IGE) | 1p34.2 | 100.00% | 11 |
| *SLC9A6* | XLD | solute carrier family 9 member A6 | Membrane transport proteins | Mental retardation | Xq26.3 | 100.00% | 16 |
| *SPTAN1* | AD | spectrin, alpha nonerythrocytic 1 | Cytoskeletal protein | Early infantile epileptic encephalopathy (EIEE) | 9q34.11 | 99.71% | 57 |
| *SRPX2* | UN | sushi repeat containing protein X-linked 2 | Enzyme | Rolandic epilepsy, speech dyspraxia, and mental retardation | Xq22.1 | 100.00% | 10 |
| *ST3GAL3* | AR | ST3 beta-galactoside alpha-2,3-sialyltransferase 3 | Enzyme | Early infantile epileptic encephalopathy (EIEE) | 1q34.1 | 100.00% | 13 |
| *STXBP1* | AD | syntaxin-binding protein 1 | Membrane trafficking | Early infantile epileptic encephalopathy (EIEE) | 9q34.11 | 99.52% | 20 |
| *SYN1* | XLR, XLD | synapsin I | Membrane trafficking | X-linked epilepsy with variable learning disabilities and behavior disorders | Xp11.3-p11.2 | 99.67% | 13 |
| *TBC1D24* | AR | Tre2-Bub2-Cdc16/TBC1 domain family, member 24 | Enzyme modulator | Familial infantile myoclonic epilepsy (FIME) | 16p13.3 | 100.00% | 7 |
| *TCF4* | AD | transcription factor 4 | Transcription factor | Pitt-Hopkins syndrome | 18q21.2 | 99.80% | 25 |
| *TMLHE* | XLR | trimethyllysine hydroxylase, epsilon | Enzyme | Autism | Xq28 | 99.50% | 8 |
| *TPP1* | AR | tripeptidyl peptidase 1 | Enzyme | Neuronal ceroid lipofuscinosis | 11p15.4 | 100.00% | 13 |
| *TSC1* | AD | TSC complex subunit 1 | Enzyme | Tuberous sclerosis | 9q34.13 | 100.00% | 21 |
| *TSC2* | AD | TSC complex subunit 2 | Enzyme | Tuberous sclerosis | 16p13.3 | 97.92% | 42 |
| *TSEN2* | AR | tRNA splicing endonuclease subunit 2 | Enzyme | Pontocerebellar hypoplasia | 3p25.2 | 99.56% | 12 |
| *UBE3A* | AD | ubiquitin protein ligase E3A | Enzyme | Angelman syndrome | 15q11.2 | 99.88% | 13 |
| *ZEB2* | AD | zinc finger E-box binding homeobox 2 | Transcriptional repressor | Mowat-Wilson syndrome | 2q22.3 | 100.00% | 13 |

**Tab.4** Genes and characteristic included in the 85 NGS panel.

The specific targeted gene panel has been defined by using Agilent Sure Design tool (Agilent Technologies, CA, USA), the total amplicons obtained were 14.971, with a targeted region of 268,42 Kbp corresponding to the 85 selected genes protein-coding exons (CCDS) plus 25 bp of exons/introns boundaries. This design ensures an overall coverage greater than 98.5% of the targeted region.

### 4.2.2 Library preparation

Genomic DNA of each patients enrolled for the study was extracted from the peripheral blood leukocytes by using BioRobot EZ1 (Qiagen, Solna, Sweden).

The quality of DNA was tested on 1% electrophorese agarose gel, and the DNA concentration was measured using a Qubit DNA BR Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, San Diego, CA, USA).

A library of all coding exons and intron-exon boundaries was prepared using a HaloPlex target enrichment kit following the manufacturer's instructions (Agilent, Santa Clara, USA). The experimental workflow is represented in Figure 11.

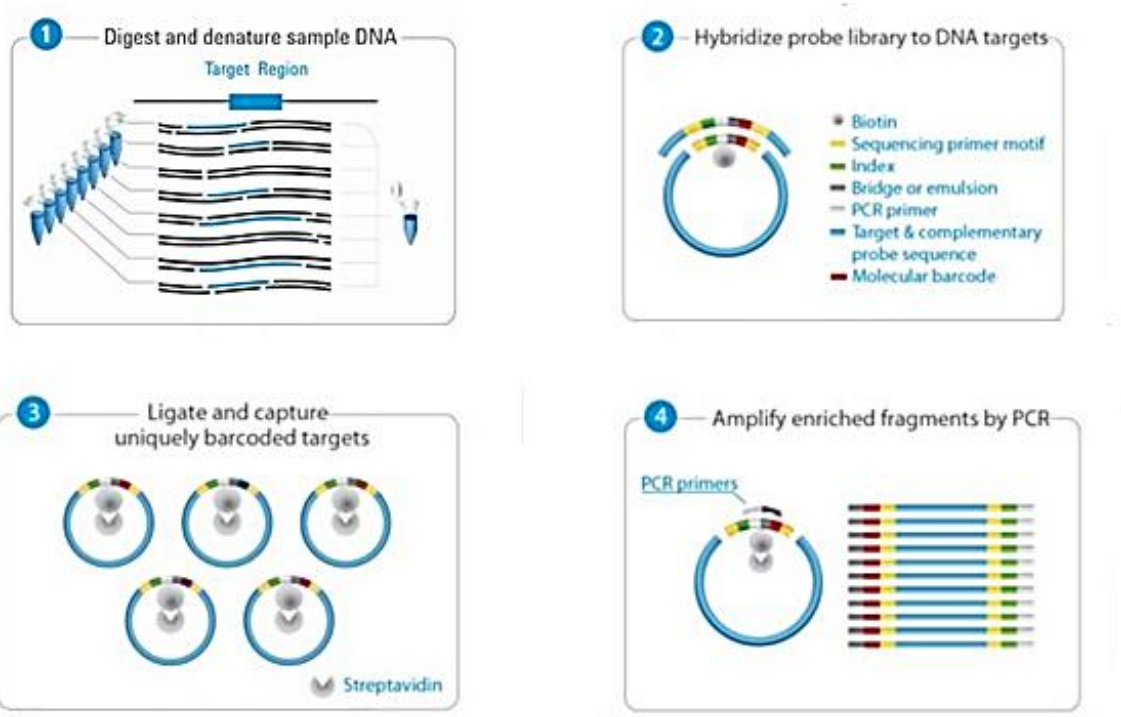


**Fig.11** HaloPlex target enrichment workflow.

Briefly, we fragmented the human genome (the samples were digested by 16 different restriction enzymes to create a library of gDNA restriction fragments) and enriched for the coding regions of genes by using complementary highly specific biotinylated probes. HaloPlex probes are designed to hybridize selectively to fragments originating from target regions of the genome and to direct circularization of the targeted DNA fragments.

Hybridized probes were captured with magnetic beads and target fragments were ligated to create circular DNA molecules. Subsequently, libraries were amplified by PCR, introducing unique index sequences that allow all pools to be sequenced together. Sequencing was performed using the NGS MiSeq Illumina sequencer (Illumina, Inc.). We selected a Q-score of 30, corresponding to a 1:1000 error rate, as an acceptance threshold value.

### 4.2.3 Sequencing

The libraries were pooled, and sequencing was performed on a Miseq sequencer (Illumina, San Diego, CA, USA) using Miseq Reagent Kit V3 300 cycles flow cell. For the Illumina platform, adapter modified library fragments are automatically dispensed onto a glass slide flow cell that displays oligonucleotides complementary to Illumina adapter sequences. Subsequently, a process called bridge amplification is used in order to generate clonal "clusters" of approximately 1000 identical molecules per cluster. Single-stranded, adapter-ligated fragments are bound to the surface of the flow cell exposed to reagents for polymerase-based extension. Priming occurs as the free/distal end of a ligated fragment "bridges" to a complementary oligo on the surface. Repeated denaturation and extension result in localized amplification of single molecules in "clusters". The Illumina sequencing platform is based on a sequencing-by-synthesis approach, in which all four nucleotides are added simultaneously to the flow cell channels, along with DNA polymerase, for incorporation into the oligo-primed cluster fragments. The nucleotides carry a base unique fluorescent label and the 3'-OH group is chemically blocked (Figure 12), so that each incorporation is a unique event.

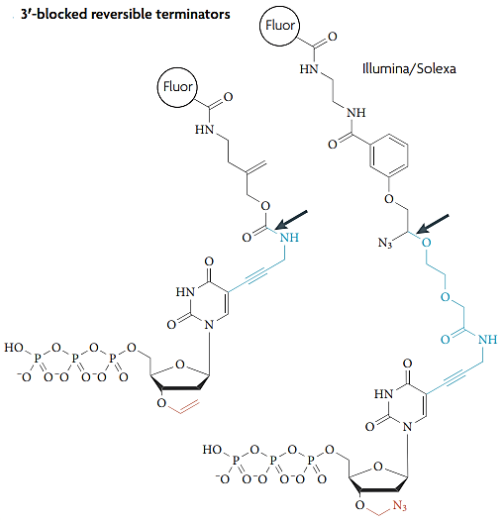


**Fig.12** 3'-blocked reversible terminator.

Once the base is incorporated, a fluorescence signal is emitted and this event is captured by a fluorescence detector. Subsequently, the 3' blocking group is chemically removed to prepare each strand for the next incorporation. The cycle is repeated, one base at a time, generating a series of images each representing a single base extension at a specific cluster.

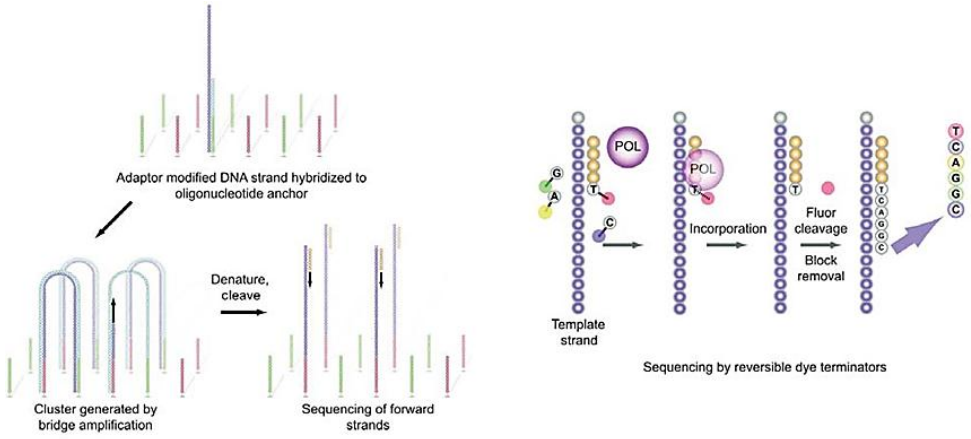


**Fig.13** Schematic representation of Illumina platform.

### 4.2.4 Bioinformatic analysis and variants prioritization

The analysis of NGS sequencing data was performed according standard bioinformatics procedures. Raw individual sequence files (*.fastq) were checked for their quality through the FastQC tool (Andrews, 2010), in order to detect trends in base call quality scores according to read position, adapter content, base compositional bias, etc. Quality-checked reads were mapped against the hg19 version of the human reference genomic sequence through the BWA align (Li et al., 2009); the produced alignments (*.bam) were also processed through the GATK suite (McKenna et al., 2010). Refined *.bam alignments were used to calculate on-target and off-target sequencing coverage statistics (in particular, average target depth of coverage and proportion of target sites at 20X minimal coverage) by using the TEQC R Package (Hummel et al., 2011). Finally, variants were detected by means of the GATK v3.7 Haplotype Caller, and annotated by using ANNOVAR package respect to the hg19 RefSeq gene and transcript annotation (Wang et al., 2010). Functionally annotated variants were also checked for their presence in public variant collections, like dbSNP v151 and its ClinVar specialized database (Sherry et al., 2001), ExAC v0.3 (Exome Aggregation Consortium 2016) and gnomAD (Konrad et al., 2019). Furthermore, variants with a missense functional impact were also annotated with pathogenicity predictors, by querying the dbNSFP resource (Liu et al., 2011).

In order to prioritization variants the following pipeline was applied:

- Allele frequency. The use of a MAF threshold >1% allowed a removal of a large number of variants not connected with the phenotype.
- Predicted consequence. Predicted effect on protein function: this step is particularly critical for non-synonymous or missense variants in which the amino acid sequence is determined the functional consequences and of the biochemical conservation respect to the replaced amino acid. During this step we used SIFT (Kumar et al., 2009), PolyPhen2 (Adshubei et al., 2010), Mutation Taster (Schwarz JM et al., 2014) FATHMM (Rogers FM et al., 2018) and CADD (Rentzsch P et al., 2019). Splice-site variants were predicted by Human Splicing Finder (Desmet FO et al., 2009).
- Inheritance. Variants were evaluated based on the family history of the disease and the inheritance of the gene (dominant/recessive/X-linked), including *de novo* mutations in dominant and X-linked genes and variants in recessive genes (homozygous and compound heterozygous). Inherited missense variants predicted to be benign were excluded.

In order to exclude the presence of mosaic variants, we focused our attention on the following values: Total Depht (filtered), Genotype Depht (filtered) and Ref/Alt (filtered reads). We used these parameters to evaluate the heterozygous or homozygous state for each patient.

For patients in which no variants of interest were found, the filtering criteria were relaxed in order to include variants with an MAF up to 0.05, distant from the canonical splicing site for around 8 bp, and potentially pathogenic synonymous variants based on the annotations predicted by the TraP (Gelfman S. et al., 2017) and absent in dbDSM database. (Database of Deleterious synonymous mutation).

All variants were visualized through tool the Integrated Genomics Viewer (Thorvaldsdottir et al., 2013) and confirmed through Sanger sequencing in order to detecting false positives.

# 5. RESULTS

In total, we recruited 49 patients with a phenotype of epilepsy and NDD. All samples have been analyzed through targeted resequencing of a gene panel containing 85 genes (10 X-linked and 75 autosomal genes, respectively) related to various form of epilepsy and NDD, and selected on the most updated literature survey.

All the sequencing run obtained more than 90% of targeted regions covered at least 100X and a minimum average coverage of 250x. For the first variants prioritization, we excluded intronic variants outside +/- 8 bp from the splice sites , as well as synonymous exonic variations outside the highly conserved acceptor and donor splice site. The candidates were filtered by frequency and submitted to an in silico prediction analysis of their functional effect at the amino acid level (i.e. protein folding, evolutionary conservation, loss of function or gain of function, etc.) and confirmed by Sanger sequencing.

This approach allowed us to identify variants in 25 of 49 patients (51%), including sequence alterations in frequently as well as in less commonly affected genes. In the remaining 24 affected individuals, the analysis revealed no significant variant at all.

In particular, we obtained a molecular diagnosis (pathogenic or likely pathogenic variants) in 10/49 probands (diagnostic yield 20.4%) while we identified variants of uncertain significance in 15/49 patients (30.6%). In details, causative variants (pathogenic or likely pathogenic) were related to 9 genes (*ARX, GAMT, KCNQ2, MECP2, POLG, SCN1A, SPTAN1, STXBP1, TCF4*) while the variants of uncertain significance were related to 19 genes (*SCN9A, TSC1, SCN2A, SCN1A, GRIN2B, GRIN2A, CLN3, CLN6, CACNA1H, CNTN4, LIAS, PRICKLE2, PNKP, SPTAN1, POLG, CACNB4, ATP1A2, CNTNAP2, KCNMA1*).

In total, we found 31 rare exonic variants, 7 were *de novo* of which 6 mutations in autosomal genes (*GAMT, KCNQ2, SCN1A, STXBP1, TCF4*) and 1 mutation in X-linked gene (*MECP2*), 7 inherited variants and 17 with unknown inheritance since parents DNA was not available. Furthermore, of these 31 variants, 10 were new, i.e. not reported in literature or public database, and 21 were previously reported.

X-linked disorders were diagnosed in 2 patients (1 male and 1 female), including *ARX*, epileptic encephalopathy, early infantile (#308350) and *MECP2*, encephalopathy, neonatal severe (#300673), respectively.

With regard to the types of the variants, we identified: 26 missense variant, 4 frameshif, 1 nonsense.

Frameshift variants were more common in patients with mutations in autosomal dominant genes (such as *TCF4*, *SPTAN1*, *KCNMA1*).

Missense variants were found to be disease causing in both dominant and recessive disorders.

The 31 variants were related to 25 genes. The genes included *ARX* (n = 1), *ATP1A2* (n = 1), *CACNA1H* (n = 2), *CACNB4* (n = 2), *CLN3* (n = 1), *CLN6* (n = 1), *CNTN4* (n = 1), *CNTNAP2* (n = 1), *GAMT* (n = 1), *GRIN2A* (n = 1), *GRIN2B* (n = 1), *KCNMA1* (n = 1), *KCNQ2* (n = 2), *LIAS* (n = 1), *MECP2* (n = 1), *POLG* (n = 2), *TSC1* (n = 1), *PRICKLE2* (n = 1), *PNKP* (n = 1), *STXBP1* (n = 2), *SCN1A* (n = 2), *SCN2A* (n = 1), *SCN9A* (n = 1), *SPTAN1* (n = 1), *TCF4* (n = 1). These genes identified in our cohort were classified into the following ten categories related to the epilepsy-causing mechanism: ion channels (*ATP1A2, CACNB4, CACNA1H, GRIN2A, GRIN2B, KCNQ2, KCNMA1, SCN1A, SCN2A, SCN9A*), enzyme (*GAMT, MECP2, LIAS, PNKP, STXBP1, TSC1*) , nucleic acid binding (*ARX*), cell adhesion molecules (*CNTNAP2, CNTN4*),), transcription factor (*TCF4*), cytoskeletal protein (*SPTAN1*), membrane trafficking (*STXBP1*), lysosomal function (*CLN3*, CLN6), nuclear receptor (*PRICKLE2*) (Figure 14).

From a clinical point of view, we identified variants in 10/25 (40%) patients with a diagnosis of epileptic encephalopathy (4 pathogenic/likely pathogenic and 8 VUS), 7/25 (28%) patients with a diagnosis of syndromic epilepsy (epilepsy with other symptoms such as mental retardation) (2 pathogenic/likely pathogenic and 6 VUS), 1/25 (4%) with a diagnosis of Lennox-Gastaut syndrome (1 likely pathogenic), 1/25 (4%) with a diagnosis of GEFS/SMEI (1 pathogenic), 1/25 (4%) with a diagnosis of generalized epilepsy (4 VUS) and 5/25 (20%) with a diagnosis of epilepsy (2 pathogenic/likely pathogenic and 3 VUS).

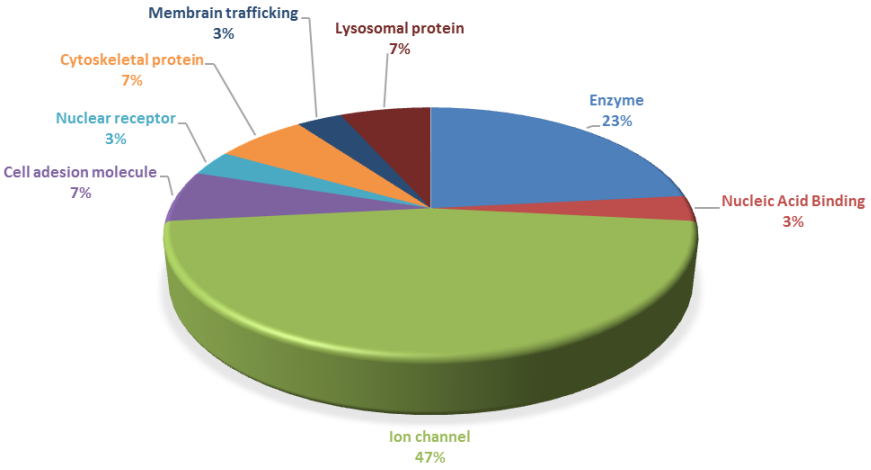


**Fig**.14 Categories in percentage of genes in our cohort.

In table 5 all mutation identified in our cohort are reported.

| ID Samples | Sex | Age | Diagnosis | Gene | Mutation | SIFT/ Polyphen2 | MutationTaster/ FATHMM | CADD | ExAC/ gnomAD | Novel/ reported | Type | Parental origin | ACMG pathogenicity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TRS_EPIL_18-0371 | F | 14 years | Epilepsy | *POLG* | NM_002693:exon4:c.926G>A:p.R309H | D/D | D/D | 35 | 2.501e-05/ 1.646e-05 | rs780953863 | missense | pat (het) | LP |
| TRS_EPIL_18-1775 | M | 1 year | Epilepsy | *KCNQ2* | NM_172107:exon8:c.1058G>C p.R353P | D/D | D/D | 34 | None | novel | missense | de novo (het) | LP |
| TRS_EPIL_18-2045 | F | 23 years | Lennox-Gastaut syndrome (LGS) | *SPTAN1* | NM_001130438:exon53:c.6940_6943G | - | - | - | None | novel | frame-shift | nd (het) | LP |
| TRS_EPIL_18-3914 | M | 1 year | Epileptic Encephalopathy (EEs) | *ARX* | NM_139058:exon2:c.989G>T:p.R330L | D/D | D/D | 25.3 | None | novel | missense | mat (homo) | LP |
| TRS_EPIL_18-2398 | M | 6 years | Epileptic Encephalopathy (EEs) | *STXBP1* | NM_003165:exon3:c.130T>C:p.C44R | D/D | D/D | 27.8 | None | novel | missense | de novo (het) | LP |
| TRS_EPIL_18-4727 | F | 29 years | Mental retardation, epilespy microcephaly | *GAMT* | NM_000156:exon6:c.650C>T:p.P217L | D/D | D/D | 22.1 | 3.417e-05/ 3.295e-05 | rs139890971 | missense | de novo (het) | LP |
| TRS_EPIL_18-1559 | M | 18 years | GEFS+/SMEI | *SCN1A* | NM_001165963:exon15:c.2807A>G:p.D936G | D/B | D/D | 27.4 | None | novel | missense | de novo (het) | PATH |
| TRS_EPIL_18-2161 | M | 7 years | Mental retardation, epilespy ataxia | *TCF4* | NM_001348218 exon10:c.1025insCAGGATCTCATGCTACCAGGATC TCA | - | - | - | None | novel | delins | de novo (het) | PATH |
| TRS_EPIL_18-4187 | F | 2 years | Early Infantile Epileptic Encephalopathy (EIEE) | *MECP2* | NM_001110792:exon3:c.952C>T:p.R318C | D/D | D/D | 32 | None | rs28935468 | missense | de novo(het) | PATH |
| TRS_EPIL_19-0610 | F | 1 year | Early Infantile Epileptic Encephalopathy (EIEE) | *KCNQ2* | NM_004518:exon4:c.602G>A:p.R201H | D/D | D/D | 34 | None | rs1057516085 | missense | de novo (het) | PATH |
| TRS_EPIL_18-1136 | F | 2 years | Epileptic Encephalopathy (EEs) | *SCN9A* | NM_002977:exon27:c.5107G>A:p.D1703N | D/D | D/D | 25.2 | 3.306e-05/ 2.031e-05 | rs200733722 | missense | pat (het) | VUS |
| TRS_EPIL_18-1259 | M | 18 years | Epilepsy | *TSC1* | NM_000368:exon23:c.3133C>G:p.L1045V | T/P | D/D | 11.95 | 0.0001/ 7.741e-05 | rs747162992 | missense | nd (het) | VUS |
| TRS_EPIL_18-1289 | F | 1 year | Epilepsy | *SCN2A* | NM_021007:exon18:c.3454G>A:p.A1152T | T/B | D/D | 17.53 | 5.767e-05/ 4.468e-05 | rs766523968 | missense | pat (het) | VUS |
| TRS_EPIL_18-1709 | F | 2 years | Epileptic Encephalopathy (EEs) | *SCN1A* | NM_001165963:exon16:c.3386C>T:p.T1129M | D/D | D/D | 28.4 | 6.595e-05 4.064e-05 | rs779989776 | missense | mat (het) | VUS |
| TRS_EPIL_18-2078 | M | 44 years | Myoclonic syndrome, cognitive impairment | *GRIN2B* *GRIN2A* | NM_000834:exon13:c.3076G>A:p.G1026S NM_000833:exon14:c.3578T>G:p.L1193W | ./P T/D | ./D D/T | 24.4 23.6 | 0.0002 0.0001 | rs201963596 rs75761674 | missense missense | nd (het) | VUS |
| TRS_EPIL_18-2084 | F | 21 years | Epilepsy, paroxysmal dystonia | *CLN3* | NM_001286105:exon3:c.22C>T:p.R8X | T/D | M/D | 12.63 | 0.0006 | rs137906617 | stopgain | nd (het) | VUS |
| TRS_EPIL_18-2293 | M | 16 years | Mental retardation, epilespy | *CACNB4* | NM_000726:exon14:c.1550G>A:p.R517Q | T/D | D/T | 25.5 | 1.667e-05 8.158e-05 | rs760115429 | missense | pat (het) | VUS |
| TRS_EPIL_18-3244 | M | 22 years | Epileptic Encephalopathy (EEs) | *GRIN2B* | NM_000834:exon2:c.92G>C:p.S31T | ./B | ./D | 13.3 | None | novel | missense | nd (het) | VUS |
| TRS_EPIL_18-3998 | F | 34 years | agenesis of corpus callosum, mental retardation, epilepsy and dysmorphic features | *ATP1A2* | NM_000702:exon11:c.1410C>G:p.D470E | T/B | ./D | 9.578 | None | rs533400580 | missense | nd (het) | VUS |
| TRS_EPIL_18-4006 | M | 15 years | Epileptic Encephalopathy (EEs) | *LIAS* *PRICKLE2* | NM_001278590:exon10:c.973_977A NM_198859:exon8:c.1907A>T:p.H636L | _ T/D | _ D/D | _ 11.46 | _ 4.946e-05 | novel rs145422223 | frameshift missense | nd (het) nd (het) | VUS |
| TRS_EPIL_18-4514 | M | 4 years | Epileptic Encephalopathy (EEs) | *POLG* *CNTNAP2* | NM_002693:exon10:c.1720C>T:p.R574W NM_014141:exon4:c.479G>A:p.R160H | D/D D/D | D/D D/D | 35 31 | 8.302e-06 0.0004 | rs774474723 rs138738227 | missense missense | nd (het) | VUS |
| TRS_EPIL_18-4527 | F | 20 years | Generalized Epilepsies | *CLN6* *CACNA1H* *KCNMA1* *PNKP* | NM_017882:exon4:c.308G>A:p.R103Q NM_021098:exon6:c.733G>A:p.G245S NM_001014797:exon1:c.57delinsCGGCGGA NM_007254:exon6:c.601C>T:p.R201C | D/D T/D _ D/D | D/D D/D _ M/T | 34 32 _ 22.9 | 3.314e-05 8.316e-06 9.911e-05 4.061e-06 | rs154774634 rs774687054 rs760628050 _ | missense missense delins missense | nd (het) | VUS |
| TRS_EPIL_18-4538 | M | 13 years | Epilepsy | *CACNA1H* | NM_021098:exon35:c.6374T>C:p.V2125A | T/B | N/D | None | None | novel | missense | nd (het) | VUS |
| TRS_EPIL_18-2500 | F | 3 years | Epileptic Encephalopathy (EEs) | *CNTN4* | NM_001206956:exon8:c.850G>A:p.A284T | D/D | D/D | 32 | 8,13E-03 | rs764462108 | missense | pat (het) | VUS |
| TRS_EPIL_18-4568 | F | 30 years | Epilesy, Focal Cortical Dysplasia | *SPTAN1* | NM_001195532:exon36:c.4773C>A:p.D1591E | T/B | D/D | 14.18 | None | novel | missense | nd (het) | VUS |

**Tab.5** Summary of the pathogenic, likely pathogenic and VUS variants identified in this study.
F: female; M: male; Het: heterozygous; Homo: homozygous;
SIFT and FATHMM prediction: D: deleterious; T: tolerated;

Pholyphen2 prediction: D: probably damaging; P: possibly damaging; B: benign;
Mutation Taster prediction: D: disease causing; A: disease causing automatic; N: polymorphism; P: polymorphism; automatic;
CADD_phred prediction: Phred-Scaled CADD score. Basically, a score of 10 means that variant is placed within the 10% percentile highest (more deleterious) scores, 20 means is for 1% percentile highest scores, and 30 is 0.1% percentile highest scores and so on.

In details:

- TRS_EPIL_18-1559 is a 18 years old male with genetic epilepsy with febrile seizures plus (GEFS+). Target resequencing analysis revealed a heterozygous mutation (c.2807A>G; p.Asp936Gly) in *SCN1A*. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its *de novo* origin (Figure 15).

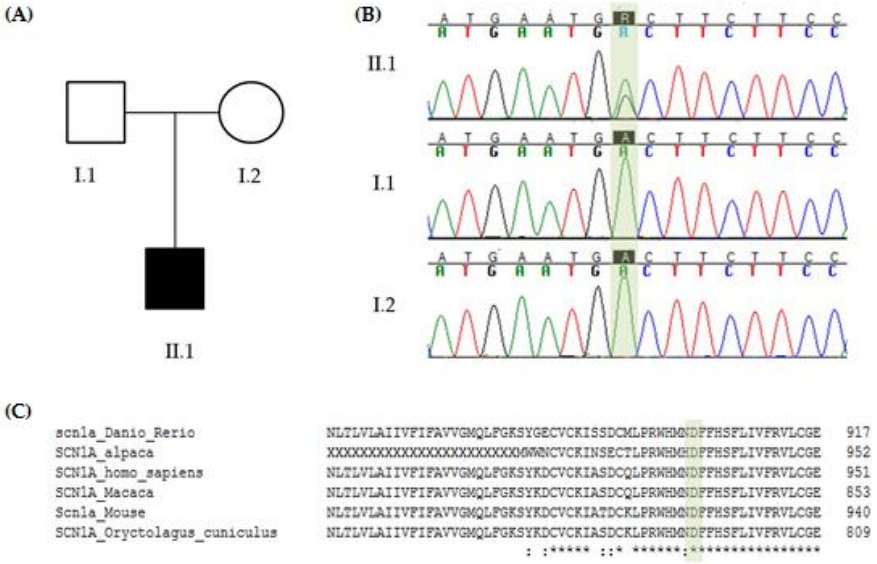


**Fig. 15 (A)** Family pedigree of the TRS_EPIL_18-1559 patient. **(B)** Family electropherogram of the c.2807A>G variant in *SCN1A* gene**. (C)** Evolutionary conservation of aspartic acid in position 936 of SCN1A protein.

*SCN1A* is the $\alpha$-subunit of the neuronal sodium channel, a voltage-sensitive channel necessary for normal activity of neurons, which consists of four identical domains of six transmembrane segments each. S5-S6 forms the pore region, the functionally important part of the channel, whereas S4 is the voltage sensor and in that way important for controlling the normal functioning of the channel (Escayg and Goldin, 2010) (figure 16). *SCN1A* is considered one of the most relevant epilepsy-related genes in the clinical setting (Gonsales et al., 2019). Mutations in *SCN1A* gene have been found to be

responsible for at least 80% of patients with Dravet syndrome, and 90% of these mutations arise *de novo*. Variable clinical phenotypes is commonly observed among these patients with *SCN1A* mutations, suggesting that genetic modifiers may influence the phenotypic expression of Dravet syndrome (Jiang et al., 2018).

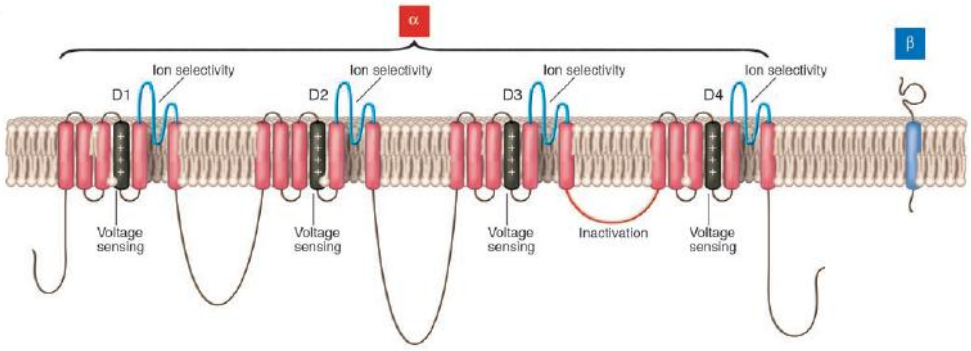


**Fig.16** The sodium channel α and β subunits encoded by *SCN1A* gene.

In particular, this variant were predicted as pathogenetic by applying the ACMG guidelines. This novel c.2807A>G transversion resulted in the substitution of aspartic acid for a aliphatic glycine p.(Asp936Gly). The pathogenic role was supported by the evidence that the variation involved the pore region DIIS5–S6 linkers of the sodium channel a-1 subunit and was not found in her parents. Zuberi et al (2001) found that significant polarity changes are more frequently seen in the voltage sensor and pore region of the *SCN1A* protein. These are areas of crucial importance for channel function and contain several charged amino acids. Any significant change in polarity in these areas may alter voltage sensing or lead to an alteration of charge in the pore region. Our results show, once more, that amino acid changes in the functionally important domains S4 and S6 are associated with a severe phenotype, whereas changes in the transmembrane segments S1–S3 are not, so we may assert that the areas significantly affected by polarity changes are the voltage sensor and pore segments, but not the whole transmembrane domain.

- TRS_EPIL_18-2161 is a 7 years-old male patient that presented unclassified EE, agenesis of corpus callosum, speech impairment and motor retardation. Through TRS, novel frameshift disease-causing variant in *TCF4*

(c.1025_1026insCAGGATCTCATGCTACCAGGATCTCA) was found. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its *de novo* origin (Figure 17).

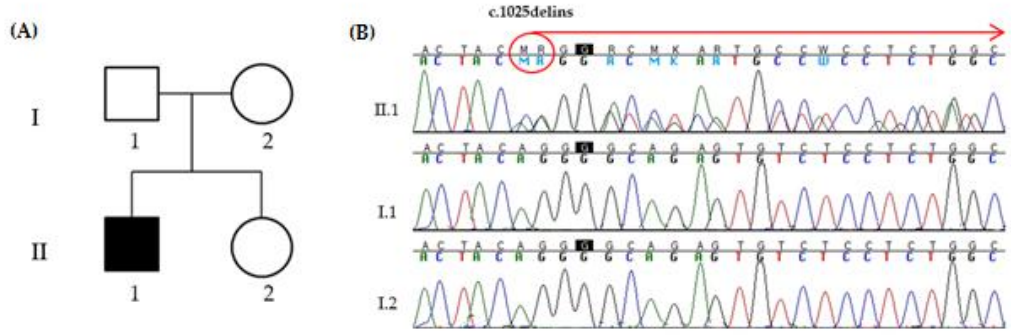


**Fig.17 (A)** Family pedigree of the TRS_EPIL_18-2161 patient. **(B)** Family electropherogram of the c.1025_1026insCAGGATCTCATGCTACCAGGATCTCA variant in *TCF4* gene.

This variant generate a truncated protein p.(Gln342Hisfs*42).

*TCF4* gene encodes for the transcription factor 4, a basic helix-loop-helix transcription factor. It is broadly expressed, and may play an important role in nervous system development. Defects in this gene are cause of Pitt-Hopkins syndrome (PTHS), an autosomal dominant syndromic encephalopathy characterized by specific dysmorphic features, severe motor retardation, intellectual disability, speech delay, stereotypic movements, breathing abnormalities (hyperventilation episodes, apnea), ophthalmological disorders (strabismus, myopia), hypotonia and seizures (Sepp et al., 2012). The patient reported here showed a lot of the core features of the PTHS (Rosenfeld et al., 2009) including dysmorphic features, intellectual disability, speech delay, stereotypic movements, hypotonia and seizures. This variant not observed in the general population databases (1000 Genomes Project, Exome Variant Server, Genome Aggregation Database).

Given this clinical and molecular evidence, we assign a pathogenetic role to the detected variant.

- TRS_EPIL_18-4187 is a female children of 2 years with a clinical diagnosis of EOEE. Thanks to TRS we detected a previously reported *MECP2* heterozygous variant

(c.916C>T, p.(Arg306Cys), rs28935468). The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its *de novo* origin (Figure 18).

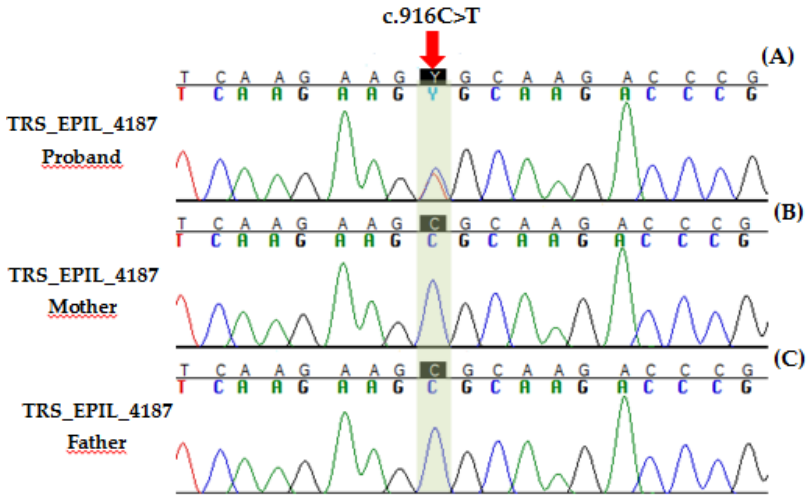


**Fig.18 (A)** Electropherogram of variant c.916C>T in *MECP2* gene in TRS_EPIL_18-4187 patient. **(B)** Electropherogram of the mother. **(C)** Electropherogram of the father.

This variant is listed as pathogenic in ClinVar, and not observed in the general population databases (1000 Genomes Project, Exome Variant Server, Genome Aggregation Database).

*MECP2* is a multifunctional protein which influences gene expression and metabolism on many levels. The main function of *MECP2* is to recognize and bind specifically methylated cytosine residues in the DNA (namely 5MeCyt) that are enriched with A/T bases adjacent. *MECP2* binds also but with lesser affinity to hydroxymethylated DNA (namely 5-hydroxy methylated cytosine, 5OHMeCyt). Mutations in *MECP2* are known to cause Rett syndrome (Ehrhart et al., 2016) (figure 19).

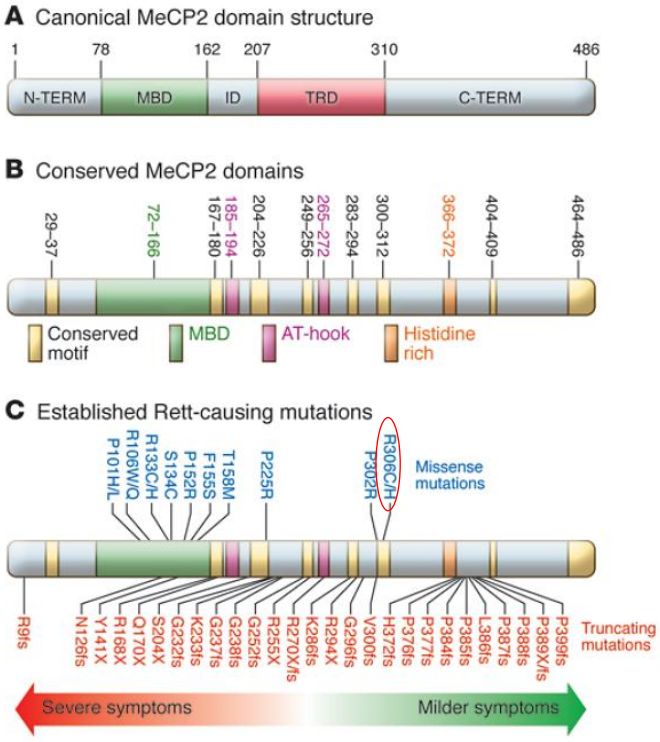


**Fig.19** The *MECP2* gene strurcture (A), conserved domain (B) and variants (C).

The *MECP2* c.916C>T, p.(Arg306Cys) variant (rs28935468) is a recurrent alteration in individuals diagnosed with Rett syndrome, and often found as a *de novo* change (Cheadle et al., 2000; Wan et al., 1999). Transgenic mice expressing the variant protein show developmental and behavioral phenotypes reminiscent of the clinical symptoms found in human patients (Brown et al., 2016; Heckman et al., 2014). Functional characterization of the *MECP2* protein indicates disruption in its association with co-repressors, such as HDAC3 and the NCoR complex (Ebert, 2013; Heckman et al., 2014; Lyst et al., 2013), and reduction in in-vivo DNA occupancy (Heckman et al., 2014). This results in a failure of the *MECP2* protein in mediating transcriptional repression at its targets (Ebert et al., 2013; Lyst et al., 2013). Another missense variant in the same residue, p.(Arg306His), has also been implicated in Rett syndrome (Cheadle et al., 2000).

- TRS_EPIL_19-0610 is a female children of 1 year-old with a clinical diagnosis of EE, burst suppression and appearance of seizures during the first week of life.

TRS allowed us to detected the variant c.602G>A p.(Arg201His) in *KCNQ2* gene. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its *de novo* origin (data not showed). The variant is show in figure 20.

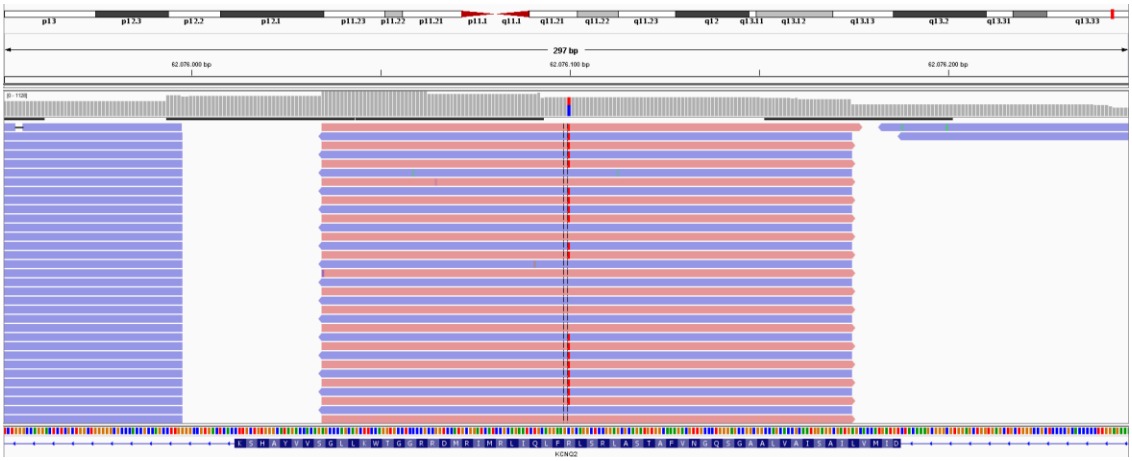

**Fig.20** The IGV view for the variant c.602G>A p. (Arg201His) in *KCNQ2* gene at locus 20q13.33.

*KCNQ2* is expressed predominantly in the brain and encode for voltage-gated potassium channel subunits. KCNQ2 subunit consists of hetero multimeric channels with 6 transmembrane domains (S1-S6), including a voltage sensor in S4, a loop between S5-S6 that builds the ion channel pore, a cytoplasmic N-terminal, and a long C-terminal region of mostly unknown function.

Mutations of this gene have shown to cause both benign familial neonatal seizures (BFNS) (Biervert et al., 1998; Singh et al., 1998) and neonatal epileptic encephalopathy (Weckhuysen et al., 2012), confirming the association of *KCNQ2* mutations with intractable seizures. Some cases showed a suppression-burst pattern on EEG, tonic seizures, and profound intellectual disability (Kato et al., 2013).

In our cases, this sequence change replaces arginine with histidine at codon 201 of the KCNQ2 protein p.(Arg201His). The arginine residue is highly conserved and this variant is not present in population databases (ExAC, gnomAD, CADD). This variant has been reported in literature as *de novo* in individuals affected with early infantile epileptic encephalopathy (Carvill et al., 2013). Furthermore, experimental studies have shown that this missense change results in a protein that has a significant increase in current density and loss of voltage-dependent gating (Miceli et al., 2015). For these reasons, the variant c.602G>A has been classified as pathogenic

- in a 14-year-old female patient (TRS_EPIL_18-0371) with epilepsy, we detected a variant (c.926G>A; p.(Arg309His), rs780953863) in *POLG* gene (figure 21).

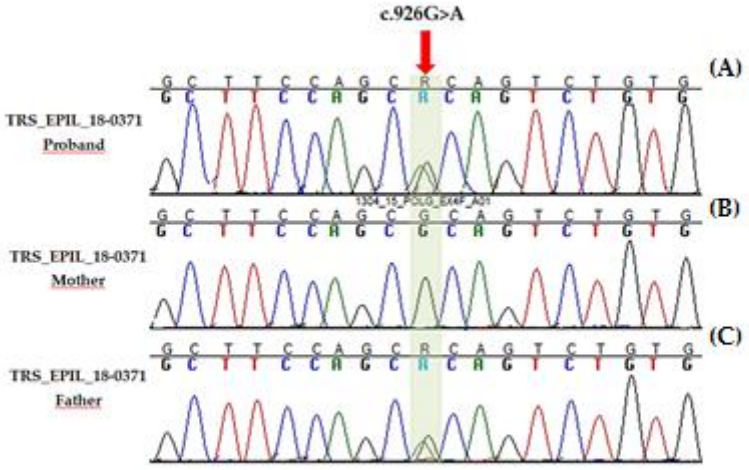


**Fig.21 (A)** Electropherogram of variant c.926G>A in *POLG* gene in TRS_EPIL_18-0371 patient. **(B)** Electropherogram of the mother. **(C)** Electropherogram of the father.

*POLG* is a nuclear gene encoding the catalytic subunit of DNA polymerase gamma which is responsible for the replication of the mitochondrial DNA. Mutations in *POLG* are among the most common causes of mitochondrial disease. Pathogenic mutations in *POLG* can cause a diverse spectrum of diseases (Stumpf et al., 2013). Our patient carried a heterozygote c.926G>A mutation in exon 4 predicting a p.Arg309His amino acid substitution.

The Arg309His variant has been reported previously in an individual with encephalopathy (Szczepanowska and Foury, 2010). Functional studies suggest that the Arg309His variant results in mtDNA instability (Szczepanowska and Foury, 2010) and this substitution occurs at a position that is conserved across species supporting the functional importance of this region of the protein. In silico analysis predicts this variant is probably damaging to the protein structure/function. Given this clinical and molecular evidence, we assign a likely pathogenetic role to the detected variant. Due to the autosomal recessive inheritance of *POLG*, and due to the lack of a second single nucleotide variations on the other allele, we cannot exclude the presence of a deletion on the other allele of the gene.

- in a 1-year-old male patient (TRS_EPIL_18-1775) with unclassified EE, we detected a variant c.1058G>C p.(Arg353Pro) in *KCNQ2* gene. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its *de novo* origin (Figure 22).

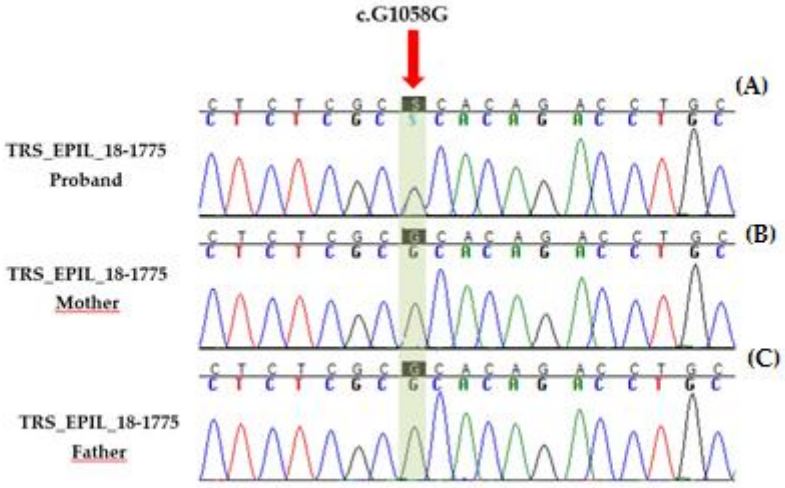


**Fig.22 (A)** Electropherogram of variant c.1058G>C in *KCNQ2* gene in TRS_EPIL_18-1775 patient. **(B)** Electropherogram of the mother. **(C)** Electropherogram of the father.

In the *KCNQ2* gene, mutations can cause haploinsufficiency or a more severe dominant-negative effect (Inn-Chi Lee et al., 2017). Our patients presented a novel *de novo* mutation in *KCNQ2*, p.(Arg353Pro), and this substitution occurs at a position that is highly conserved across species, supporting the functional importance of this region of the protein. Given this evidence, we strongly support the hypothesis of a likely pathogenetic effect of the detected variants.

- in a 23-year-olf female (TRS_EPIL_18-2045) with Lennox Gastaut Syndrome (LGS) we detected of a frame-shift variant (c.6871_6873del, p.(Gln2291del)) in *SPTAN1* gene. The variant was confirmed by Sanger sequencing, (Figure 23).

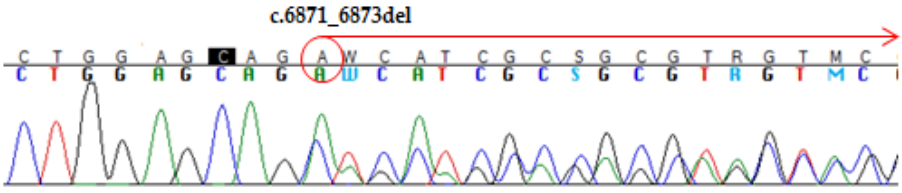


**Fig.23** Electropherogram of variant c.6871_6873del in *SPTAN1* gene in TRS_EPIL_18-2045 patient.

We did not confirm inheritance of variant because parental DNA sample were not available. *SPTAN1* encodes for the membrane structural protein alpha-II-spectrin, which forms heterotetramers with beta spectrin proteins. These spectrin assemblies are important for the maintenance of the cytoskeleton. In neurons, spectrin complexes bind with syntaxins to play a putative role in presynaptic vesicular release and interact directly with Shank proteins that localize glutamate receptors in the post synaptic densities. *SPTAN1* expression is highest in the temporal and cerebellar cortices, explaining the decreased volumes of these structures in affected patients, and the consequent occurrence of early onset epilepsy and poor motor coordination. (Gartner et al., 2018). This variant results in the deletion of 1 amino acid of the SPTAN1 protein (p.Gln2291del), but otherwise preserves the integrity of the reading frame. Experimental studies and prediction algorithms are not available for this variant, but this kind of molecular variation is known as a pathogenic alteration of *SPTAN1*, so we classified this variant likely pathogenic. We reported the details of the case in discussion section.

- in a 1 year old male (TRS_EPIL_18-3914) with epileptic encephalopathy we detected variant a pathogenic variant in *ARX* gene c.989G>T; p.(Arg330Leu), inherited from the unaffected mother (Figure 24) and have been predicted as deleterious by most pathogenicity prediction tools integrated within the dbNSFP database.

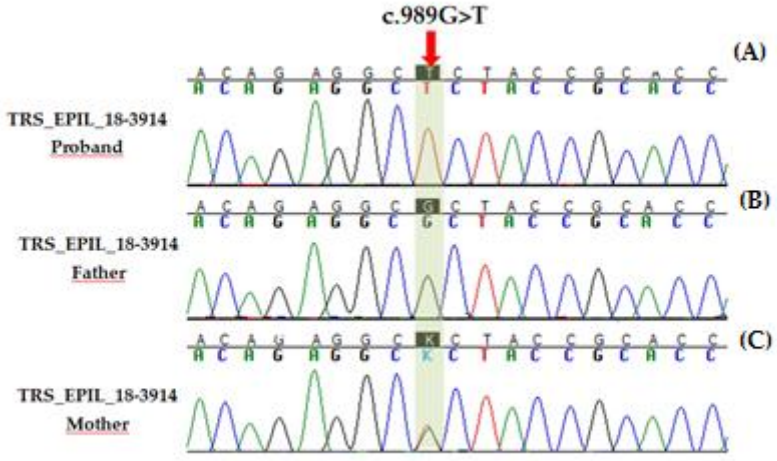


**Fig.24 (A)** Electropherogram of variant c.989G>T in ARX gene in TRS_EPIL_18-3914 patient**.** **(B)** Electropherogram of the father. **(C)** Electropherogram of the mother.

The Aristaless-related homeobox gene (*ARX*) is located in the short arm of the X chromosome (Xp21.3). *ARX* is expressed predominately in the fetal and adult brain, testis, skeletal muscle, and pancreas and comprises five exons. The pathogenic sequence variations are frequently located in exon 2, and they involve the expansion of a poly alanine tract, and also missense mutations (Tapie et al., 2017). Our missense mutation is located in homeobox domain and it has not been published as a pathogenic variant, nor has it been reported as a benign variant to our knowledge. This substitution occurs at a position that is conserved across species and in silico analysis predicts this variant is probably damaging to the protein structure/function. Missense variants in nearby residues (Arg332Cys, Arg332His, Arg332Pro, Thr333Ser, Thr333Asn) have been reported in the Human Gene Mutation Database in association with ARX-related disorders (Stenson et al., 2014), supporting the functional importance of this region of the protein. Therefore, this variant was classified as likely pathogenic.

- in a 6 year old male (TRS_EPIL_18-2398) with epileptic encephalopathy we detected variant in *STXBP1* c.130T>C p.(Cys44Arg). The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its *de novo* origin (Figure 25).

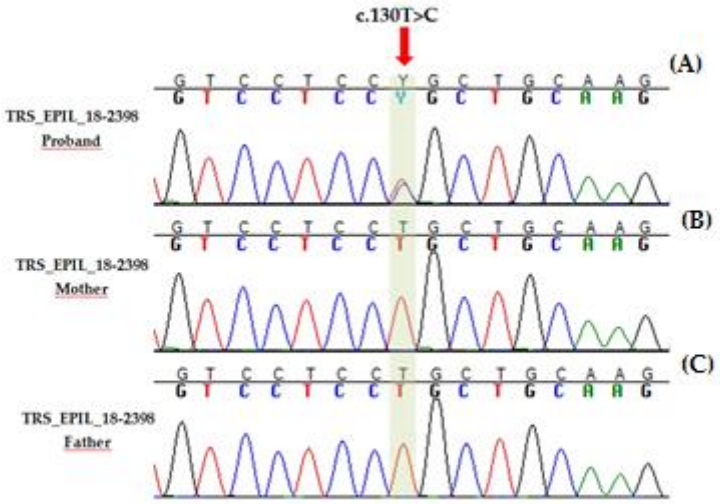


**Fig.25 (A)** Electropherogram of variant c.130T>C in TRS_EPIL_18-2398 patient**. (B)** Electropherogram of the mother. **(C)** Electropherogram of the father.

Syntaxin binding protein 1 (STXBP1 or Munc18.1) is encoded by the gene *STXBP1* located on chromosome 9q34.11. This well-conserved protein plays an important role in presynaptic vesicular docking and fusion through interaction with the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex proteins and as such has an essential function in neurotransmitter release (Stamberger et al., 2017). This variant affects the syntaxin-binding protein 1 domain, which present other pathogenic variants (Carvill et al., 2013). The variant is absent in population databases (ExAC, gnomAD) and have been predicted as deleterious by most pathogenicity prediction tools. For these reasons we classified this variant as likely pathogenic.

- in a 29 year old female (TRS_EPIL_18-4727) with diagnosis of mental retardation, epilepsy, microcephaly we reported a *de novo* mutation c.650C>T p.(Pro217Leu) in *GAMT* gene. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its *de novo* origin (Figure 26).

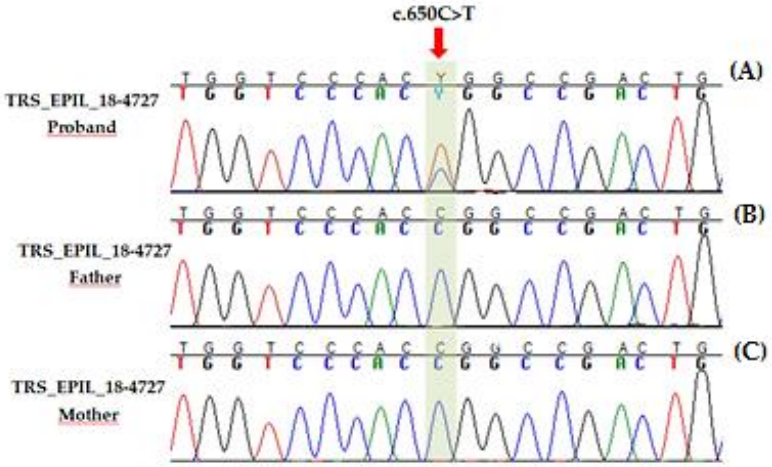


**Fig.26 (A)** Electropherogram of variant c.650C>T in TRS_EPIL_18-4727 patient. **(B)** Electropherogram of the father. **(C)** Electropherogram of the mother.

Guanidinoacetate methyltransferase (*GAMT*) deficiency (MIM 601240) is an autosomal recessive inborn error of creatine synthesis, which results in global developmental delay/intellectual disability (DD/ID). Affected individuals exhibit marked impairment of expressive speech, autistic features, and varying neurological manifestations, including epilepsy and movement disorders (Stockler-Ipsiroglu et al., 2014). To the best

of our knowledge, this variant has not been reported in the literature in individuals with a GAMT-related disease. The variant, presented as a *de novo*, was reported as pathogenic by several prediction tools (SIFT, PolyPhen, CADD, Mutation Assessor, Mutation Taster) and its frequency is lower than expected for this clinical condition (MAF 0.00003). For these reasons, following the ACMG guidelines, we classified the variant as likely pathogenic.

- TRS_EPIL_18-1136 patient, a 2-years-old female with epileptic encephalopathy, presented a variant c.5170G>A p.(Asp1703Asn) (figure 27) in *SCN9A* gene. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its paternal origin (data not showed).

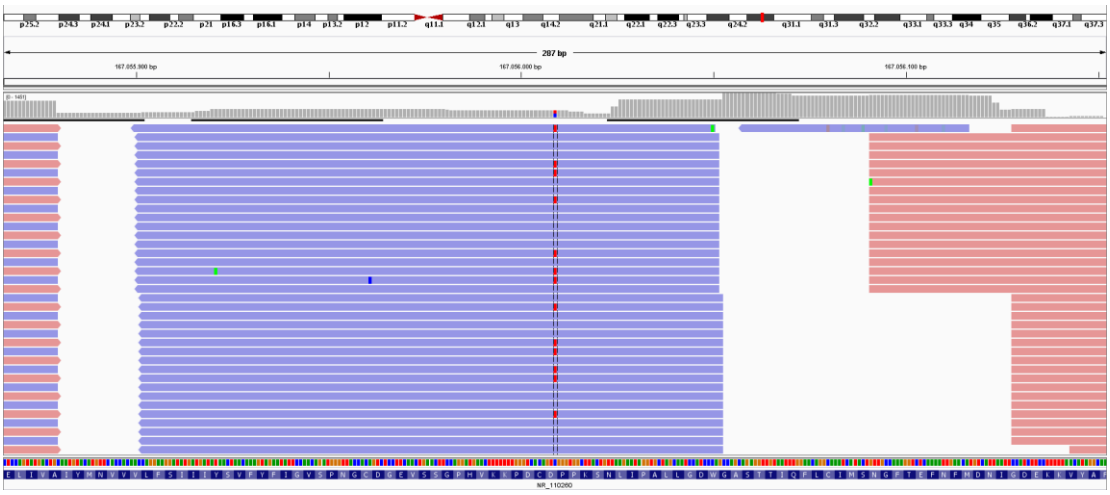

**Fig.27** The IGV view for the variant c.5170G>A p.(Asp1703Asn) in *SCN9A* gene at locus 2q24.3.

This gene encodes a voltage-gated sodium channel which plays a significant role in nociception signaling. Assuming opened or closed conformations in response to the voltage difference across the membrane, the protein forms a sodium-selective channel through which Na(+) ions may pass in accordance with their electrochemical gradient. Mutations in this gene have been reported in patients with FS-related epilepsies. A mouse model with knock-in of Asn641Tyr presents susceptibility to epileptic seizures, suggesting that *SCN9A* may be a modifier or susceptibility gene of epilepsy (Wei et al., 2017).

Our variant replaces aspartic acid with asparagine at codon 1703 of the SCN9A protein (p.Asp1703Asn). The aspartic acid residue is highly conserved and there is a small physicochemical difference between aspartic acid and asparagine. This variant is present in population databases with a low frequence (rs200733722, ExAC 0.006) and has not been reported yet in the literature in individuals with a *SCN9A*-related disease. Algorithms developed to predict the effect of missense changes on protein structure and function do not agree on the potential impact of this missense change. In summary, this variant is a rare missense change with uncertain impact on protein function. Therefore, we classified as a variant of uncertain significance.

- TRS_EPIL_18-1259 patient, a 18 years-old male, with diagnosis of generalized epilepsy. Through targeted resequencing we identified a variant c.3133 C>G p.(Leu1045Val) in *TSC1* gene. The variant is show in figure 28.

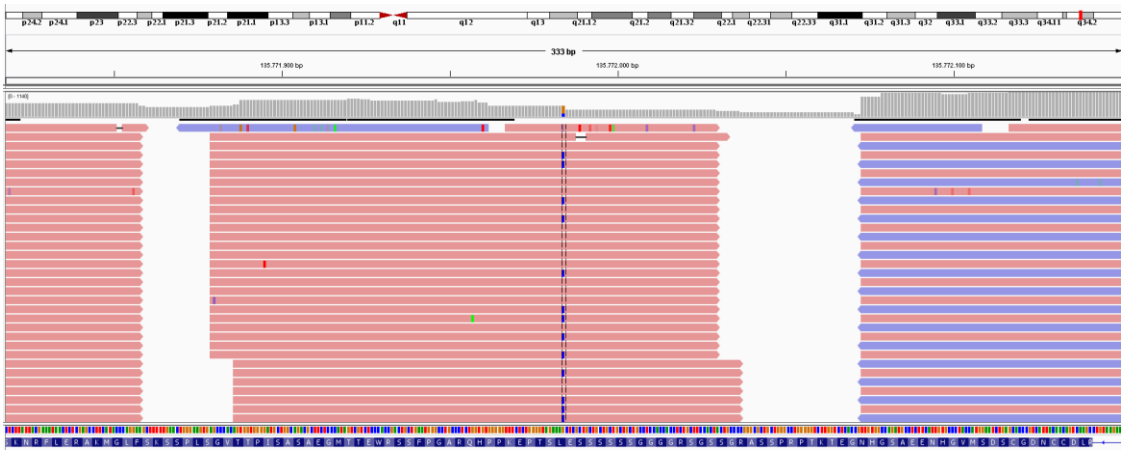

**Fig.28** The IGV view for the variant c.3133C>G p.(Leu1045Val) in *TSC1* gene at locus 9q34.13.

The protein encoded by this gene is mainly involved in cell proliferation, division and cell adhesion, and in recent years it was observed that TSC1 protein can interact with TSC2 protein. When TSC1 coding abnormality occurs, it will lead to inhibition of cell proliferation processing, causing accelerated cell proliferation (Howard and Lu, 2014). In addition, by detecting *TSC1* gene in patients with epilepsy, skin pigmentation, and vascular fibroma, it was found that the above patients had different degree of TSC1 gene mutation, leading to abnormal protein function (Liu et al., 2012; Jiang et al., 2017).

This variant is extremely rare (MAF of 0.00007 in the gnomAD), is never reported in ClinVar, and is never described before in medical literature. Furthermore, predictions tools do not agree on the potential impact of this missense change. Based on these information, we classified the variant as VUS.

- TRS_EPIL_18-1289 patient, a 2-years-old female with epileptic encephalopathy, presented a variant c.3454G>A p.(Ala1152Thr) in *SCN2A* gene. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its paternal origin (data not showed). The variant is show in figure 29.

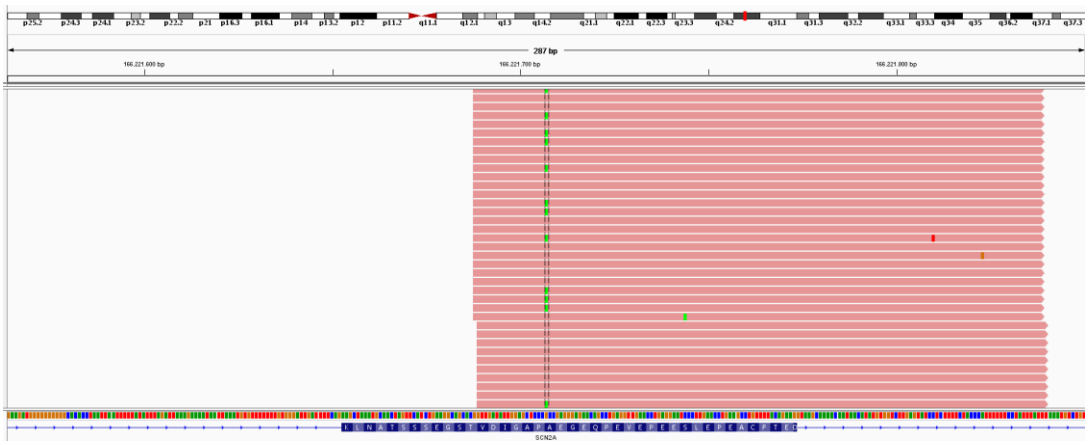

**Fig.29** The IGV view for the variant c.3454G>A p.(Ala1152Thr) in *SCN2A* gene at locus 2q24.3.

The *SCN2A* gene encodes the voltage-gated sodium channel Nav1.2, one of the major neuronal sodium channels that play a role in the initiation and conduction of action potentials. Nav1.2 is expressed in axon initial segments and nodes of Ranvier of myelinated nerve fibres in early development, and in the adult brain in the axon initial segment and unmyelinated axons. Accordingly, *SCN2A* mutations have been mainly shown to affect the early developmental period, but some mutations have also been found as causes of later onset neurological diseases, or a combination of both (Wolff et al., 2017). Our variant is located in a hot spot mutational and well-established functional domain (sodium ion transport-associated) without benign variation, but has been reported to date (rs766523968) as variant with uncertain significance.

- TRS_EPIL_18-1709 patient, a 2 years-old female, with epileptic encephalopathy, with a c.3386C>T p.(Thr1129Met) variant in *SCN1A* gene. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its paternal origin (Figure 30).

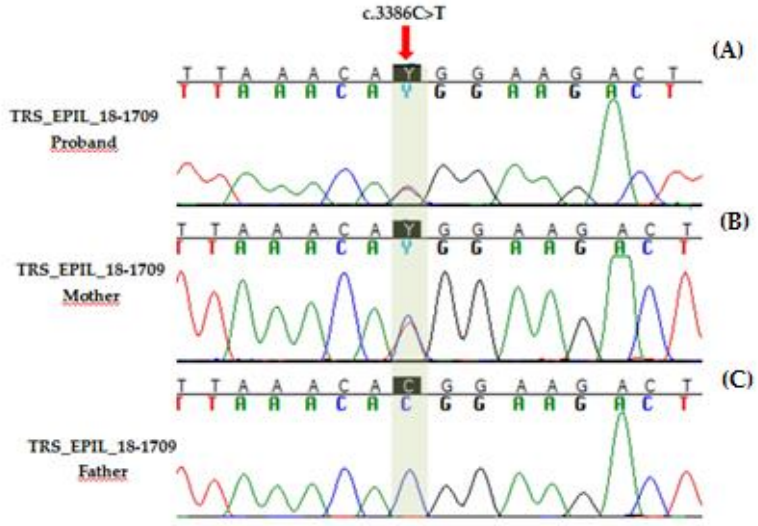


**Fig.30 (A)** Electropherogram of variant c.3386C>T in *SCN1A* gene TRS_EPIL_18-1709 patient. **(B)** Electropherogram of the mother. **(C)** Electropherogram of the father.

This variant is located in a critical functional domain (sodium ion transport-associated), was never reported in public databases and was described as pathogenic in several prediction tools. The variant detected was inherited from an healthy mother, but mechanisms of incomplete penetrance for *SCN1A* was described (Depienne et al., 2010). In conclusion, we classified this variant as VUS.

- TRS_EPIL_18-2078 patient, a 44 years old male, with myoclonic epilepsy and cognitive deficit presented a variant in *GRIN2A* (c.3578T>G ; p.(Leu1193Trp)) and a variant in *GRIN2B* (c.3076G>A; p.(Gly1026Ser)) genes. The variant was confirmed by Sanger sequencing (Figure 31).

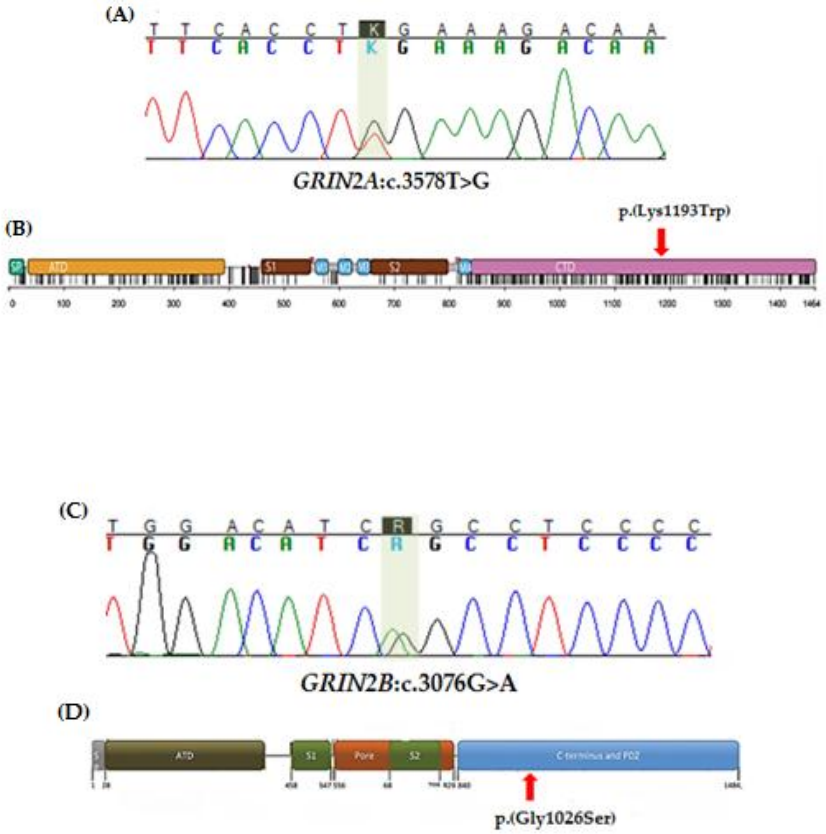


**Fig.31 (A)** Electropherogram of c.3578T>G variant in *GRIN2A* gene in TRS_EPIL_2078 patient.

**(B)** Rappresentation of c.3076G>A in *GRIN2B* gene in TRS_EPIL_2078 patient.

*GRIN2A* encodes for the $\alpha$2 subunit of the N-methyl-d-aspartate (NMDA) receptor, a cation channels that are gated by the major excitatory neurotransmitter glutamate. NMDAR-mediated signaling is involved in normal development, plasticity, learning, memory and high cognitive functions. NMDARs play important role in temporal integration of neuronal network activity and long-term alterations in synaptic structure and function *GRIN2A* mutations of various types have been found in the very last years in patients and families presenting with disorders of the epilepsy-aphasia spectrum. (Burnashev and Szepetowski, 2015).

The GluN2B, another subunit of NMDA receptor, encoded by GRIN2B is highly expressed prenatally, and its expression level starts to decline after birth in most brain regions Over 60 variants in the *GRIN2B* have been reported in the literature identified in individuals from patient cohorts with a different set of phenotype including epileptic encephalopathy (Akazawa et al., 1994).

Both the variant are present in population databases with a frequency of 0.0002% and 0.0001 respectively (ExAC). Algorithms developed to predict the effect of missense changes on protein structure and function do not agree on the potential impact of these missense change, and both variant were never reported before as benign. In summary, these variants are rare missense changes with uncertain impact on protein function, and the available evidence is currently insufficient to prove their contribution to clinical phenotype.

- TRS_EPIL_2084, a 21 years-old female, with epilepsy and paroxysmal dystonia, presented a variant c.22C>T; p.R8X in *CLN3* gene. The variant was confirmed by Sanger sequencing (Figure 32).

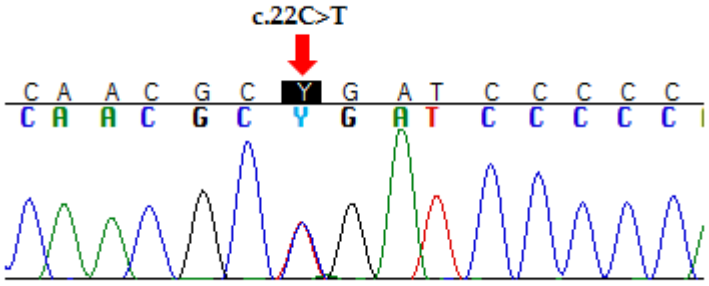


**Fig. 32** Electropherogram of c.22C>T variant in *CLN3* gene TRS_EPIL_2084 patient.

*CLN3* gene encodes an endosomal/lysosomal membrane protein. Although *CLN3* function is not fully understood, numerous studies suggest that *CLN3* plays a major role in post-Golgi, endocytic, autophagic, and lysosomal trafficking. Despite intensive research, the primary function of the CLN3 protein remains elusive (Cotman and Staropoli, 2012). The presenting symptom in *CLN3* disease is a loss of visual acuity, usually between 4 and 7 years old (Mole et al., 2005) leading to blindness within 2 to 10 years (Haltia, 2003). Cognitive decline, behavioural and psychiatric problems follow. Epileptic seizures develop between 7 to 18 years and are predominantly of the tonic-clonic type, although partial complex and myoclonic seizures are also observed. Seizures worsen and may frequently be, or become over time, refractory to medication (Schulz et al., 2013; Shahwan et al., 2005).

This variant is present in population databases with a frequence of 0.0006 (gnomAD, ExAc). We classified this variant as uncertain clinical significance since the patient's phenotype is not completely compatible with emerging literature data.

- TRS_EPIL_18-2293, a 16 years-old male, with epilepsy and mental retardation, presented a variant c.1550G>A; p.(Arg517Gln) in *CACNB4* gene. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its paternal origin (Figure 33).

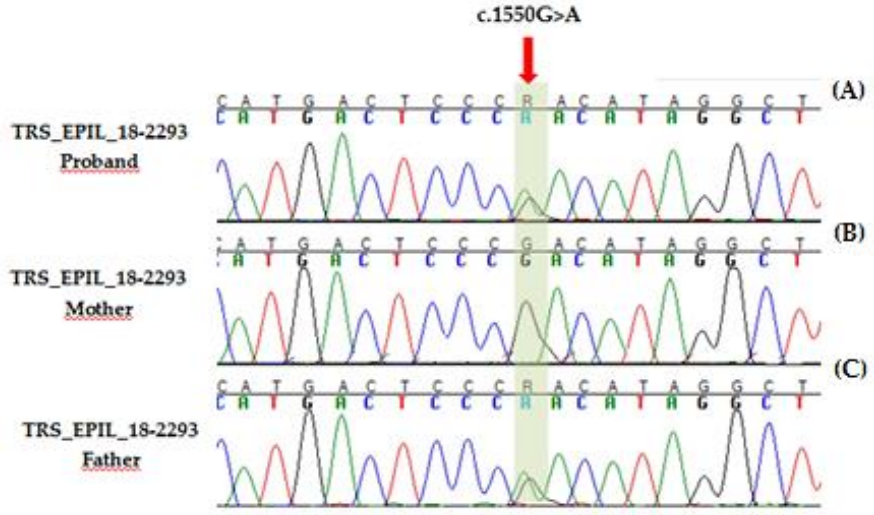


**Fig.33 (A)** Electropherogram of variant c.3386C>T in *CACNB4* gene TRS_EPIL_18-2293 patient**. (B)** Electropherogram of the mother. **(C)** Electropherogram of the father.

This gene encodes a member of the beta subunit family of voltage-dependent calcium channel complex proteins. Mutations in this gene have been associated with idiopathic generalized epilepsy (IGE), juvenile myoclonic epilepsy (JME), and episodic ataxia, type 5 (Greenberg et al., 1998).

Our variant c.1550G>A p.(Arg517Gln) occurs at a position that is conserved across species, and in silico analysis predicts this variant is probably damaging to the protein structure/function. The p.(Arg517Gln) variant is a semi-conservative amino acid substitution, which may impact secondary protein structure as these residues differ in some properties. Therefore, based on the currently available information, it is unclear

whether this variant is a pathogenic mutation or a rare benign variant. For this reason, we classified this variant as variant of uncertain significance.

- TRS_EPIL_3244 patient, a 22 years-old male, with epileptic encephalopathy, presented a novel variant c.92G>C; p.(Ser31Thr) in *GRIN2B* gene. The variant show in figure 34. This variant is never reported in public databases (ExAC, gnomAD, dbSNP) or in medical literature, thus is classified as VUS.

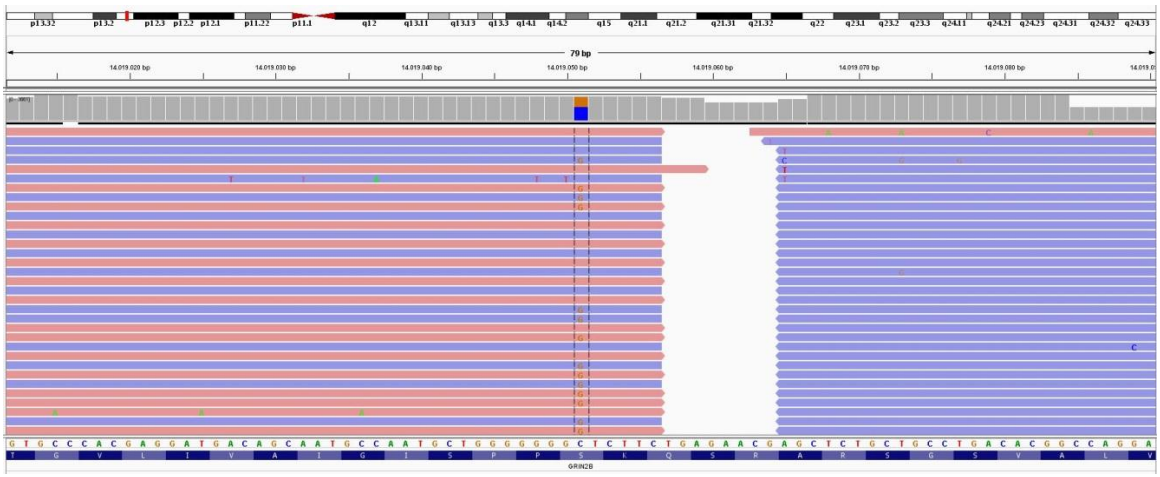

**Fig.34** The IGV view for the variant c.92G>C; p.(Ser31Thr) in *GRIN2B* gene at locus 16p13.1.

- TRS_EPIL_18-3998 patient, 34 years-old female, with agenesis of corpus callosum, mental retardation, epilepsy and dysmorphic features. We reported a variant c.1410C>G ;p.(Asp470Glu) in *ATP1A2* gene. The variant show in figure 35.

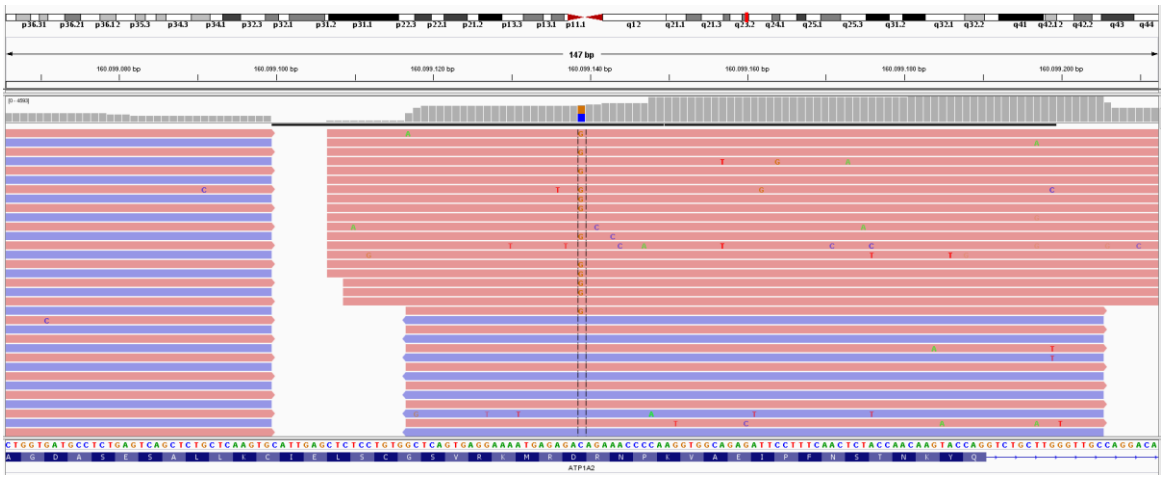

**Fig.35** The IGV view for the variant c.1410C>G ;p.(Asp470Glu) in *ATP1A2* gene at locus 1q23.2.

*ATP1A2* gene, coding for the $\alpha$2 subunit of the Na ,K -ATPase and the mutation might change the role of the ATPase enzyme in the regulation of the neuronal membrane potential and hence neuronal firing to induce seizure. The c.1410C>G variant in the *ATP1A2* gene has not been reported previously as a pathogenic variant or as a benign variant, to our knowledge. The Asp470Glu variant is a conservative amino acid substitution, which is not likely to impact secondary protein structure as these residues share similar properties. In silico analysis is inconsistent in its predictions. This variant is absent in in population databases (ExAC, gnomAD, dbSNP) and in literature mutations in *ATP1A2* have been reported with incomplete penetrance for seizure (Costa et al., 2014). For these reason we classified c.1410C>G as a variant of uncertain significance.

- TRS_EPIL_18-4006 patient, a 15 years-old male, with epileptic encephalopathy (EEs), presented a variant c.974_977del p.(Thr326Lysfs*22) in *LIAS* gene and a variant c.1907A>T p.(His636Leu) in *PRICKLE2* gene. These variant was confirmed by Sanger sequencing (Figure 36).

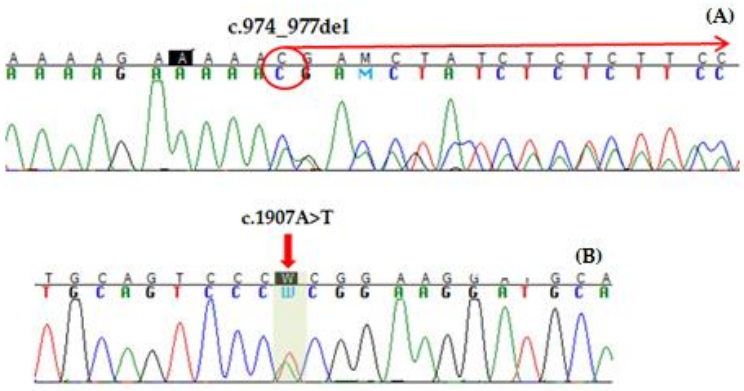


**Fig.36 (A)** Electropherogram of c.974_977del variant in *LIAS* gene. **(B)** Electropherogram of c.1907A>T variant in *PRICKLE2* gene.

*LIAS* (enzyme lipoic acid synthetase) encoded a highly conserved enzyme found in prokaryotes and eukaryotes. It localizes in mitochondrion and plays an important role in alpha-(+)-lipoic acid synthesis. It may also function in the sulfur insertion chemistry in lipoate biosynthesis (Folbergrová and Kunz, 2012). Mutations in *LIAS* have benne

associated to neonatal-onset epilepsy, muscular hypotonia, lactic acidosis, and elevated glycine concentration in plasma and urine (Mayr et al., 2011). Our variant was never reported before in public databases or medical literature, thus was classified as VUS.

The *PRICKLE2* gene encodes a postsynaptic protein involved in neuronal architecture and function (Sowers et al., 2013) and mutations in this gene have been associated with myclonic epilepsy (Tao et al., 2011). The variant (c.1907A>T) produces substitution p.(His636Leu). This variant was never reported before in public databases or medical literature, thus was classified as VUS.

- TRS_EPIL_18-4514 patient, a 4 years-old male, with epileptic encephalopathy showed variant c.1720C>T; p.(Arg574Trp) in *POLG* gene and variant c.479G>A p.(Arg160His) *CNTNAP2* gene. These variant was confirmed by Sanger sequencing (Figure 37).

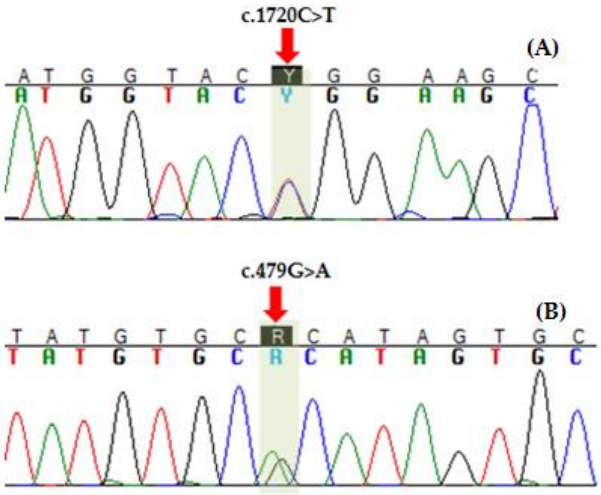


**Fig.37 (A)** Electropherogram of c.1720C>T variant in *POLG* gene. **(B)** Electropherogram of c.479G>A variant in *CNTNAP2* gene.

The enzyme polymerase gamma (*POLG*), the only DNA polymerase thought to exist in mammalian mitochondria, plays a vital role in replication and repair of mitochondrial DNA (mtDNA). In humans, POLG exists as a heterotrimer (195 kDa), consisting of a catalytic subunit (p140, encoded by *POLG* on chromosome 15q25) and two identical accessory subunits (p55, encoded by *POLG2* on chromosome 17q25), which are absolutely essential for DNA synthesis. The *POLG* gene consists of 22 coding exons and performs two distinct functions including an N-terminal 3′–5′ exonuclease (proofreading) connected to a C-terminal 5′–3′ polymerase activity (synthesising and

lyase function) through a linker region that structurally supports and enhances the processivity of the holoenzyme (Anagnostou et al., 2016). This variant was extremely rare (MAF 0.000008) and is predicted as damaging by prediction tools, but was never reported before in public databases or medical literature, thus classified as VUS. *CNTNAP2* encodes CASPR2, a transmembrane protein member of the neurexin superfamily, which forms molecular bridges at synapses and plays multiple roles in brain development and function (Leonardi et al., 2018). The variant has been previously reported (rs138738227) but was never published as a pathogenic variant, nor has it been reported as a benign variant to our knowledge. In silico analysis predicts this variant is probably damaging to the protein structure/function, so we classified the variant as VUS.

- TRS_EPIL_18-4527 patient, a 20-year-old female, with generalized epilepsy, showed the variants c.308G>A p.(Arg103Glu*)* in *CLN6* gene, c.601C>T p.(Arg201Cys*)* in *PNKP* gene, c.733G>A p.(Gly245Ser) in *CACNA1H* gene, c.57delinsCGGCGGA in *KCNMA1* gene. These variant was confirmed by Sanger sequencing (Figure 38).

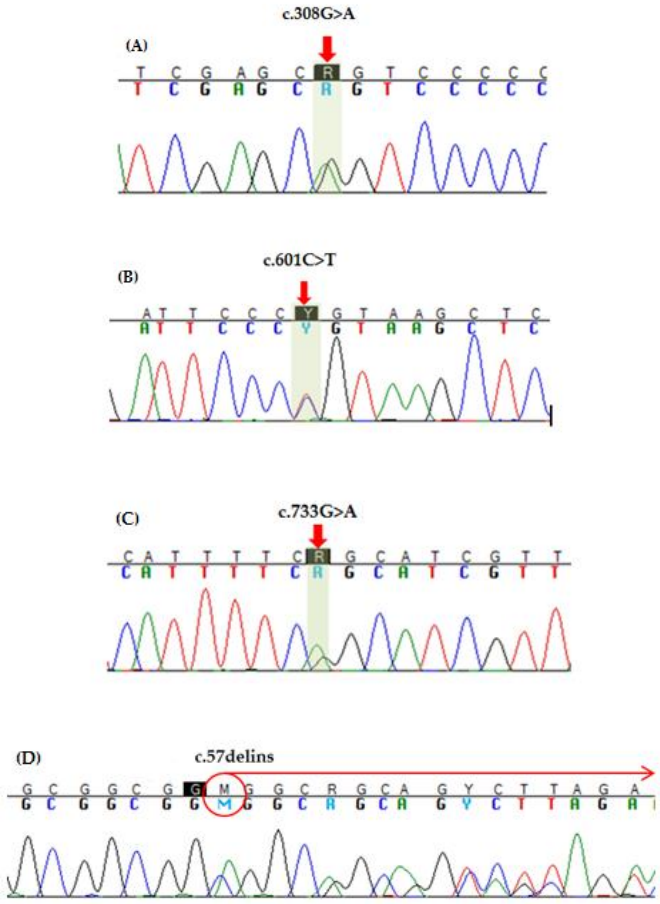


**Fig.38 (A)** Electropherogram of c.308G>A variant in *CLN6* gene. **(B)** Electropherogram of c.601C>T in *PNKP* gene **(C)** Electropherogram of c.733G>A in *CACNA1H* gene**. (D)** Electropherogram of c.57delinsCGGCGGA in *KCNMA1* gene**.**

*CLN6*, which plays a role in lysosomal function, is an ER membrane protein with seven transmembrane (TM) domains, an amino terminal domain facing the cytosol, a carboxyl terminal domain facing the ER lumina, and six loops connecting the TM domains. The TM domains are TM1 to TM7 from the N-terminus to the C-terminus (Sun et al., 2018). Mutations in this gene are responsible for neuronal ceroid-lipofuscinoses (NCLs), a group of inherited, neurodegenerative, lysosomal storage disorders characterized by progressive intellectual and motor deterioration, seizures, and early death. The variant c.308G>A p.(Arg103Gln) is located in Ceroid-lipofuscinosis neuronal protein 6 domain, is predicted as damaging by prediction tools and been reported as variant of uncertain significance in public databases.

The polynucleotide kinase 3′-phosphatase gene (*PNKP*) encodes for an enzyme that is involved in DNA repair networks and maps to 19q13.33. Through a genome-wide linkage analysis in seven consanguineous families, Shen et al. (2010) associated mutations in this gene with an autosomal recessive syndrome involving early-onset, drug-resistant seizures, microcephaly, developmental delay, and behavioral disorders. This variant has been interpreted as a variant with uncertain significance for the following characteristics: variant with a lower frequency (MAF 0.000004 in gnomAD) and damaging in most of the simulation programs selected for interpretative use. It is known that, at the moment, in the Database of digenic interactions DIDA (http://dida.ibsquare.be/) there is no evidence of digenic or oligogenic interaction between the other genes found and the *PNPK* gene responsible for epilepsy phenotype.

*CACNA1H* encodes a T-type member of the alpha-1 subunit family, a protein in the voltage-dependent calcium channel complex. Calcium channels mediate the influx of calcium ions into the cell upon membrane polarization and consist of a complex of alpha-1, alpha-2/delta, beta, and gamma subunits (Vitko et al., 2005). This sequence change replaces glycine with serine at codon 245 of the *CACNA1H* protein p.(Gly245Ser). The glycine residue is highly conserved and there is a small physicochemical difference between glycine and serine. Algorithms developed to predict the effect of missense changes on protein structure and function do not agree on the potential impact of this missense change. Algorithms developed to predict the effect of sequence changes on RNA splicing suggest that this variant may create or strengthen a splice site, but this prediction has not been confirmed by published transcriptional studies. In summary, the available evidence is currently insufficient to determine the role of this variant in disease. Therefore, it has been classified as a VUS.

This variant, c.54_56dupCGG, results in the insertion of 1 amino acid to the KCNMA1 protein p.(Gly20dup), but otherwise preserves the integrity of the reading frame. The variant is extremely rare (0.00009 in ExAC), and was never described in medical literature. Experimental studies and prediction algorithms are not available for this variant, and the functional significance of the duplicated amino acid is currently unknown. In summary, this variant has uncertain impact on *KCNMA1* function. The available evidence is currently insufficient to determine its role in disease. Therefore, it has been classified as a VUS.

- TRS_EPIL_18-4538 patient, a 13 years-old male, with generalized epilepsy, presented variant c.6374T>C; p.(Val2125Ala) in *CACNA1H* gene. The variant is show in figure 39. This variant was never reported in public databases (ExAC, gnomAD, dbSNP) or in medical literature, thus was classified as VUS.

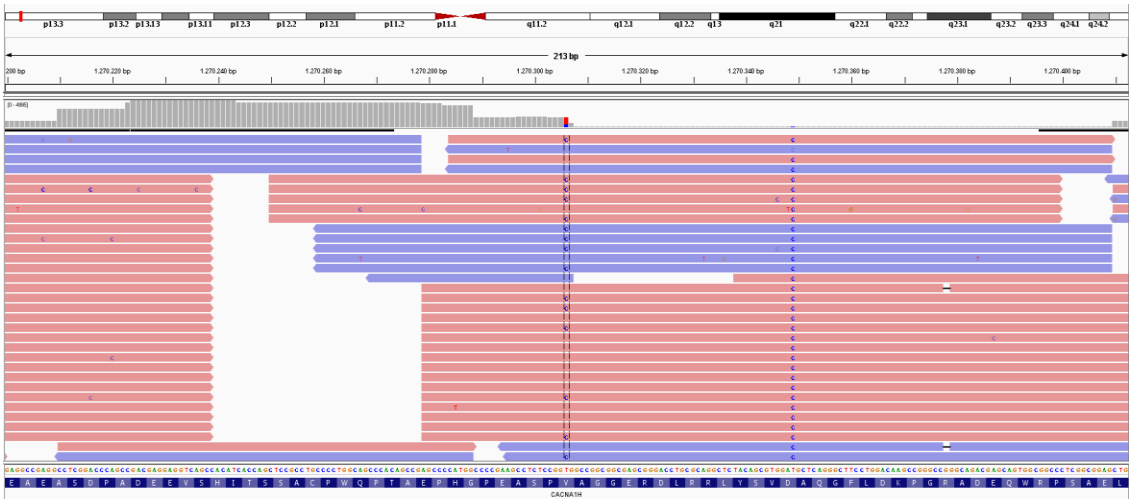

**Fig.39** The IGV view for the variant c.6374T>C; p.(Val2125Ala) in *CACNA1H* gene at locus 16p13.3.

- TRS_EPIL_18-2500 patient, a 3 years-old female, with epileptic encephalopathy and showed variant c.850G>A p.(Ala284Thr) in *CNTN4* gene. The variant was confirmed by Sanger sequencing, and parental DNA analysis showed its paternal origin (figure 40).

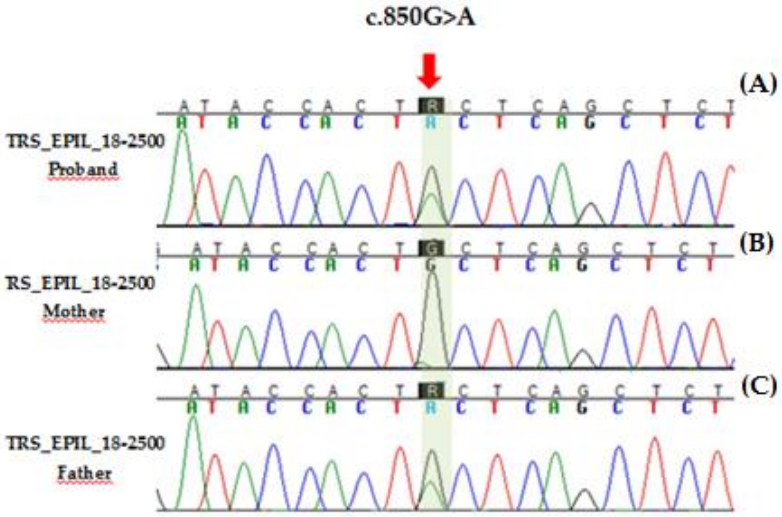


**Fig.40 (A)**Electropherogram of variant c.850G>A in CNTN4 gene in TRS_EPIL_18-2500 patient. **(B)** Electropherogram of the mother. **(C)** Electropherogram of the father.

This gene encodes a member of the contactin family of immunoglobulins. Contactins are axon-associated cell adhesion molecules that function in neuronal network formation and plasticity. The encoded protein is a glycosylphosphatidylinositol-anchored neuronal membrane protein that may play a role in the formation of axon connections in the developing nervous system. Deletions or mutations of this gene may play a role in 3p deletion syndrome and autism spectrum disorders (Zeng et al., 2002). The detected variant was reported as probably pathogenic by predictions tools, and was absent in public databases. Considering all evidences, the significance of this variant remains uncertain. We reported the details of the case in discussion section.

- The patient TRS_EPIL_18-4568, 30-years-old female, displaying epilepsy and focal cortical dysplasia. Targeted resequencing analysis revealed a mutation c.4773C>A p.(Asp1591Glu) in *SPTAN1* gene. This variant is show in figure 41.

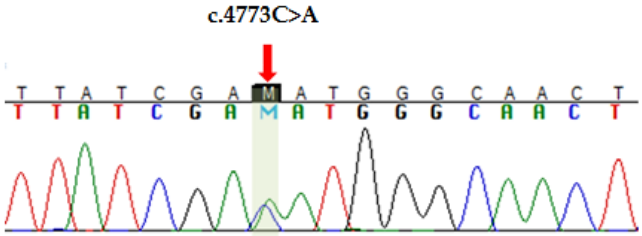


**Fig.41** Electropherogram of variant c.4773C>A in *SPTAN1* gene in TRS_EPIL_18-4568 patient.

*SPTAN1* which consists of 53 exons and encodes α-II spectrin, essential for proper myelination in zebrafish. The phenotypes of patients with *SPTAN1* mutations are not well recognized because the number of identified patients is small despite extensive genetic testing of epileptic encephalopathy cases (Tohyama et al., 2015). This variant was never reported in public databases or medical literature, and predictions tools are uninformative, thus we assign a variant of uncertain significance role to the detected variant.

There are two main reasons for the negative results. First, the patients were recruited for research purposes and in most cases the clinical indication was generic and not useful to suggest a specific syndrome and to address the molecular studies towards a specific clinical condition. Second, the

absence of any presumed disease-causing variant in 24/49 patients (49%) with intensive follow-up and without relevant clinical changes could probably be due to the fact that the causative gene was not present in our gene panel. On the other hand, it should be noted that the percentage of negative findings revealed in our study is in accordance with data reported by other authors who followed a similar approach (Lemke et al., 2012; Della Mina et al., 2015).

The molecular analyses also identified 10 synonymous variants (table 6 ).

| Patient | Chr | position | Synonymous variant | TraP | CADD |
|---|---|---|---|---|---|
| TRS_EPIL_18-1289 | 16 | 2134643 | TSC2:NM_001318831:exon30:c.3688A>C:p.R1230R | 0.045 | 0.003 |
| TRS_EPIL_18-1709 | 1 | 44386081 | ST3GAL3:NM_001270460:exon9:c.657A>C:p.A219A | 0.144 | 2.788 |
| TRS_EPIL_18-1725 | 11 | 792162 | SLC25A22:NM_001191060:exon9:c.798T>C:p.S266S | 0.01 | 0.067 |
| TRS_EPIL_18-2080 | 17 | 44248283 | KANSL1:NM_001193466:exon2:c.1233T>G:p.S411S | O.145 | 11.59 |
| TRS_EPIL_18-2398 | 2 | 145162572 | ZEB2:NM_001171653:exon4:c.351A>C:p.T117T | 0.071 | 3.487 |
| TRS_EPIL_18-2824 | X | 25031671 | ARX:NM_139058:exon2:c.441A>C:p.A147A | 0.023 | 0.659 |
| TRS_EPIL_18-4006 | 8<br>2 | 68536435<br>51253552 | CPA6:NM_020361:exon2:c.168G>A:p.L56L<br>NRXN1:NM_001135659:exon3:c.828A>G:p.Q276Q | 0.003<br>---- | 11.45<br>0.019 |
| TRS_EPIL_18-4294 | 18 | 53254303 | TCF4:NM_001083962:exon2:c.45G>A:p.E15E | 0.247 | 10.62 |
| TRS_EPIL_18-4946 | 8 | 27327497 | CHRNA2:NM_000742:exon3:c.75T>A:p.G25G | 0.277 | 5.067 |

**Tab.6** List of synonymous variants identified in our cohort.
TraP prediction: score range 0-1
CADD prediction: Phred-Scaled CADD score. Basically, a score of 10 means that variant is placed within the 10% percentile highest (more deleterious) scores, 20 means is for 1% percentile highest scores, and 30 is 0.1% percentile highest scores and so on.

Finally, we reanalyzed the identified variants in order to carry out a reclassification. Variants can be reclassified over time, as new insights regarding their phenotypic effects are discovered. A change in variant reclassification can have profound implications for patient care (table 7).

| Variant | Starting classification | Reclassification |
|---|---|---|
| *TSC1:* NM_000368:exon23:c.3133C>G:p.L1045V | VUS | B |
| *SCN2A:* NM_021007:exon18:c.3454G>A:p.A1152T | VUS | B |
| *GRIN2B* : NM_000834:exon13:c.3076G>A:p.G1026S<br>*GRIN2A* : NM_000833:exon14:c.3578T>G:p.L1193W | VUS<br>VUS | B<br>B |
| *ATP1A2:* NM_000702:exon11:c.1410C>G:p.D470E | VUS | B |
| *PRICKLE2:* NM_198859:exon8:c.1907A>T:p.H636L | VUS | B |
| *POLG* : NM_002693:exon10:c.1720C>T:p.R574W<br>*CNTNAP2* :NM_014141:exon4:c.479G>A:p.R160H | VUS<br>VUS | LP<br>B |
| *CLN6* :NM_017882:exon4:c.308G>A:p.R103Q | VUS | LP |

**Tab.7** Vus: variant with uncertain significance; LP: likely pathogenic; B: benign.

# 6. DISCUSSION

Our study involved 49 patients with a clinical diagnosis of epilepsy and neurodevelopmental disorders.

This work was aim to identify novel variants in epilepsy genes useful to expand our knowledge about the molecular mechanisms of the disease, as well as for the enlargement of the epilepsy mutational spectrum and to characterize, both from molecular than from clinical point of view, novel syndromic and non-syndromic forms of the disease useful for diagnosis, counseling and management of affected patients and their families, thorough a broad genetic test.

The employed high throughput sequencing technology enabled the generation of large amounts of sequence data, and determined the need of an accurate assessment of the identified genetic variations.

Likewise, segregation analysis of the DNA changes within families could be hindered by incomplete penetrance and variable expressivity. A high number of bioinformatics solutions for the annotation and prioritization of variants are currently available in order to predicting the functional consequences of a variant on epilepsy-phenotype. Our results show the importance, for correct variant prioritization, of incorporating different computational tools in order to predict the impact of the variants result of a NGS approach, and to identify functionally significant or clinically relevant variants.

For the first step we evaluated the quality of the sequencing reads on their alignment with the reference genome (hg19/Grh37), and the subsequent variant calling; the resulting DNA changes were annotated for facilitating the filtering and prioritization steps. We used online databases such as 1000 Genome Project and dbSNP138 which were helpful in the annotation process. Based on those datasets, initially we considered only variants with MAF $\leq$ 0.01 and we confirmed this variants with Sanger sequencing in order to excluded false positive. One of the greatest challenges in NGS data is the ability to distinguish pathogenic mutations from a large number of background variations.

After these analyses, we obtained two groups of variants: the first group is represented by variants with of pathogenic or likely pathogenic significance, and the second group is a list of variants with uncertain significance that presenting several data interpretation challenges. These nucleotide changes were thoroughly reviewed on a case by case basis. Despite the uncertain predictions according to bioinformatics tools, the rarity of the variants, the biological relevance of the

affected domain, and the suggestive phenotype of the studied subjects supported their possible pathogenic role. These variants are being periodically reanalyzed using recent advances in the scientific literature. For increasing the probability that variants selected are disease-relevant we used several online tools (i.e. SIFT, Polyphen2, Mutation Taster, FATHMM, CADD) for predicting the pathogenicity of substitution based on different information of the variant, such as its sequence homology, protein structure and evolutionary conservation.

Regarding our set of DNA changes, Polyphen2 and FATHMM were the methods resulting respectively in the greater and in the fewer number of prediction in case of variants with uncertain significance.

We obtained a diagnostic yield of 20%, if considering pathogenic and likely pathogenic variants, and of 51% considering all mutations identified (pathogenic, likely pathogenic and VUS). Our diagnostic yield could be associated to numbers of genes selected. In fact, a study of the diagnostic yield of epilepsy gene panels in 2015 found the numbers varied between 35 and 265 genes investigated, with the diagnostic yields reported to be between 10 and 48.5% (Mercimek-Mahmutoglu et al., 2015). The largest epilepsy panel (265 gene) yielded a diagnostic rate of 48.5% from a cohort of 33 patients (Lemke et al., 2012; Mercimek-Mahmutoglu et al., 2015). Although this may seem to indicate that an increased diagnostic yield may be associated with a larger number of genes, another study utilizing a 67 epilepsy gene panel was identified to have a diagnostic yield of 47% (9/19) of patients (Della Mina et al., 2015). This result, although from a small sample size, indicates that a higher number of genes in a panel may not always be needed to obtain a diagnosis. (Paul Dunn et al., 2018).

Previous studies show that NGS of multiple disease-related genes is an efficient tool for the diagnosis of epilepsy-related disorders. This finding is particularly true for early onset epileptic encephalopathy, which has been reported to have a higher proportion of monogenic causes than other epilepsies (Lindy et al., 2018). We identified a variants in 10/25 patients with a diagnosis of epileptic encephalopathy. Compared with patients with later onset, in patients with epileptic encephalopathies with onset in the first years of life, the overall positive rate was 40%, which is in line with recent smaller studies examining the diagnostic yield of targeted NGS panels (Mercimek-Mahmutoglu S et al., 2015). This finding suggests that the increased yield in patients with epilepsy is driven largely by patients with neonatal and infantile onset. Even though several genes, (e.g., *KCNQ2*, *MECP2*, *SCN1A*, *SCN2A*, and *STXBP1*) are well established for severe early-onset

epilepsies, in our cohort we identified variants in *CNTN4*, *CNTNAP2*, *LIAS, PRICKLE2* and *POLG* as probably associated to epileptic encephalopathy.

In particular, the patient TRS_EPIL-18-2500 with a diagnosis of epileptic encephalopathy, presented a missense mutation (c.850G>A; p.Ala284Thr) in *CNTN4* gene. This gene encodes a member of the contactin family of immunoglobulins. Contactins are axon-associated cell adhesion molecules that function in neuronal network formation and plasticity, and mutations in this gene are associated to autism. To the best of our knowledge, this is the first association between *CNTN4* gene and epileptic encephalopathy phenotype. Recently, Zhang et al (2019) performed clinical and genetic characterization of three individuals from unrelated families with copy number variants (CNV) (one deletion and two duplications) within *CNTN4*. In particular, the patients exhibited cognitive delay (3/3), growth restriction (3/3), motor delay (2/3), and febrile seizure/epilepsy (2/3). From a clinical point of view, in this study the clinical trait more frequently observed was developmental delay or the intellectual disability. We observed some variability among patients, even if they have the same genetic anomaly. This finding is quite common in NDDs and could be explained by variable expressivity and/or incomplete penetrance or differential effects on the transcript by different molecular events such as deletions and/or point mutations.

This newly identified missense variant is the first to be reported in a patient with epileptic encephalopathy phenotype in *CNTN4* gene. Obviously the identification of the same variant in other patients with epileptic encephalopathy and functional studies are needed to reinforce the proposed association.

Accordingly, our study may help shed some light on the genetic architecture of this patient population.

The number of de novo and inherited disease-causing variants was calculated for all genes where parental testing was possible. A total of 7/31 (22.6%) variants *de novo* was found in our cohort. In particular, we found de novo variants in 5 autosomal genes (*GAMT, KCNQ2, SCN1A, STXBP1* and *TCF4*), and 1 in X-linked gene (*MECP2*). More recent studies suggest that many severe epilepsy types begin in infancy or childhood, especially those with psychomotor retardation and epileptic encephalopathies are often due to *de novo* mutations (Sato K et al., 2002; Kurahashi H et al., 2018).

Knowledge of the mode of inheritance of variants is important when assessing their involvement in a patient's clinical phenotype. In cases of dominant severe diseases, it is essential to determine whether the detected variant is an inherited or *de novo* mutation. *De novo* mutations are the most extreme form of rare genetic variation. In general, they are more deleterious than inherited

variations; because they have been subjected to less stringent evolutionary selection; these mutations are prime candidate causative genes underlying genetic diseases that occur sporadically. Intolerant dominant genes are those that are most likely to be affected by *de novo* mutations (Roca I et al., 2018).

Interesting, we identified 10 novel variants, i.e. not reported in literature or public database. Although these variants are not functionally characterized yet, are useful to expand our knowledge on the mutational spectrum of specific genes and to shed light on new molecular mechanisms underlying the phenotype. Obviously functional studies and or animal models are needed to corroborate our hypothesis/findings.

All variants identified (pathogenic, likely pathogenic and VUS) were categorized into different groups according to genetic causes of epilepsy to help us to obtain a better understanding of the underlying pathogenesis in each case. Ion channel-related genes, especially sodium and potassium ion channels, accounted for a large proportion of the variants, and in most of these genes, variants produce either a dominant-negative loss-of-function or gain-of-function protein (Cestele S et al., 2013; Wolff M et al., 2017). In particular we identified 14 (47%) variants in ion channel (*SCN1A, SCN2A, ATP1A2, GRIN2A, GRIN2B, KCNQ2*, *CACNA1H*, *KCNMA1*, *SCN9A* and *CACNB4*), 7 (22,5%) variants in enzyme (*POLG, LIAS, MECP2, GAMT, TSC1, PNKP*), 2 (6,5%) variants in cell adhesion molecule (*CNTNAP2, CNTN4*), 2 (6,5%) variants in lysosomal protein (*CLN3, CLN6*), 1 (3,2%) variant in transcriptor factor (*TCF4*), 2 (6,5%) variant in cytoskeletal protein (*SPTAN1*), 1 (3,2%) variant in membrane trafficking (*STXBP1*) and 1 (3,2%) variant in nucleic acid binding (*ARX*). Ion channels (voltage-gated and ligand-gated) accounted for 47% in total, confirmed that dysfunction of ion channels plays critical roles in the pathogenesis of epilepsy.

In case TRS_EPIL_18-2045, a patient with a diagnosis of Lennox-Gastaut syndrome, we identified a novel variant in *SPTAN1* (c.6940_6943del). Lennox-Gastaut syndrome (LGS # 606369) is one of the most severe epilepsies, that usually begins in childhood and is associated with developmental and epileptic encephalopathy (Warren AEL et al., 2019). *SCN1A, CHD2, FOXG1* and *DNM1* genes, in addition to rare CNVs, have been reported to be implicated in the pathogenesis of LGS (Verrotti A, et al., 2018). These findings are usually reported in single cases. To date, to the best of our knowledge, a precise correlation between genotype and phenotype is not reported in the literature. *SPTAN1* is among the genes whose mutations are associated with EIEE development (OMIM# 613477). This gene encodes $\alpha$II spectrin, one of the proteins involved in the stabilization of cell membranes. Two $\alpha$- and five $\beta$ spectrin subunits have been identified. These subunits are

assembled in an antiparallel side-by-side manner into heterodimers that can form end-to-end tetramers that integrate into the membrane cytoskeleton. As heterotetramer integrity is essential for neuronal process development and inhibitory synapse formation, mutation of *SPTAN1* can lead to significant neurological disorders and most cases of *SPTAN1* mutations are associated with EIEE. However, in literature there have been described Mild cases have been reported, suggesting that different *SPTAN1* variants can be associated with different clinical manifestations (Rapaccini V et al., 2018). In particular, Syrbe et al. analysed characteristics of 20 patients with pathogenic or likely pathogenic *SPTAN1* mutations and compared them with those of subjects with the same genetic abnormality in previously published studies. These authors reported that 62% of affected children presented with EIEE. These patients typically presented with early onset of recurrent, intractable seizures associated with severe developmental delay. Several patients had West syndrome, with epilepsy and developmental delay. Most of the severe cases had in-frame deletion/duplication mutations located in the last two αII spectrin repeats in the C-terminal region. The variant detected in our patient falls into this domain, and as approximately 20% (Ali A. Asadi-Pooya 2018) of patients diagnosed with West syndrome can evolve into the more severe Lennox-Gastaut syndrome phenotype, our finding may be the first associated with LGS and for this reason useful to expand our knowledge on the mutational spectrum of *SPTAN1* suggesting that this gene could play a role in the etiology of Lennox-Gastaut syndrome.

Among patients with diagnosis of Rett syndrome we didn't identified a candidate variant in *MECP2* gene or in *CDKL5* and *FOXG1*. Mutations in *MECP2*, located on Xq28, account for 95% of typical Rett syndrome cases and 73.2% of atypical Rett syndrome. Rett syndrome was diagnosed also in 3%–5% patients who were negative for *MECP2* mutations (Neul et al., 2008). *CDKL5* and *FOXG1* are other genes identified as causative genes in atypical forms of Rett syndrome.(Ariani et al., 2008; Weaving et al., 2004). In the last few years, using whole exome sequencing, many other uncommon genetic mutations in 69 new genes have been identified as causative for Rett phenotype (classic or variant) (Ehrhart et al., 2018;) (Operto FF et al., 2019), some of these genes are not present in our panel, this could justify the negative result of patients with Rett syndrome.

We also identified 10 synonymous rare variants that might act as phenotype modifiers. Additional studies about their function on mRNA transcription, splicing, transport, translation or modification are required for determining their possible non-silent role. This synonymous variants may contribute as a disease modifier to the genetic background of a patient's disorder, but at present we are unable to estimate such contribution.

Variant interpretation is not always straightforward, and requires close cooperation between molecular geneticist, bioinformatician, neurologist and genetic counsellor. We identified a large number of VUS (n =21), which represent a source of uncertainty for clinical interpretation as well as for families. VUS arise for a number of reasons, e.g., lack of functional data, missing segregation, or incomplete knowledge of genotype-phenotype correlation. In this scenario, segregation information on a novel variant only contributes to diagnostic certainty when there is confidence about the bioinformatic prediction and the associated epilepsy phenotype. If the evidence is scant, then proving that the change is *de novo*, or segregates with disease in an affected parent will, in reality, make very little difference to the patient or family until further evidence establishes the VUS as likely pathogenic, or benign.

Finally, we reanalyzed the identified variants in order to carry out a reclassification. Variants can be reclassified over time, as new insights regarding their phenotypic effects are discovered. A change in variant reclassification can have profound implications for patient care. In particular, the variants initially classified as pathogenic or likely pathogenic have not undergone changes. While, 21 of the variants classified as VUS have undergone changes. Specifically, 7 VUS have been reclassified as benign and 2 VUS have been reclassified as likely pathogenic. These reclassifications involved 9 genes (*ATP1A2*, *CLN6*, *CNTNAP2*, *GRIN2B*, *GRIN2A*, *PRICKLE2*, *POLG*, *SCN2A*, *TSC1*). Among the prediction tools, SIFT is the method with the most accurate predictions. In fact, the variants reclassified as benign had previously been predicted with the same result by SIFT. If pathogenic or likely pathogenic variants are initially classified as VUS, likely benign, or benign variants, the resulting delay in the identification of a clinically actionable variant can have profound effects on patient care. Recently, it has been observed that the majority of VUS category variants will be downgraded over time. It is important, because the reclassification could lead to a re-evaluation in the genotype-phenotype correlation.

In summary, our results reinforce the importance and feasibility of precise genetic diagnosis for epilepsy, with the hope that in future, this will both aid in understanding the molecular pathophysiology and lead to new treatment targets.

# 7. CONCLUSIONS

Molecular genetic etiologies in epilepsy have been become better understood in recent years, creating important opportunities for precision medicine. In this study, we have developed and tested a panel of 85 epilepsy-related genes based on next generation sequencing technology to identify the molecular etiology of epilepsy. A total of 49 patients were studied. This work was aimed the discovery genetic variations that cause disease in order to enlargement the knowledge of the epilepsy genes mutational spectrum.

We identified pathogenic (*SCN1A, TCF4, MECP2, KCNQ2*), likely pathogenic (*POLG, KCNQ2, SPTAN1, ARX, STXBP1, GAMT*) and variants uncertain clinical significance (*SCN9A, TSC1, SCN2A, SCN1A, GRIN2B, GRIN2A, CLN3, CACNB4, ATP1A2, LIAS, PRICKLE2, POLG, CNTNAP2, CLN6, CACNA1H, KCNMA1, PNKP, CNTN4, SPTAN1*) in 25/49 screened patients (51%). Although some of the identified variants need further investigation to be considered disease causing, our results demonstrate that the developed panel is suitable and helpful to be applied in the diagnosis of epilepsy contributing to a more detailed understanding of genotype-phenotype correlations. Functional characterization of uncertain clinical significance variants may further increase the yield. The early identification of the underlying causative genetic alteration provides essential information of prognosis, recurrence risk and could be used to influence therapeutic decisions enabling precision medicine approaches in a significant number of individuals with epilepsy.

Also, the identification of novel variants in candidate genes is useful to expand our knowledge about the molecular mechanisms of the disease.

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