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NEXT GENERATION SEQUENCING IN RARE CHILDHOOD EPILEPSY OF SUSPECTED GENETIC ETIOLOGY

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1. **Introduction**

Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure. [1] It is estimated that 50 million people actually suffer from epilepsy in the world; in Italy they are estimated in about 500.000. The incidence is 30-50 per 100.000 in the general population (WHO Data). [2]

The distribution by age has a "U" trend, with two peaks in infancy and childhood and in senile age.

In 2001, the ILAE (International League Against Epilepsy) [3] had classified epilepsy by etiology in:

- ***Idiopathic epilepsy***: epilepsy of predominately genetic or presumed genetic origin and in which there is no gross neuroanatomic or neuropathological abnormality. Included here are epilepsies of presumed multigenic or complex inheritance, but for which currently the genetic basis has not been elucidated.

- ***Symptomatic epilepsy***: epilepsy of an acquired or genetic cause, associated with gross anatomic or pathologic abnormalities, and/or clinical features, indicative of an underlying disease or condition. We thus include in this category developmental and congenital disorders where these are associated with cerebral pathologic changes, whether genetic or acquired (or indeed cryptogenic) in origin.

- ***Provoked epilepsy***: epilepsy in which a specific systemic or environmental factor is the predominant cause of the seizures and in which there are no gross causative neuroanatomic or neuropathological changes. The reflex epilepsies are included in this category (which are usually genetic) as well as the epilepsies with a marked seizure precipitant.

- ***Cryptogenic epilepsy***: epilepsy of presumed symptomatic nature in which the cause has not been identified. The number of such cases is diminishing, but currently this is still an important category, accounting for at least 40% of adult-onset cases of epilepsy.

In 2011, Berg & Scheffer [4] proposed a new classification in:

- *Genetic*: the epilepsy is a direct result of a genetic cause, in terms of a pathological mechanism determined by genic mutations: channelopathies are the best example of genetic epilepsies.

  However, this term would also apply to electroclinical syndromes for which twin or family segregation studies reproducibly show clinical evidence of a genetic basis (e.g., in the case of the genetic generalized epilepsies).

- *Structural-Metabolic*: the epilepsy is the secondary result of a separate structural or metabolic condition.

It is not possible to strictly split epilepsies in these categories: structural brain lesions, including many malformations of cortical development, often have genetic causes [5] and most metabolic disorders are of genetic origin.

In the following section, main genes involved in different epileptic phenotypes will be illustrated.

Epileptic Encephalopathies

The term epileptic encephalopathy refers to a condition in which the epileptic activity *itself* may contribute to severe cognitive and behavioral impairments above and beyond what might be expected from the underlying pathology alone (e.g., cortical malformation), and that these can worsen over time. [6]

The general characteristics of *Epileptic Encephalopathies are:* the early onset (usually in the first year of life), the high frequency of seizures that may be of different type in an individual patient, the highly expressed EEG paroxysmal activity which varies according to the age (primarily burst–suppression patterns in the neonatal period, hypsarrhythmia in infancy and slow generalized spike-wave discharges (GSWD) in early childhood). In most cases, cognitive, behavioral and neurological deficits are associated. [6] The following epileptic encephalopathies have their onset in the neonatal period, infancy and early childhood: early myoclonic encephalopathy, Ohtahara syndrome, West syndrome, Dravet syndrome (severe myoclonic epilepsy in infancy- SMEI), Lennox–Gastaut syndrome, Landau–Kleffner syndrome, epilepsy with continuous spike-and-waves during slow-wave sleep (ESES - other than Landau-Kleffner syndrome), myoclonic status in non-progressive encephalopathies. [6]

For many of these electro-clinical syndromes, a genetic etiology has been identified.

Ohtahara syndrome (also called "Early Infantile Epileptic Encephalopathy1" – EIEE1) is related to mutations in *ARX*, *STXBP1* and *SPTAN*. [7, 8, 9]

In West Syndrome mutations in *CDKL5*, *STXBP1*, *KCNQ2*, and *GRIN2A* have been reported [10]; mutations in the latter have been also reported in patients with Landau–Kleffner syndrome. [11]

Dravet syndrome is mostly due to mutations, deletions or insertions in *SCN1A*, but recently other genes are associated with Dravet *phenotype* such as *SCN2A*, *GABRG2, GABRA1, PCHD19* and *HCN1*. [12, 13, 14]

It is in early infantile epileptic encephalopathy (EIEE) that the largest genetic heterogeneity is found; in OMIM more than 50 phenotypes are classified as EIEE, associated to mutations in the following genes. (https://www.omim.org/phenotypicSeries/PS308350):

AARS, ALG13, AP3B2, ARHGEF9, ARV1, CACNA1A, CAD, CDKL5, DENND5A, DNM1, DOCK7EEF1A2, FGF12, FRRS1L, GABRA1, GABRB1, GABRB3, GNAO1, GRIN2B, GRIN2D, GUF1, HCN1, ITPA, KCNA2, KCNB1, KCNQ2, KCNT1, NECAP1, PCDH19, PIGA, PLCB1, PNKP, SCN1A, SCN2A, SCN8A, SCN9A, SIK1, SLC12A5, SLC13A5, SLC1A2, SLC25A12, SLC25A22, SLC35A2, SPTAN1, STXBP1, SZT2, ST3GAL3, TBC1D24, UBA5, WWOX.

Progressive myoclonic epilepsies

Progressive myoclonic epilepsies (PMEs) comprise a group of rare, heterogeneous genetic disorders, mainly with autosomal recessive inheritance, characterized by cortical myoclonus, other types of epileptic seizures, and progressive neurocognitive impairment.

PMEs usually present in late childhood or adolescence, which distinguishes them from epileptic encephalopathies that start with polymorphic seizures in early infancy. [15]

PMEs share common neurological signs that include progressively worsening cortical myoclonus and epileptic seizures, with classic onset in late childhood and adolescence. Other neurological symptoms, namely dementia and ataxia, are typically associated with myoclonus-epilepsy syndromes, and occasionally further signs and symptoms are due to the specific impairment of nervous or other systems. [16]

The 'core' symptom of PMEs is multifocal reflex (action-induced) myoclonus. This type of myoclonus has cortical origin, since it is typically associated with 'subtle' central EEG changes that can be studied using EEG-EMG relationship analysis (including jerk-locked back-averaging and other techniques). Moreover, cortical myoclonus is coupled with neurophysiological features reflecting neocortical hyperexcitability, such as 'giant' evoked potentials and enhanced long-loop reflexes. [15]

PMEs are derived from heterogeneous genetic disorders, probably with distinct pathological mechanisms, including neural degeneration (Unverricht-Lundborg and dentatorubralpallidoluysian atrophy- *DRPLA*), storage disorders (Lafora disease, neural-ceroid-lipofuscinoses, sialidoses, Gaucher III, Niemann Pick type C, and action myoclonus-renal failure syndrome), mitochondrial disorders (myoclonic epilepsy associated with ragged red fibers), and ion channel dysfunction. [15]

Table 1: Distinguishing features of some of the more common inherited progressive myoclonus epilepsies and relative genes (from Turnbull, 2016) [17]

Progressive Myoclonic Epilepsies	Inheritance	Onset (years)	Suggestive clinical signs	Pathologic features	Gene(s)
Unverricht-Lundborg disease (EPM1)	AR	6-15	Slow progression; mild and late cerebellar impairment; late or absent dementia	None	*CSTB*
Lafora disease (EPM2)	AR	6-19	Visual symptoms	Polyglucosan inclusions (Lafora bodies)	*EPM2A* *EPM2B*
Myoclonic epilepsy with red ragged fibres (MERRF)	Maternal	Any age	Lactic acidosis	Ragged red fibres	*MTTK (tRNALys)*
Neuronal ceroid lipofuscinoses (NCLs)	AR, AD	Variable	Macular degeneration and visual impairment (except adult form)	Lipopigment deposits; granular osmiophilic, curvilinear or fingerprints inclusions	*CLN1–CLN14*
Sialidoses	AR	8-15	Gradual cerebellar impairment; cherry-red spot maculopathy	Urinary oligosaccharides, fibroblast neuraminidase deficit	*NEU* *PPGB*

Idiopathic epilepsies

The term "*idiopathic epilepsies"* has been recently replaced by "*genetic generalized epilepsy"* (GGE) [4] to underscore their likely etiology.

Several twin studies, starting with those by William G. Lennox in the 1940s, suggest that the concordance in identical twins exceeds 90%. This, compared with the much lower concordance in non-identical twins, suggests

that on a population level, the vast majority of causative factors are genetic. This important role of genetic factors is reflected in genetic epidemiological studies, which suggest that the recurrence in siblings for Childhood Absence Epilepsy (CAE), Juvenile Absence Epilepsy (JAE), and Juvenile Myoclonic Epilepsy (JME) is higher than in other epilepsies. [18]

Genetic studies in large families with GGE identified mutations in *GABRG2* in families with absence epilepsy as the predominant phenotype [19, 20] and *GABRA1* in families with juvenile myoclonic epilepsy. [21] Variants in *CACNA1H*, coding for a T-type calcium channel important in the thalamocortical circuitry, may predispose to GGE. [22]

The term *GEFS+* (*Genetic epilepsy with febrile seizures plus*) refers to a familial constellation of clinical symptoms, in which the febrile seizures are the main feature. However, in contrast to familial febrile seizures, affected individuals and/or family members have additional seizures types or epilepsy syndromes. The clinical recognition of the diverse GEFS+ phenotypes in a single family as the variable expression of a single genetic disease rather than random association of diverse phenotypes was the key step to identifying *SCN1A, SCN1B*, and *GABRG2* as genes for monogenic epilepsies. [18] *SCN1A* and *SCN1B* code for subunits of voltage-gated sodium channels; *GABRG2* is the gene for the gamma-2 subunit of the GABA-A receptor. All three findings were pivotal in establishing the channelopathy concept of human epilepsies, postulating ion channel alterations as the main pathological correlate of human seizure disorders. Recently, whole-exome sequencing in independent large pedigrees identified co-segregating *STX1B* mutations in in fever-associated epilepsy syndromes. [23]

Copy number variations (CNVs) emerged as significant risk factors for the GGE. Three microdeletions are established as risk factors: microdeletion of

15q13.3, 15q11.2, and 16p13.11 in GGE and intellectual disability. [24, 25, 26, 27]

Genome-wide studies revealed significant associations for GGEs at 2p16.1 and 17q21.32. The search for syndrome-related susceptibility alleles identified significant associations for Absences epilepsies at 2q22.3 and at 1q43 for JME. Suggestive evidence for an association with GGEs was found in the 2q24.3 region, nearby the *SCN1A* gene, which is currently the gene with the largest number of known epilepsy-related mutations. The associated regions harbor high-ranking candidate genes: *CHRM3* at 1q43, *VRK2* at 2p16.1, *ZEB2* at 2q22.3, *SCN1A* at 2q24.3 and *PNPO* at 17q21.32. [26]

CHD2 mutation is the first identified cause of eyelid myoclonia with absences (Jeavon's Syndrome) the archetypal generalized photosensitive epilepsy syndrome. [29] The same gene has been described in myoclonic-atonic epilepsy (MAE). [30] Recent evidences showed that MAE is associated to a heterozygous mutation in the *SLC6A1* gene on chromosome 3p25. [31]

Mutations in *SLC2A1* were identified in patients with rare generalized epilepsies including frequencies of up to 10% in early-onset absence epilepsy (EOAE) and MAE. [32, 33]

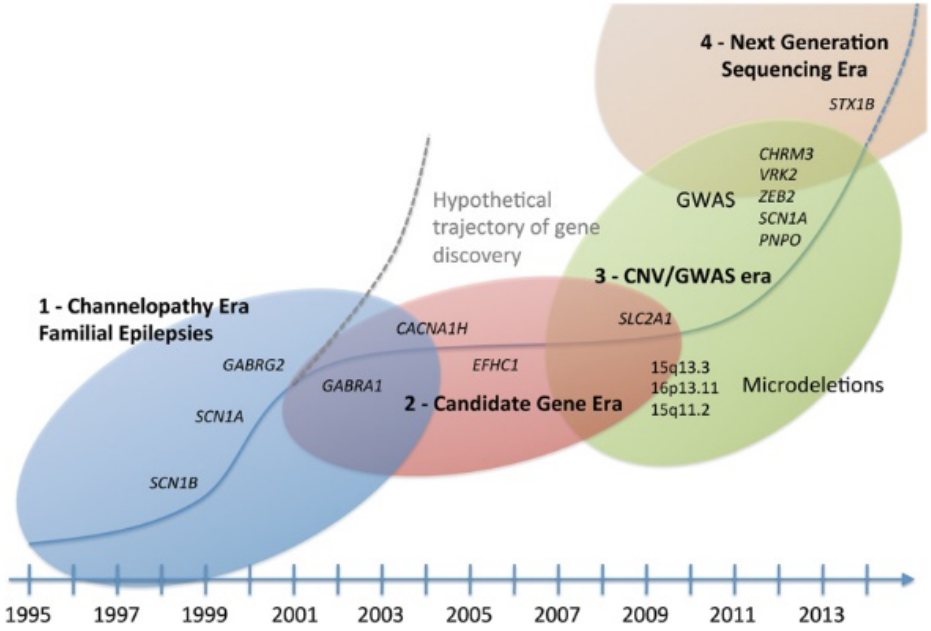

Figure 1: Gene discovery in generalized epilepsy; 1: linkage era; 2: gene sequencing era; 3: CNV and GWAS era; 4: Next generation sequencing era. [18]

Focal epilepsies

Few focal epilepsies are related to gene mutations. Using exome sequencing, mutations in *DEPDC5* gene, involved in mTOR pathway, in GATOR complex, are found in autosomal dominant familial focal epilepsy with variable foci (FFEVF), in which family members have seizures originating from different cortical regions. [34] Mutations in *NPRL3*, one of three genes of the same complex together with *NPRL2*, have recently been reported to cause focal cortical dysplasia with focal epilepsy. [35]

Mutations in nicotinic acetylcholine receptor (nAChR) subunits *CHRNA4*, *CHRNB2*, and *CHRNA2* determine autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [36]. For the same phenotype, but more severe and with early onset, mutations in *KCNT1* have also been reported. [37]

Epilepsy in epilepsies amenable to specific treatment.

SLC2A1 or *GLUT1* codes for the major glucose transporter in the mammalian blood–brain barrier. Initially described in patients with severe epileptic encephalopathies it is now clear that the mutation, which impairs the transport of glucose from blood to the brain, is associated with a wide spectrum of phenotypes, which may also include less severe epilepsies. [38, 39] An early diagnosis is critical for the patient because it allows a prompt initiation of the ketogenic diet, which improves symptoms, providing ketone bodies as an alternative source of fuel for the brain. [40, 41]

Mutations in the *PNPO* gene cause pyridoxamine 5-prime-phosphate oxidase deficiency (PNPOD), an autosomal recessive inborn error of metabolism resulting in vitamin B6 deficiency that manifests as neonatal-onset severe seizures and subsequent encephalopathy. [42] Same metabolic pathway is involved in pyridoxine-dependent epilepsy (PDE), caused by mutations in the *ALDH7A1* gene. These syndromes are characterized by a combination of various seizure types, usually occur in the first hours of life and are unresponsive to standard anticonvulsants, but they respond only to immediate administration of pyridoxine hydrochloride or pyridoxal-5′-phosphate. There are some reports that affirm that early treatment can be associated with normal neurodevelopment in childhood in PNPOD. [43]

Malformation of cortical development

These pathologies have been classified in three large categories: a) malformations secondary to abnormal neuronal and glial proliferation or apoptosis (microcephaly and megalencephaly); b) malformations due to abnormal neuronal migration (periventricular heterotopia, lissencephaly,

subcortical heterotopia) c) malformations due to abnormal postmigrational development (polimicrogyria, focal cortical dysplasia).

Progress has been made in understanding neuronal migration at the intracellular level. The importance of microtubule transport, centrosomal positioning, nuclear transport (associated with *LIS1*), microtubule stabilization (associated with *DCX*), vesicle trafficking and fusion (*ARFGEF2* and *FLNA*), and neuroependymal integrity (*MEKK4* and *FLNA*) in neuronal migration is well known. Mutations affecting microtubule proteins *TUBA1A*, *TUBA8*, *TUBB2B* and *TUBB3* are associated with abnormal neuronal migration (lissencephaly) and postmigrational development (polymicrogyria or polymicrogyria-like dysplasias). [5]

Schizencephaly is associated to mutations in *COL4A1* gene, also described (together with mutation in *COL4A2*) in familial and sporadic porencephaly. [44, 45].

Epilepsy associated to other conditions

In numerous pathologies, epilepsy is associated with other symptoms such intellectual disability, autism and/or movement disorders.

A paradigmatic example is Rett Syndrome, an X-linked neurodevelopmental disorder that manifests in early childhood with developmental stagnation, loss of speech and hand use, followed by the appearance of characteristic hand stereotypies, severe cognitive impairment, and autistic features. About 60% of patients have epilepsy, that occurs before the age of 3 years. [46] *MECP2* gene alterations are present in >90% of patients with typical Rett syndrome but only in 50-70% of atypical cases. Over the last years, intragenic or genomic alterations of the *CDKL5* and FOXG1 genes have been associated with severe cognitive impairment, early

onset epilepsy and, often, dyskinetic movement disorders, which have variably been defined as Rett variants. [46]

Other genetic syndromes in which epilepsy occur, associated to developmental delay and dysmorphic features are Kabuki Syndrome, caused by mutations in *KMT2D* [47] and *KMD6A* gene [48], and Pitt-Hopkins Syndrome, due to mutation in the *TCF4* gene. [49]

Genetic study in epilepsy

The importance of identifying genetic background in epileptic patients is essential for many reasons:

- The definitive diagnosis allows to avoid a series of laborious, expensive, and often stressful diagnostic procedures for patient and family;
- It is possible to provide genetic counseling to patient and relatives concerning the risk of recurrence;
- In some instances, the recognition of a causative gene mutation allows a tailored treatment in some syndromes (e.g. ketogenic diet in Glut1 deficiency syndrome, Na^+ channel blockers in *SCN2A* mutations) and to avoid potentially worsening drugs in others (e.g. Na^+ channel blockers in Dravet syndrome due to *SCN1A* mutation);

When a genetic etiology is suspected, there are numerous tests to be performed, from single-gene sequencing to genome-wide techniques.
Karyotype and Comparative Genomic Hybridization (CGH) Array allow to exclude structural chromosomic abnormalities and *copy number variations* (CNV), i.e. deletions and duplications. If the diagnostic suspect is directed to a single gene, it is possible to perform specific sequencing, with PCR

(Polymerase Chain Reaction) or *Sanger*, that they amplify the DNA, allowing the sequence study of the investigated gene.

Contribute of Next Generation Sequencing in the Genetic Diagnosis of Epilepsies

During the last decade, *Next Generation Sequencing* (NGS) technologies such as targeted gene panels, whole exome sequencing and whole genome sequencing have led to an explosion of gene identifications in monogenic epilepsies including both familial epilepsies and severe epilepsies, often referred to as epileptic encephalopathies. The increased knowledge about causative genetic variants has had a major impact on diagnosis of genetic epilepsies and has already been translated into treatment recommendations for a few genes. [50] Furthermore, these techniques allow the analysis of a large number of genes in a single experiment, shortening the time to reach a definite diagnosis, and contribute to save costs.

In 2001, the International Human Genome Sequencing Consortium reported a draft sequence of the euchromatic portion of the human genome, and the results of this process have been published in 2004. [51] This important study allowed the *whole genome sequencing* (also known as **WGS**, full genome sequencing, complete genome sequencing, or entire genome sequencing), the process of determining the complete DNA sequence of genome at a single time.

Whole exome sequencing (**WES** or **WXS**) is a technique applied to sequence all of the genes expressed in the genome (known as *"exome"*). It consists of two steps: the first step selects only the portion of DNA that encodes proteins; these regions are known as *exons*: (humans have about

180,000 exons, constituting about 1% of the human genome, or approximately 30 million base pairs). The second step is the sequence of the exonic DNA by means of any high-throughput DNA sequencing technology. [52] *Targeted sequencing* (by use of "gene panels") is aimed to isolating and sequencing a subset of genes or regions of the genome, which are known to be relevant for a specific disease (e.g. genes associated with epileptic encephalopathies or genetic epilepsies with seizures triggered by fever). This technique had a cost-efficient advantage over WGS/WES since it screens only the relevant genes related to the putative disease. Moreover, the "gene panel" approach reduces the risk of "unexpected finding", a relevant ethical issue for WES.

Diagnostic rate of Targeted Next-Generation Sequencing

Targeted next-generation sequencing panels increased the genetic diagnostic yield between less than 10% to over than 25% in patients with epileptic encephalopathy. [53]
Diagnostic efficacy reaches 50% when patients have a putative genetic epilepsy and a very high number of genes are analyzed [54]. In early-onset epileptic encephalopathy, using targeted next generation sequencing analysis, the genetic background was identified in 20-40 % of probands [55, 56, 57]

Selection of genes based on phenotypic definition and current genetic knowledge

As summarized, a large number of genes is related to epileptic encephalopathies, epileptic syndromes or pathological conditions in which

epilepsy is associated with other symptoms, e.g. intellectual disability or movement disorders. Moreover, some genes encode for "accessory proteins" that are associated to genes/proteins involved in epileptogenesis (e.g. PEX5L is associated to HCN), and therefore worth to be included. To set up a "gene panel", the first step is the choice of genes to be included.

Selection of resulting variants

The high-throughput sequencing imposes a great effort in the interpretation of the results, in which not only geneticists but also the clinicians who request that analysis are involved.

For each subject analyzed, all resulting gene variants must be studied, first performing a comparison with the variants already described in specific databases. The variant is a *polymorphism*, mutation that falls within the interindividual variability, if its frequency is > 1% in the general population. The variant can be identified as "*benign*" if it does not alter the structure and the function of the transcribed protein; conversely, if it is already described for its deleterious function, it will be defined as "*causative or pathogenic*" or "*variant of unclear significance*" (VUS) (see Figure 2 [14]). The nature of the last variant is not currently attributable to a pathogenic or non-pathogenic category: it may have a not yet proven causative meaning, predisposing the onset of the clinical phenotype, or simply to be a polymorphic variant.

Figure 2: Classification of genetic variants (from Ream, 2015)

Aim of the research

The DNA from 81 pediatric epileptic patients was analyzed with a gene panel, set up by child epileptologists, neurophysiologists and geneticists. The gene panel included 55 genes, later extended to 91, whose mutations had been described in patients with epilepsy, associated or not to intellectual disability or neurological involvement, and complex malformations. Screened patients were diagnosed and are currently followed up by the Operative Unit of Neuropsychiatry; the genetic analysis was performed in the Unit of Genetics of Neurodegenerative and Metabolic Diseases, at the "Fondazione IRCCS Istituto Neurologico Carlo Besta", Milan.

The aim of this research was to identify gene variants underlying epilepsies with a challenging etiologic classification.

2. Materials and Methods

Cohort

Patients who present epileptic encephalopathy, generalized epilepsy and focal epilepsy, diagnosed according to ILAE (International League Against Epilepsy) criteria have been selected. [58]

Patients that did not present seizures but cerebral malformations, associated to developmental delay and/or intellectual disability, neurological signs and/or movement disorders were also included.

All patients were extensively characterized through the collection of data concerning family, perinatal and physiological history; clinical,

neurophysiological and neuropsychological evaluation were carried out at seizure onset and during the follow-up.

Genetic testing

Genetic analysis was performed between April 2015 and December 2016. Genomic DNA was extracted from peripheral blood lymphocytes, according to standard procedures.

First panel was composed by 55 genes (Table 2) analyzed through TruSeq Custom Amplicon (**TSCA**) (Illumina), with mean coverage of 88%.

The technique is based on the use of specific primers, designed with a software developed by Illumina (Studio Design), which amplified DNA fragments from 250 to 500 bp. Each of these primers hybridizes one specific genic region to amplify and sequencing and they contain target regions for univocal identification of the samples.

We used the NGS MiSeq Illumina sequencer (Illumina Inc.). The obtained sequences have been aligned to the reference genome (GRCh38.p5), using the software MiSeq Reporter (Illumina Inc.). The resulting variants were filtered based on their recurrence in the population (rare polymorphism or novel variants, never described). Possible effects on mutated protein have been predicted by in *silico* simulations (Polyphen-2, SIFT).

Table 2: Genes analyzed with TruSeq Custom Amplicon (TSCA)

EPILEPTIC ENCEPHALOPATHIES				
Gene	**Protein**	**Locus**	**Inheritance**	**Phenotype-Gene Relationships - #OMIM**
ARX	ARISTALESS-RELATED HOMEOBOX	Xp21.3	XLR	EIEE1 Ohtahara sdr# 308350
CDKL5	CYCLIN-DEPENDENT KINASE-LIKE 5	Xp22.13	XLD	EIEE2 Atypical Rett sdr#300672
CHD2	CHROMODOMAIN HELICASE DNA-BINDING PROTEIN 2	15q26.1	AD	EEOC # 615369
FOXG1	FORKHEAD BOX G1	14q12	IC	Rett syndrome, congenital variant #613454
GABRB3	GAMMA-AMINOBUTYRIC ACID RECEPTOR, BETA-3	15q12	AD	ECA5- Epilepsy, childhood absence 5 # 612269
GRIN2A	GLUTAMATE RECEPTOR, IONOTROPIC, N-METHYL-D-ASPARTATE, SUBUNIT 2A	16p13.2	AD	Epilepsy, focal, with speech disorder and with or without mental retardation # 245570
GRIN2B	GLUTAMATE RECEPTOR, IONOTROPIC, N-METHYL-D-ASPARTATE, SUBUNIT 2B	12p13.1	AD	EIEE27 # 616139
HCN1	HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED POTASSIUM CHANNEL 1	5p12	AD	Epileptic encephalopathy, early infantile, 24 #615871

KCNQ2	POTASSIUM CHANNEL, VOLTAGE-GATED, KQT-LIKE SUBFAMILY, MEMBER 2	20q13.33	AD	Epileptic encephalopathy, early infantile, 7 #613720
KCNQ3	POTASSIUM CHANNEL, VOLTAGE-GATED, KQT-LIKE SUBFAMILY, 3	8q24.22	AD	Epileptic encephalopathy, early infantile [59]
KCNT1	POTASSIUM CHANNEL, SUBFAMILY T, MEMBER 1	9q34.3	AD	Epileptic encephalopathy, early infantile, 14 #614959
MECP2	METHYL-CPG-BINDING PROTEIN 2	Xq28	XLD	Rett sdr, preserved speech variant # 312750
PLCB1	PHOSPHOLIPASE C, BETA-1	20p12.3	AR	Epileptic encephalopathy, early infantile, 12 # 613722
STXBP1	SYNTAXIN-BINDING PROTEIN 1	9q34.11	AD	Epileptic encephalopathy, early infantile, 4 # 612164
UBE3A	UBIQUITIN-PROTEIN LIGASE E3A	15q11.2	IC	Angelman Sdr # 105830
MAGI2	MEMBRANE-ASSOCIATED GUANYLATE KINASE, WW AND PDZ DOMAINS-CONTAINING, 2	7q21.11		Infantile spasms [10]
«BENIGN» EPILEPSY				
ATP1A2	ATPASE, NA+/K+ TRANSPORTING, ALPHA-2 POLYPEPTIDE	1q23.2	AD	familial hemiplegic Migraine, 2 (associated to Epilepsy) [60]
KCNQ2	POTASSIUM CHANNEL, VOLTAGE-GATED, KQT-LIKE SUBFAMILY, MEMBER 2	20q13.33	AD	Seizures benign neonatal 1 #121200

KCNQ3	POTASSIUM CHANNEL, VOLTAGE-GATED, KQT-LIKE SUBFAMILY, MEMBER	8q24.22	AD	Benign familial neonatal Seizures, 2 ## 121201
PRRT2	PROLINE-RICH TRANSMEMBRANE PROTEIN 2	16p11.2	AD	Benign familial infantile Seizures, 2 #605751 Convulsions, familial infantile, with paroxysmal choreoathetosis #602066
EPILEPSY AMENABLE TO SPECIFIC TREATMENT				
Gene	**PROTEIN**	**Locus**	**Inheritance**	**Phenotype-Gene Relationships**
ALDH7A1-A16	ALDEHYDE DEHYDROGENASE 7 FAMILY, MEMBER A1	5q23.2	AR	Epilepsy, pyridoxine-dependent #266100
PHGDH	PHOSPHOGLYCERATE DEHYDROGENASE	1p12	AR	Neu-Laxova syndrome - neurometabolic disorder associated with reduced L-serine biosynthesis [61]
PNPO	PYRIDOXAMINE 5-PRIME-PHOSPHATE OXIDASE	17q21.32	AR	Pyridoxamine 5-prime-phosphate oxidase deficiency
SLC2A1	SOLUTE CARRIER FAMILY 2 (FACILITATED GLUCOSE TRANSPORTER), MEMBER 1	1p34.2	AD AR	GLUT1 deficiency syndrome 1, infantile onset
SLC6A8	SOLUTE CARRIER FAMILY 6 (NEUROTRANSMITTER TRANSPORTER, CREATINE), MEMBER 8	Xq28	XLR	Cerebral creatine deficiency syndrome 1
EPILEPSY IN PROGRESSIVE DISEASES				
Gene	**PROTEIN**	**Locus**	**Inheritance**	**Phenotype-Gene Relationships**

CTSD	CATHEPSIN D	11p15.5	AR	Ceroid lipofuscinosis, neuronal, 10 610127
POLG	POLYMERASE DNA, GAMMA	15q26.1	AR AD	Mitochondrial DNA depletion syndrome 4A (Alpers type) Progressive external ophthalmoplegia, autosomal dominant 1
PPT1 (CLN1)	PALMITOYL-PROTEIN THIOESTERASE 1	1p34.2	AR	Ceroid lipofuscinosis, neuronal, 1 #256730
TWINKLE C10ORF2	CHROMOSOME 10 OPEN READING FRAME 2	10q24.31	AD	Progressive external Ophthalmoplegia with mitochondrial DNA deletions, autosomal dominant 3 #609286
EPILEPSY AND EPILEPTIC ENCEPHALOPATHIES WITH FEBRILE SEIZURES				
Gene	**PROTEIN**	**Locus**	**Inheritance**	**Phenotype-Gene Relationships**
CACNA1A	CALCIUM CHANNEL, VOLTAGE-DEPENDENT, P/Q TYPE, ALPHA-1A SUBUNIT	19p13.13	AD	Epileptic encephalopathy, early infantile, 42 #617106
GABRG2	GAMMA-AMINOBUTYRIC ACID RECEPTOR, GAMMA-2	5q34	AD	Epilepsy, generalized, with febrile seizures plus, type 3
HCN2	HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED POTASSIUM CHANNEL 2	19p13.3		GEFS+; Idiopathic Generalized epilepsy
PCDH19	PROTOCADHERIN 19	Xq22.1	XL	Epileptic encephalopathy, early infantile, 9 #300088
SCN1A	SODIUM CHANNEL, NEURONAL TYPE 1, ALPHA SUBUNIT	2q24.3	AD	Dravet Sdr #607208

SCN1B	SODIUM CHANNEL, NEURONAL TYPE I, BETA SUBUNIT	19q13.11	AD	Epilepsy, generalized, with febrile seizures plus, type 1 #604233
SCN2A	SODIUM CHANNEL, NEURONAL TYPE 2, ALPHA SUBUNIT	2q24.3	AD	Epileptic encephalopathy, early infantile, 11 613721
SCN8A	SODIUM CHANNEL, NEURONAL TYPE 8, ALPHA SUBUNIT	12q13.13	AD	Epileptic encephalopathy, early infantile, 13 #614558

NEURONAL MIGRATING DISORDERS				
Gene	**PROTEIN**	**Locus**	**Inheritance**	**Phenotype-Gene Relationships**
COL4A1	COLLAGEN, TYPE IV, ALPHA-1	13q34	AD	Porencephaly 1 #175780
COL4A2	COLLAGEN, TYPE IV, ALPHA-2	13q34	AD	Porencephaly 2 #614483
DCX	DOUBLECORTIN	Xq23	XL	Subcortical laminal heteropia, X-linked #300067
EMX2	EMPTY SPIRACLES, DROSOPHILA, 2, HOMOLOG OF	10q26.11	?	Schizencephaly #269160
FLNA	FILAMIN A	Xq28	XLD	Heterotopia, periventricular #300049
GPR56	G PROTEIN-COUPLED RECEPTOR 56	16q21	AR	Polymicrogyria, bilateral frontoparietal #606854
HESX1	HOMEOBOX GENE EXPRESSED IN ES CELLS	3p14.3	AD, AR	Septooptic dysplasia #182230
PAFAH1B1/LIS1	PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE, ISOFORM 1B, ALPHA SUBUNIT	17p13.3	IC	Lissencephaly 1 #607432
RELN	REELIN	7q22.1	AR AD	Lissencephaly 2 (Norman-Roberts type) #257320 Epilepsy, familial temporal lobe, 7 #616436
SRPX2	SUSHI REPEAT-CONTAINING PROTEIN, X-LINKED, 2	Xq22.1	XL	Polymicrogiria, Rolandic epilepsy, mental retardation, and speech dyspraxia #300643

TUBA1A	TUBULIN, ALPHA-1A	12q13.12	AD	Lissencephaly 3 #611603
TUBB2B	TUBULIN, BETA-2B;	6p25.2	AD	Polymicrogyria, symmetric or asymmetric #610031
TUBB3	TUBULIN, BETA-3	16q24.3	AD	Cortical dysplasia, complex, with other brain malformations 1 #614039
TUBB8	TUBULIN, BETA-8	10p15.3	AD	Oocyte maturation defect 2 #616780
VLDLR	VERY LOW DENSITY LIPOPROTEIN RECEPTOR	9p24.2	AR	Cerebellar hypoplasia and mental retardation with or without quadrupedal locomotion 1 #224050
OTHER GENES ASSOCIATED TO EPILEPSY				
CAV3	Caveolin 3 (Channel protein)	3p25.3		Long QT #611818 [62]
HCN4	HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED POTASSIUM CHANNEL 4	15q24.1	AD	Brugada Syndrome #613123 Sick sinus syndrome 2 #163800
APBA2	AMYLOID BETA A4 PRECURSOR PROTEIN-BINDING, FAMILY A, MEMBER 2	15q13.1		Autism spectrum disorder #209850
ATP1A3	ATPASE, NA+/K+ TRANSPORTING, ALPHA-3 POLYPEPTIDE	19q13.2	AD	Alternating hemiplegia of childhood #614820

GRASP	TAMALIN	12q13.13		Protein associated to membrane trafficking [63]
KCNE2	POTASSIUM CHANNEL, VOLTAGE-GATED, ISK-RELATED SUBFAMILY, MEMBER 2	21q22.11	AD	Long QT syndrome 6 #13693
PEX5L (TRIP8b)	PEROXISOME BIOGENESIS FACTOR 5-LIKE Auxiliary subunit of the HYperpolarization-activated cyclic-nucleotide-gated (HCN) channels,	3q26.33	-	Absence seizures [64]
SYNGAP1	SYNAPTIC RAS-GTPASE-ACTIVATING PROTEIN 1	6p21.32	AD	Autistic spectrum disorder, generalized epilepsy, intellectual disability [65]

Legend: Early Infantile Epileptic Encephalopathy (EEIE), Childhood-onset epileptic encephalopathy (EEOC), Autosomal Dominant (AD), Autosomal Recessive (AR), X –linked Dominant (XLD), X –linked recessive (XLR)

Successively, the panel has been enlarged to 91 genes (all TSCA genes plus other 33, see Table 3) and another technique, Nextera Rapid Capture (Illumina), with mean coverage of 96%, has been selected. This technology is based on enzymatic fragmentation and tagmentation of selected DNA regions, the genes that compose the panel. *Tagmentation* is a procedure that combines fragmentation, end-polishing, and adaptor-ligation steps (Protocol in supplementary materials). In our experiment, 12 samples are simultaneously analyzed.

Table 3 – Genes analyzed with Nextera Rapid Capture

EPILEPSY IN PROGRESSIVE DISEASES				
Gene	**Protein**	**Locus**	**Inheritance**	**Phenotype-Gene Relationships #OMIM**
AFG3L2	ATPase FAMILY GENE 3-LIKE 2	18p11.21	AR	Spastic ataxia 5, autosomal recessive #614487 (associated to myoclonic epilepsy - Pierson T. 2011)
PROGRESSIVE MYOCLONIC EPILEPSIES				
CERS1	CERAMIDE SYNTHASE 1	19p13.11	AR	PME, 8 #616230
CLN5	CEROID LIPOFUSCINOSIS, NEURONAL, 5	13q22.3	AR	Ceroid lipofuscinosis, neuronal, 5 #256731
CLN6	CEROID LIPOFUSCINOSIS, NEURONAL, 6	15q23	AR AR	Ceroid lipofuscinosis, neuronal, 6 #601780 Ceroid lipofuscinosis, neuronal, Kufs type, adult onset #204300
CSTB	CYSTATIN B	21q22.3	AR	PME 1A (Unverricht and Lundborg) #254800
EPM2A	LAFORIN	6q24.3	AR	Epilepsy, progressive myoclonic 2A (Lafora) #254780

GBA	GLUCOSIDASE, BETA, ACID	1q22	AR	Gaucher disease, type III (neuropathic) #231000
GOSR2	GOLGI SNAP RECEPTOR COMPLEX MEMBER 2	17q21.32	AR	PME 6 #614018
KCNC1	POTASSIUM CHANNEL, VOLTAGE-GATED, SHAW-RELATED SUBFAMILY, MEMBER 1	11p15.1	AD	PME 7 #616187
KCTD7	POTASSIUM CHANNEL TETRAMERIZATION DOMAIN-CONTAINING PROTEIN 7	7q11.21	AR	PME 3, with or without intracellular inclusions #611726 CLN14
NEU1	NEURAMINIDASE 1	6p21.33	AR	Sialidosis, type I and II #256550
NHLRC1 (EPM2B)	NHL REPEAT-CONTAINING 1	6p22.3	AR	PME, 2B (Lafora) #254780
NPC1	NPC1	18q11.2	AR	Niemann-Pick disease, type C1 #257220
NPC2	EPIDIDYMAL SECRETORY PROTEIN; HE1	14q24.3	AR	Niemann-pick disease, type C2 #607625
PRICKLE1	EPILEPSY, PROGRESSIVE MYOCLONIC, 1B	12q12	AR	Epilepsy, progressive myoclonic 1B #612437
SCARB2	SCAVENGER RECEPTOR CLASS B, MEMBER 2	4q21.1	AR	PME 4, with or without renal failure # 254900

TPP1	TRIPEPTIDYL PEPTIDASE I	11p15.4	AR	Ceroid lipofuscinosis, neuronal, 2 #204500
GENERALIZED and FOCAL EPILEPSY				
Gene	**Protein**	**Locus**	**Inheritance**	**Phenotype-Gene Relationships**
CHRNA2	CHOLINERGIC RECEPTOR, NEURONAL NICOTINIC, ALPHA POLYPEPTIDE 2	8p21.2	AD	Epilepsy, nocturnal frontal lobe, type 4 #610353
CHRNB2	CHOLINERGIC RECEPTOR, NEURONAL NICOTINIC, BETA POLYPEPTIDE 2	1q21.3		Epilepsy, nocturnal frontal lobe, 3 #605375
DEPD5	DEP DOMAIN-CONTAINING PROTEIN 5	22q12.2-q12.3	AD	Epilepsy, familial focal, with variable foci 1 #604364
TBC1D24	TBC1 DOMAIN FAMILY, MEMBER 24	16p13.3	AR	EEIE, 16 #615338 Myoclonic epilepsy, infantile, familial #605021
EPILEPSY AND COGNITIVE DEFICIT				
Gene	**PROTEIN**	**Locus**	**Inheritance**	**Phenotype-Gene Relationships**
KDM6A	LYSINE-SPECIFIC DEMETHYLASE 6	Xp11.3	XLD	Kabuki syndrome 2 #300867

MBD5	METHYL-CpG-BINDING DOMAIN PROTEIN 5	2q23.1	AD	Mental retardation, autosomal dominant 1 #156200
MEF2C	MADS BOX TRANSCRIPTION ENHANCER FACTOR 2, POLYPEPTIDE C	5q14.3	AD	Mental retardation, stereotypic movements, epilepsy, and/or cerebral malformations #613443
MLL2 (KMT2D)	LYSINE-SPECIFIC METHYLTRANSFERASE 2D	12q13.12	AD	Kabuki syndrome 1 #147920
PIGA	PHOSPHATIDYLINOSITOL GLYCAN ANCHOR BIOSYNTHESIS CLASS A PROTEIN, PSEUDOGENE 1, INCLUDED	Xp22.2	XLR	Multiple congenital anomalies-hypotonia-seizures syndrome 2 #300868
ROGDI	DROSOPHILA, HOMOLOG OF ROGDI	16p13.3	AR	Epilepsy, dementia, and amelogenesis imperfecta Kohlschutter syndrome #226750
SLC9A6	SOLUTE CARRIER FAMILY 9, MEMBER 6	Xq26.3	XLD	Mental retardation, X-linked syndromic, Christianson type #300243
SMS	SPERMINE SYNTHASE	Xp22.11	XLR	Mental retardation, X-linked, Snyder-Robinson type #309583
TCF4	TRANSCRIPTION FACTOR 4	18q21.2	AD	Pitt-Hopkins syndrome #610954

WWOX	WW DOMAIN-CONTAINING OXIDOREDUCTASE	16q23.1-q23.2	AR	EEIE, 28 #616211
ZEB2	ZINC FINGER E BOX-BINDING HOMEOBOX 2	2q22.3	AD	Mowat-Wilson syndrome #235730

Legend: Autosomal Dominant (AD), Autosomal Recessive (AR), X –linked Dominant (XLD), X –linked recessive (XLR), Progressive Myoclonic Epilepsy (PME), Epileptic encephalopathy, early infantile (EEIE)

The obtained sequences have been aligned to the reference genome (GRCh37/hg19) using MiSeq software. Data analysis was obtained using the following software: Illumina MiSeq Reporter vs 2.4.60, Illumina Variant Studio vs 2.2, Qiagen CLC Genomics Workbench vs 7.0.

Variants with MAF> 1% reported in the dbSNP database, 1000 Genome, EVS were considered benign variants and excluded from the report.

At the end of the experiment, if the coverage of each gene is lower to 95% (at depth of 20X for each nucleotide), it was suggested to repeat the analysis.

The variants at 3' and 5'UTR, intronic, synonymous and high frequency are excluded from the study.

In order to predict the functional effect of the novel variants we queried PolyPhen software (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/). ASSP algorithm (http://wangcomputing.com/assp/) was used for alternative splice site prediction and ESE Finder Software 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) for evaluating disruption of putative exonic splicing enhancers (ESEs).

The novel detected variations were studied by searching in EVS database (http://evs.gs.washington.edu/), 1000 Genomes (http://www.1000genomes.org/) and dbSNP server (http://www.ncbi.nlm.nih.gov/SNP).

Additional support for the functional/pathological significance of each variant came from the study of degree of evolutionary conservation of the amino acid residues involved in various orthologous related proteins

The obtained variants were confirmed with Sanger and, when possible, the family have been enrolled for segregation analysis.

All results were discussed by geneticists, clinicians and neurophysiologists, in order to determine the genotype - phenotype relationship and eventually to schedule other investigations.

3. Results

NGS was performed in 81 patients affected by: epileptic encephalopathy (34,5 % - 28 pts), generalized epilepsy (30,9 % - 25 pts), focal epilepsy (27,1% - 22 pts), focal and generalized seizures (not epileptic encephalopathy, 3,7% - 3 pts); cerebral malformations associated to developmental delay, neurological signs and/or movement disorders without seizure (3,7% - 3 patients).

Most patients (77,7%) had developmental delay or intellectual disability of mild to severe degree.

Seventy-nine pts (97,5%) underwent brain MRI. In 53 (65%) patients MRI was normal; pathological findings, including polymicrogyria (6 patient), cerebellar malformations or atrophy (5 patients), isolated lyssencephaly (1 patient) periventricular heterotopia (1 patient), subcortical band heterotopia (2 patients) focal cortical dysplasia (1 patient) basal ganglia abnormalities (1 patient)and cortical-subcortical abnormalities (6 patients).

Previous genetic investigations had been performed in 90,5% of patients: karyotype analysis in 62,9%, CGH array in 70,3% and preliminary screening of the most common genes associated with specific phenotypes - gene-target (e.g. *SLC2A, SCN1A, MECP2, UBE3A*) in 67,8%.
Thirty-five patients have been analyzed by TSCA, 50 patients by Nextera, (including 4 cases for which TSCA was unrevealing).

Table 4: Patient clinical, radiologic and genetic data. TSCA was performed in patients from S1654 to NMD298, from GLUT38 was performed Nextera. In GLUT39, S1775, S1776 and GLUT183 was perfomed both.

ID patient, gender	clinical features	DD/DI	MRI	MRI alterations	Epilepsy	Kariotype	alterations	CGH	alterations	Other genetic analysis	Movement disorders
S1654, F	Epileptic encephalopathy with tonic seizuires and epileptic spasms	YES	YES	NONE	EE	YES	none	YES	none	no	
S1560, F	Focal seizures, dysmorphism	YES	YES	NONE	FOC	YES	none	YES	none	met UBE3A	walking ataxia and distal hyperkinesias upper limb s
GLUT145, M	Febrile and afebrile seizures	YES	YES	NONE	GEN	YES	none	YES	none	SCN1A SLC2A1 - Xfra - SNRPN (Angelman)	
H1622, F	Early onset epileptic encephalopathy	YES	YES	NONE	EE	YES	none	YES	none	MECP2, FOXG1, telomeres, CDKL5	
H1887, F	Myoclonic epilepsy and EPC	YES	YES	CEREBELL	GEN	YES		YES	none	SCA7 - PCDH19 - MECP2	ataxia
GLUT34, M	Generalized seizures and microcephaly	YES	YES	NONE	GEN	YES	none	YES	none	no	
GLUT 94, F	Absences and ESES, behavior disorder	YES	YES	NONE	EE	YES	none	YES	dup12p13.31mat	SLC2A1-FRAXA, telomeres, PRRT2	Dystonic posture
GLUT2, F	Early onset myoclonic encephalopaty, photosensitivity, febrile-induced seizures	YES	YES	NONE	EE	YES	none	YES	none	POLG1, SCN1A, CLN10, MECP2	Cerebellar signs
S1687, F	Landau - Kleffner syndrome (Acquired aphasia)	NO	YES	NONE	EE	NO		NO	none		
H1897, M	Focal seizures with sec gen and psycomothor regression	YES	YES	NONE	EE	YES	none	YES	none	SLC2A1, fra X, UBE3A	Camptocormia

ID patient, gender	clinical features	DD/DI	MRI	MRI alterations	Epilepsy	Kariotype	alterations	CGH	alterations	Other genetic analysis	Movement disorders
H3170, M	Early onset epilepsy with status epilepticus, skeletal malformations	YES	YES	NONE	EE	YES	none	YES	dup 6q22.31 pat	GLRA1, GLRB, SLC6A5, ARHGEF9 CRLF1, SCN1A, SCN2A, SCN4A, SCN9A, CACNA1A, PRRT2, SLC2A1, ATP1A3, CLCN1, KNQ2, CAV3, met AS/pW, POLG1	Hyperkinesias(choreoatetosis and multifocal myoclonias)
S1771, M	Focal seizures, ODD and borderline cognitive	NO	YES	NONE	FOC	YES	none	YES	none	no	Intention tremor
S1772, F	Epileptic encephalopathy with focal seizures	YES	YES	NONE	EE	YES	none	YES	dup(16)(q24.1)mat - Dup (15) (q13.3)	no	Dystonic posture
S 1622, F	Focal seizure, previous West syndrome	YES	YES	NONE	FOC	NO		YES	dup(5)(p13.2)pat	CDKL5	
S1774, F	Early onset encephalopathy , pyramidal paresis , megacisterna magna	YES	YES	CEREBELL	EE	NO		YES	del(17)(q25.3)pat	TCF4, MECP2, FOXG1, CDKL5	
S 1487, F	Ohtahara	YES	YES	NONE	EE	YES	none	YES	none	CDKL5 - MUNC18 - POLG1- CNL10	
H1235, F	Focal seizures in cluster	NO	YES	NONE	FOC	NO		YES	nonene	PCDHC19 (dup parz es1 non pat)	

ID patient, gender	clinical features	DD/DI	MRI	MRI alterations	Epilepsy	Kariotype	al terations	CGH	alterations	Other genetic analysis	Movement disorders
H443, M	Encephalopathy with generalized seizures, movement disorder cerebellar atrophy	YES	YES	CEREBELL	GEN	NO		NO	none	sca1 - sca2 - sca3- sca6 - del cln3 - cln2-DRPLA - POLG1 - PLA2G6 - APTX in eterozigosi - GBA in eterozigosi (NGS)	Bradykinesia, rigidity, tremor and myoclonus
S1722, F	Status epilepticus at onset	NO	NO	NONE	GEN	NO		NO	none	no	
S1723, M	Complex cortical malformation (no seizures)	NO	YES	PMG	NO EPI	NO		YES	none		
S1445, F	Focal seizures and status epilepticus , hemiparesis	YES	YES	PMG	FOC	YES	none	YES	del(12)(p13.32)pat - del(4)(q24)pat- dup(12)(q23.3)pat	CDKL5	
S1020, M	Early onset focal seizures and behavior disease	YES	n.d.	N.D.	FOC	NO		YES	none	no	
GLUT57, M	Focal seizures and microcephaly	YES	YES	CORT+SUBC	FOC	YES	none	YES	none	Meti 15q11q13, SLC2A1, UBE3A, TCF4, SLC9A6, MECP2	
S1449, M	Focal seizures with sec gen and EPC	YES	YES	NONE	FOC	NO		YES	dup(1)(q24.1)mat	no	
NMD378, F	Early onset epilepsy, diffuse microlissencefaly	YES	YES	LIS	EE	NO		YES	none	Met UBE3A	Choreic movement of upper limbs and stereotypies in median line

ID patient, gender	clinical features	DD/ DI	MRI	MRI alterations	Epilepsy	Kario type	alterations	CGH	alterations	Other genetic analysis	Movement disorders
S1724, F	Generalized epilepsy and borderline cognitive	NO	YES	NONE	GEN	NO		NO	none	SCN1A, GABRG2	
NMD360, F	Focal seizures, photosensitivity, cortical malformation	YES	YES	PMG	FOC	NO		YES	del(2)(p24.3)mat - del(2)(q23.3)mat	LIS1 e DCX.	
H532, M	Migrant partial epilepsy (death)	YES	YES	nd	EE	n.d		NO	none	n.d	
H533, M	Migrant partial epilepsy (death)	YES	YES	nd	EE	n.d		YES	none	n.d.	
S1773, M	Febrile and afebrile seizures, hypotonia	YES	YES	NONE	FOC/ GEN	YES	none	YES	none	Fish1p36,	
NMD298, M	Epileptic spasms and focal seizures	YES	YES	NONE	FOC	NO		YES	none	SLC2A1, fraX,	
GLUT98, M	Early onset absences, familial	YES	YES	NONE	GEN	YES	none	YES	none	SLC2A1	
S1973, M	Focal seizures	YES	YES	NONE	FOC	YES		YES	none	no	
S1620, F	Early onset encephalopathy	YES	YES	CORT+SUBC	EE	YES		YES	dup(5)(q35.3)dn	no	
S1664, M	Focal seizures	NO	YES	NONE	FOC	NO		NO	none	no	
S1938, F	Focal seizures with sec gen, cortical mlaformations	YES	YES	SBH	FOC	NO		NO	none	no	
S1688, M	Focal seizures and expressive language disorder	NO	YES	NONE	FOC	NO		NO	none	no	
S2013, M	Generalized seizures febrile - induced, ADHD	YES	YES	NONE	GEN	NO		NO	none		
S1778, F	Generalized seizures	YES	YES	PH	GEN	NO		NO	?	no	

ID patient, gender	clinical features	DD/DI	MRI	MRI alterations	Epilepsy	Kariotype	Alterations	CGH	alterations	Other genetic analysis	Movement disorders
S892, F	Early onset epileptic encephalopathy and cortical cerebellar atrophy	YES	YES	CEREBELL	EE	YES		n.d	none	MECP2, CDKL5, FOXG1	
S1776, F	Encefalopathy with microcephaly and dyskinetic palsy (no seizures)	YES	YES	NONE	NO EPI	YES		YES	none	telomeres FISH 22q11.2, met e seq UBE3A, MECP2 (seq e MLPA) - FOXG1	Dystonia, Dyskinesias
GLUT8, M	Myoclonic seizures, behavior disease	NO	YES	NONE	GEN	NO		NO	none	SLC2A1	
GLUT 183, M	Absences, borderline cognitive	YES	YES	NONE	GEN	NO		NO	none	SLC2A1 (mlpa)	Tremor
GLUT123, M	Myoclonic seizures and borderline cognitive	NO	YES	NONE	GEN	YES	None	YES	del(14)(q21.3)pat - gene MDGA2	SLC2A1	
S1973, M	Focal seizures	YES	YES	NONE	FOC	YES		YES	none	no	
S1620, F	Early onset encephalopathy	YES	YES	CORT+SUBC	EE	YES		YES	dup(5)(q35.3)dn	no	
S1664, M	Focal seizures	NO	YES	NONE	FOC	NO		NO	none	no	
S1938, F	Focal seizures with sec gen, cortical mlaformations	YES	YES	SBH	FOC	NO		NO	none	no	
GLUT115, M	Myoclonic astatic epilpesy and behavior disease	YES	NO		GEN	YES		YES	del(14)(q23.3) - dup(13)(q31.3)mat	SLC2A1	

ID patient, gender	clinical features	DD/ DI	MRI	MRI alterations	Epilepsy	Kariotype	alteration	CGH	alterations	Other genetic analysis	Movement disorders
S1688, M	Focal seizures and expressive language disorder	NO	YES	NONE	FOC	NO		NO	none	no	
GLUT39, F	EPC and alternating hemiplegia	YES	YES	NONE	FOC	YES	NONE	YES	none	ATP1A3, SLC2A1, POLG1	
S1998, M	Early onset epilepsy and language disorder	YES	YES	NONE	EE	YES	NONE	YES	del 6q12,1 (COL21A1) e del 5q14,3 (none geni noneti)	X fra, STXBP1	
S1824, M	Generalized seizures, cortical myoclonus and neuropsycological disorders	YES	YES	NONE	GEN	NO		NO	none	POLG1 - melas e merrf	
S2013, M	Generalized seizures febrile - induced, ADHD	YES	YES	NONE	GEN	NO		NO	none		
S1778, F	Generalized seizures	YES	YES	PH	GEN	NO		NO	?	no	
S2037, M	Absences, behavior disorder	YES	YES	SBH	GEN	YES	NONE	YES	none	X-fragile, test di metilazione del chr15	
NMD 375, M	Epilepsy onset with West syndrome, currently Focal EEG alterations and mood disorder	YES	YES	NONE	FOC	YES	NONE	YES	none	no	
S1843, F	Early onset epilepsy, focal seizures, pyramidal signs	YES	YES	BG	EE	NO		NO	none	cln10	Extrapyramidal hypertonia
S1469, M	Early onset epilepsy, dysmorphisms and diffuse cortical atrophy	YES	YES	CORT+SUBC	EE	YES	NONE	YES	none	ARX, CDKL5, Telomeres, FISH fot UBE3A, MECP2, STPBX (e	

ID patient, gender	clinical features	DD/ DI	MRI	MRI alterations	Epilepsy	Kariotype	alteration	CGH	alteration	Other genetic analysis	Movement disorders
S1624, M	Focal seizures, absence of speech, microcephaly and cortical atrophy	YES	YES	CORT +SUBC	FOC	YES	none	YES	none	22q11.12; UBE3A, FOXG1, TCF4, SLC9A6, MECP2	
S1791, F	Previous West syndrome, currently tonic seizures and autism	YES	YES	NONE	EE	YES	none	YES	none	CKL5, FOXG1, FISH15,met PW/AS, MECP2, STXPB1	Stereotypies
S1970, F	Generalized seizures, also febrile - induced and myoclonic seizures	NO	YES	NONE	GEN	NO	none	NO	none	PCDH19	
GLUT37, F	Epileptic encephalopathy with myoclonic, tonic and TCG and recurring status epilepticus	YES	YES	NONE	GEN	YES	none	YES	none	ring20	
S1626, F	Epileptic encephalopathy	YES	YES	NONE	EE	YES	none	YES	del(22)(q12.3)pat		
S1829, M	Focal seizures and epileptic spasms	YES	YES	PMG	FOC	YES	none	YES	none	ATP1A3	
S1777, F	Generalized seizures and autism	YES	YES	NONE	GEN	YES	none	YES	none	pcdh19 met Angelman, seq MECP2, X fragile del MECP2	
GLUT138 , F	Myoclonic seizures, psychomotor and language deterioration	YES	YES	NONE	EE	YES	none	YES	none	CDKL5, STXBP1, SCN1A, PTEN, SLC2A1, PCHD19, WDR45	
S1928, M	Focal seizures	NO	YES	NONE	FOC	NO	none	NO	none	SLC2A1	

ID patient, gender	clinical features	DD/ DI	MRI	MRI alteration s	Epilepsy	Kariotype	alterations	CGH	alterations	Other genetic analysis	Movement disorders
S1951, M	Myoclonic seizures and dysmorphisms	YES	YES	NONE	GEN	YES	none	YES	none	22q11.12; Angelman, UBE3A, Foxg1, TCF4, SLC9A6, MECP2	
H2355, M	Myoclonic astatic epilepsy and language disorder	NO	YES	NONE	GEN	YES	none	YES	del(7)(q36.1) pat - del(13)(q31.2) pat (IS)	CKL5,FOXG1, FISH15,met PW/AS, MECP2, STXPB1	Stereotypies
S1924, M	Epileptic spasm and spastic-distonic cerebral palsy	YES	YES		EE	NO	none	NO	none	PCDH19	
S1780, M	Cortical malformation (no seizures)	NO	YES	PMG	NO EPI	NO	none	NO	none	ring20	
S1631, F	Encephalopathy with microcephaly, hypoacusis, gross motor impairment	YES	YES	PMG	EE	YES	none	YES	dup 9p24.2		
S1756, F	Dravet syndrome	YES	YES	NONE	EE	YES	none	YES	none	atp1a3 (ep parossistici)	
S1673, F	Generalized seizures, hypotonia, dismorphisms	YES	YES	NONE	GEN	YES	none	YES	gene EXT1	pcdh19 (?), met Angelman, seq MECP2, X fragile	
GLUT 158, M	Myoclonic seizures	NO	YES	NONE	GEN	YES	none	YES	none	del MECP2 CDKL5, STXBP1, SCN1A, PTEN, SLC2A1, PCHD19, WDR45	
S1553, M	Focal seizures and multiple cortical malformation	YES	YES	CORT +SUBC	FOC	YES	none	YES	dup(15)(q13.3)pat	ARX, fraX, riarrang subtelomerici,	

ID patient, gender	clinical features	DD/ DI	MRI	MRI alterations	Epilepsy	Kariotype	alterations	CGH	alterations	Other genetic analysis	Movement disorders
S2012, F	Migrant partial epilepsy and EPC	YES	YES	CORT+ SUBC	FOC	YES	none	YES	none	22q11.12; Angelman, UBE3A, Foxg1, TCF4, SLC9A6, MECP2	
S1630, M	Othahara	YES	YES	CORT+ SUBC	EE	YES	none	YES	none	CKL5,FOXG 1, FISH15,met PW/AS, MECP2, STXPB1	Stereotypies
GLUT270 , F	Progressive myoclonic epilepsy	YES	YES	NONE	GEN	YES	none	NO	none	PCDH19	
S1967, M	Febrile - induced focal seizures and autism	YES	YES	NONE	FOC	YES	none	YES	microdel 7 p22.2 (mat)	ring20	
H3176, F	HHE, cerebellar atrophy	YES	YES	CEREBEL L	FOC	YES	none	YES	dup DAB1 (pat) e dup DLGAP 5 (mat)		
S1775, M	Focal and myoclonic seizures, cognitive deterioration	YES	YES	FCD	FOC/ GEN	YES	none	YES	dup(14)(q31.2)mat - dup(22)(q 13.33)dn	atp1a3	
S1997, F	Generalized seizures in cluster, infection-induced	NO	YES	NONE	GEN	NO	none	NO	none	pcdh19 (?), met Angelman, seq MECP2, X fragile	
S1833, M	GEFS+	NO	YES	NONE	GEN	NO	none	NO	none	no	
NMD347, M	Previous West syndrome	YES	YES	NONE	EE	YES	none	YES	none	ARX, fraX, riarrang subtelomerici,	
S2124, F	Focal and generalized seizures	NO			FOC/ GEN	NO	none	NO	none	SLC2A1	
GLUT175	Early onset epilepsy, West syndrome and stereotypies	YES	YES	NONE	EE	YES	none	YES	none	no	

TRUSEQ CUSTOM AMPLICON

Pathogenetic mutations have been identified in 5 patients among 35 (diagnostic rate 14,2%):

The patients are all female, affected by early onset epileptic encephalopathy. Discovered mutations involved *FOXG1, KCNQ3, KCNQ2,* and *CDKL5* in two cases (**Table 5**).

All mutations are novel, except a mutation in compound heterozygous in *KCNQ3,* and they have a prediction in *silico* as "probably damaging".

Case, gender ID	Gene	Mutation	Inheritance	Age and seizure at onset	Epileptic syndrome	Neurological and Neuroradiological Features
1, F H1622	**CDKL5**	c.400C>T; p.Arg134Ter nonsense; ***novel***	X-linked, de novo	6 months Epileptic spasms and tonic seizures	Epileptic encephalopathy with epileptic spasms Rett Syndrome	Spastic tetraparesis, microcephaly, severe intellectual disability , scoliosis, nocturnal apnea *MRI: normal*
2, F S1772	**CDKL5**	c.404-1G>T; splicing; ***novel***	X-linked, de novo	9 months Epileptic spasms	Epileptic Encephalopathy with spasms Rett syndrome	Moderate developmental delay *MRI: normal*
3, F NMD378	**FOXG1**	c.2903G>A; p.Tyr246Ter nonsense; ***novel***	AD, de novo	15 months tonic seizures	Epileptic Encephalopathy	Hypotonic-hyperreflexic syndrome, microcephaly, severe developmental delay; choreic movements, stereotypies *MRI: diffuse microlissencephaly*
4, F S 1487	**KCNQ3**	c.1624G>A; p.Asp542Asn Missense, COSM1096201 (Inherited from mother) c.1075G>T; p.Val359Leu Missense; ***novel*** (Inherited from father)	AR, compound heterozygous	1 month, tonic seizures	Ohtahara syndrome	Hypertonic syndrome, strabism, severe developmental delay *MRI: thin corpus callosum*
5, F S1445	**KCNQ2**	c.928G>A; p.Gly310Ser splicing; ***novel***	AD, de novo	At birth Status epilepticus ,	Epileptic encephalopathy	Hemiparesis, moderate developmental delay *MRI: polymicrogyria*

Table 5: Genetic and Clinical features of patients with mutations revealed with TSCA

NEXTERA RAPID CAPTURE

Pathogenetic mutations have been identified in 9 patients out 50 (diagnostic rate 18%). The following mutations were found:

- *TBC1D24*, in two patients, females, one affected by progressive myoclonic encephalopaty with epilepsia partialis continua (S2012), the other affected by epilepsia partialis continua and alternating hemiplegia (GLUT39);
- *SLC2A1,* in a female with generalized epilepsy with absences and periventricular heterotopia (S1778);
- *SCN1A* e *SCN2A*, in a patient, female, with Dravet Syndrome but cognitive impairment worse than usually expected in "classic" phenotypes (S1756);
- *MBD5*, in a male with moderate intellectual disability and epilepsy with absences (S2037);
- *GPR56*, in a male with severe cortical and subcortical malformation, associated to focal epilepsy and severe intellectual disability (S1829);
- *SCN2A*, in a patient, male, with early onset epileptic encephalopathy (S1630);
- *MEF2C*, in a male with febrile seizures, developmental delay and autism (S1967);
- *PRRT2*, in a female with myoclonic and focal seizures and normal psychomotor development (S2124).

Clinical and genetic details are specified in Table 6.

All mutations have a prediction in *silico* as "probably damaging".

Case, gender ID	Gene	Mutation	Inheritance	Age and seizure at onset	Epileptic syndrome	Neurological features *MRI*
5, F S1756	**SCN1A** **SCN2A**	c.2723A>G; p.Arg612Ter Nonsense; rs398123585 c.1834C>T p.Lys908Arg Missense; rs2228980	AD, de novo AD, inherited from mother	4 months, afebrile focal seizure	Dravet Syndrome	Mild developmental delay, Hypotonia myoclonus *MRI: Normal*
6, F S1778	**SLC2A1**	c.940G>A; p.Gly314Ser Missense; rs121909739	AD, de novo	18 months, febrile generalized seizure	Generalized epilepsy with absences	Mild cognitive delay migraine *MRI: Left frontal cortical heterotopia*
7, F GLUT39	**TBC1D24**	c.116C>T; p.Ala39Val Missense, ***novel*** (inheredited from father) c.457G>A p.Glu153Lys Missense, rs376712059 (inheredited from mother)	AR, compound heterozygous	4 months, focal seizure	Focal epilepsy with epilepsia partialis continua	Moderate developmental delay, extrapiramidal signs multifocal myoclonus Recurrent hemiplegic attacks *MRI: Normal*
8, M S2037	**MBD5**	c.2030G>A; p.Ser677Asn Missense; rs114314967	AD, inherited from mother	10 years absences	Epilepsy with absences (SW 3 Hz) and focal seizures from right frontal region	Moderate intellectual disability Obsessive compulsive personality disorder *MRI: subcortical band heterotopia*
9, M S1829	**GPR56**	c.105C>A; p.Cys35Ter Nonsense; CM131923	AR, homozygous	17 months Epileptic spasms	Focal epilepsy and epileptic spasms	Severe intellectual disability, distal hyperkinesies *MRI*: bilateral polymicrogyria, lissencephaly, cerebellar vermis hypoplasia

11, M S1630	**SCN2A**	c.4886G>A; p.Arg1629His Missense; ***novel***	AD, de novo	3 months Epileptic spasms	Early onset epileptic encephalopaty;	Severe intellectual disability; *MRI*: normal
12, M S1967	**MEF2C**	c.52_54+4delCAGGTGA splicing; **novel**	AD, de novo	9 months febrile seizures	Focal epilepsy	developmental delay and autism *MR: arachnoid cyst*
13, F S2124	**PRRT2**	c.771delG; p.Gly259ValfsTer54 nonsense; ***novel***	AD, inherited form mother	6 months myoclonic and focal seizures	Focal epilepsy	normal psychomotor development. *MRI*: normal
14, F S2012	**TBC1D24**	c.753C>G; p.Phe251Leu Missense; ***novel***	AR, omozygous	Infancy Myoclonic seizures	Progressive myoclonic epilepsy (Epilepsia partialis continua and migrating seizures)	Sever intellectual disability Pryramidal spasticity *MRI: cortical and subcortical atrophy*

Table 6: Genetic and clinical features of patients with mutations revealed with Nextera.

4. Discussion

In our cohort of 81 patients, 14 pathogenetic mutations have been revealed, with a diagnostic rate of 17.2%. The diagnostic efficiency amounts to 14.2% with TSCA and to 18% with Nextera.

On a genetic point of view all mutations involve highly-conserved amino acids; ten of these mutations have not yet been described. These two aspects, combined with the prediction data, allowed us to consider them as causative. Furthermore, the phenotype previously described as associated to the mutations, was fitting with the clinical picture of our patients.

The largest part (90,5%) of our patients had already had previous unrevealing genetic investigations (cytogenetics or at least one single-gene analysis). This indicates that our population was highly selected, since the gene panel had not been considered as the first step of genetic investigation.

The examination of gene panel results improved our diagnostic abilities and allowed us to speed up the diagnostic work-up in patients not yet studied; following the detection of a given mutation, we were able to extend the single gene analysis in patients with similar phenotypes. For example, the discovery of mutations of *TBCID24* in a patient with EPC and recurrent myoclonic status led to successfully analyze the same gene in three further patients with similar phenotype, including a first cousin of the proband.

Some "unexpected results" in our series deserve consideration: a patient with focal seizures, periventricular nodular heterotopia and borderline cognitive abilities, was investigated through NGS, to search for mutations in genes related to malformation of cortical development. The unexpected and surprising result was the detection of a pathogenic *SLC2A1* mutation

(causally related to GLUT1 deficiency syndrome) which led to re-evaluate the clinical history, diagnostic work up and therapy: besides partial seizures, the patient had had recent onset of "absences" (also recorded with EEG), and cognitive deterioration; the genetic data prompted CSF examination which revealed hypoglycorrhachia. The patient was given the ketogenic diet that led to the seizure control and improvement of cognitive performances.
Another unexpected finding was the detection of pathogenic variants in *HCN4* and *SCN1B*, which are associated with cardiac arrhythmias, in three patients. Based on this finding, the genetic analysis was extended to the family members, and appropriate cardiologic examination performed.
The absence of pathogenetic mutations in the large part of cohort indicate that the alteration may be in many other genes not present in our panel, highlighting the high genetic heterogeneity of epilepsy

Our study confirms the utility of the Next Generation Sequencing in the diagnosis of rare child epilepsy, and underscores the key role of the discussion between physicians and geneticists in selecting the candidate patients and in evaluating the implication of the results.

This concept is mostly important when the detected mutations involve genes associated with multiple phenotypes, as in the case of mutations in KCNQ2, which are associated both with benign familial neonatal seizures (BNFE), and neonatal epileptic encephalopathy (NEE). [59]
Secondary aim of our research was, to pinpoint the indications to genetic analysis by target sequencing technique. Based on our results it appears that, to save time and costs, sequencing of single gene is worth doing in patients with "classic" phenotype, suggestive of specific and well defined epileptic

syndromes, such as Dravet syndrome, PCDH19 mutations o GLUT1DS. By contrast, sequencing a large amount of genes by NGS is indicated in patients with epileptic syndromes (e.g. early onset encephalopathy) which may be associated with mutations in several putative genes.

Finally, we would once again underscore that NGS analysis must not be considered a screening examination, and that it requires multidisciplinary approach in selecting patients, and in reading into expected and unexpected results.

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6. Supplementary materials

Nextera Technology

The Nextera™ DNA Sample Prep Kit is designed to prepare genomic DNA libraries compatible with the Illumina® Genome Analyzer I and II and HiSeq™ 2000 sequencers. Nextera technology employs in vitro transposition to simultaneously fragment and tag DNA in a single-tube reaction, and prepare sequencer-ready libraries in under 2 hours.

The Nextera library preparation procedure is a significant improvement upon current procedures, which generally consist of distinct DNA fragmentation, end-polishing, and adaptor-ligation steps. The Nextera library preparation procedure combines these steps into one (tagmentation), uses only 50 ng of starting DNA, and allows incorporation of platform-specific tags and optional barcodes.

Target DNA is fragmented and tagged with Nextera Enzyme Mix containing transposon ends appended with sequencing primer sites. Limited-cycle PCR with a four-primer reaction adds bridge PCR (bPCR)-compatible adaptors to the core sequencing library. Optional bar codes can be added between the downstream bPCR adaptor and the core sequencing library adaptor (see Figure S1for preparation details).

The following diagram illustrates the workflow using a Nextera DNA Library Prep kit. Safe stopping points are marked between steps.

Figure 1 Nextera DNA Library Prep Workflow

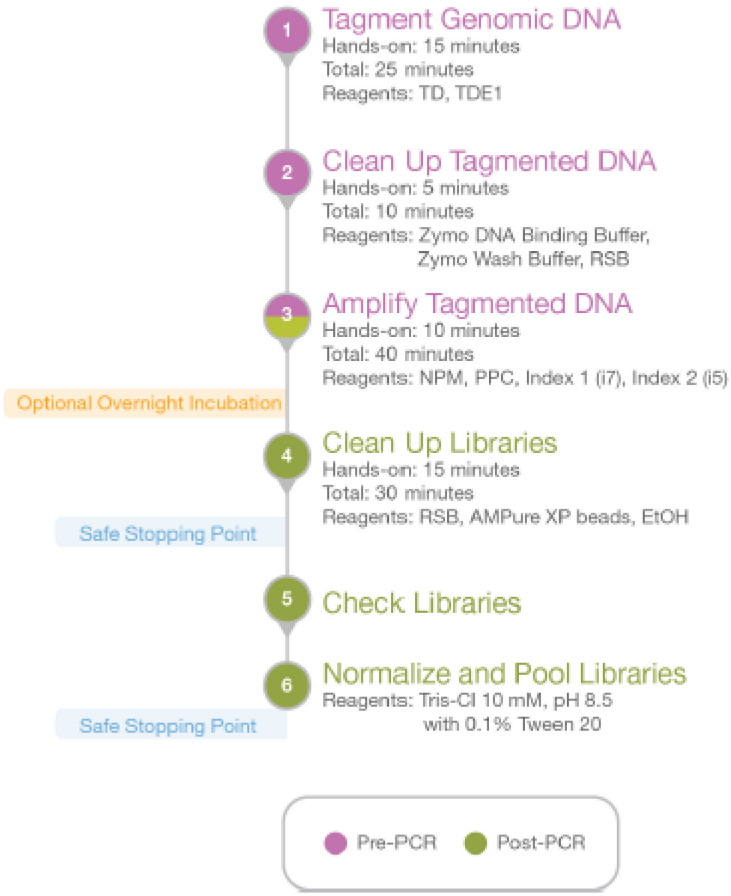

Figure S1: preparation of samples for Nextera.