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IN-DEPTH CLINICAL, GENETIC AND NEUROPSYCHOLOGICAL STUDY OF
FAMILIAL AND SPORADIC CASES WITH SLEEP-RELATED HYPERMOTOR
EPILEPSY (SHE): IDENTIFICATION OF NEW GENES BY WHOLE EXOME
SEQUENCING (WES)

Presentata da: Dr. LAURA LICCHETTA

Coordinatore Dottorato

Relatore

Prof. Gaetano D. Gargiulo

Prof. Paolo Tinuper

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INTRODUCTION

I. SLEEP-RELATED HYPERMOTOR EPILEPSY (SHE)

I.1. DEFINITION AND CLINICAL FEATURES

Sleep-related hypermotor epilepsy (SHE), previously named Nocturnal Frontal Lobe Epilepsy (NFLE), is a distinctive epilepsy syndrome characterized by clusters of paroxysmal motor events occurring predominantly during sleep (Ryvlin et al., 2006a).

SHE is a rare focal epilepsy (FE) with an estimated prevalence of 1.8/100,000 individuals (Vignatelli et al., 2016). It affects individuals of both sexes and any age, with a peak of seizure onset during childhood and adolescence (Scheffer et al., 1994; Provini et al., 1999).

The electro-clinical features of the syndrome were recently revised during an international consensus conference that provided diagnostic criteria developed with three levels of certainty (Panel 1): witnessed (possible) SHE, video documented (clinical) SHE and video-EEG documented (confirmed) SHE (Tinuper et al., 2016). The change in nomenclature emphasized the characteristic aspect of the seizures (hypermotor seizures), the relation with sleep instead of the chronobiological patterns of seizure occurrence and the possible extra-frontal origin of the seizures (Tinuper et al., 2016). In fact, the primary clinical expression of SHE consists of hypermotor events characterized by hyperkinetic features possibly associated with asymmetric tonic/dystonic posturing with or without head/eye deviation. Seizures typically show variable complexity and duration varying from brief stereotyped sudden arousals from sleep to more complex dystonic-dyskinetic seizures and, more rarely, prolonged ambulatory behavior known as “epileptic nocturnal wandering” (Provini et al., 1999; Nobili et al., 2003; Montagna, 1992; Terzaghi et al., 2008). Retained awareness during seizures is common and affected individuals may report a distinct aura.

Seizures occur predominantly during sleep non-REM (NREM) sleep and rarely during REM sleep. Seizures during active wakefulness may also occasionally occur during the patient’s lifetime.

Surface EEG and invasive intracranial stereotactic EEG recordings (SEEG) documented a frontal lobe origin of seizures in most cases (Nobili et al., 2007; Rheims et al., 2008). However, it has been widely documented that ictal discharges may arise from various extra-frontal area including temporal (Nobili et al., 2004; Vaugier et al., 2009), insulo-opercular (Ryvlin et al., 2006b; Dobesberger et al., 2008; Nguyen et al., 2009; Proserpio et al., 2011) and parietal (Montavont et al., 2013; Gibbs et al., 2016) cortices, then propagating to the frontal cortex and resulting in hypermotor seizures.

Etiology is unknown in the majority of patients. Recognized etiologies of SHE are heterogeneous and include acquired injuries, genetic causes and structural anomalies such as focal cortical dysplasia (FCD). Multiple etiologies (structural-genetic) are also possible. Non-specific clinical features distinguished different etiologies (Tinuper et al., 2016) even if SHE due to structural lesions (FCD) usually manifests with early-onset drug-resistant seizures (Nobili et al., 2009) and showed a worse long-term prognosis

(Licchetta et al., 2017). In these cases, epilepsy surgery and removal of the epileptogenic zone could represent a highly effective treatment option (Nobili et al., 2007).

Most patients (86%) are sporadic cases while 14% reported a family history for epilepsy. The familial, autosomal dominant form of SHE (ADSHE, previously ADNFL) accounts for about 5% of cases (Licchetta et al., 2017).

Panel 1 Electro-clinical features and diagnostic criteria of SHE

Clinical features

- Brief seizures (<2 minutes) with stereotyped motor patterns and abrupt onset and offset
- The most common clinical expression consists of "hypermotor" events
- Seizures occur predominantly during sleep; seizures during wakefulness may also occur

Electro-clinical features

- Interictal and ictal scalp EEG features may be uninformative
- Prolonged video-EEG is the best diagnostic test; if negative it does not rule out the diagnosis (seizures may not be recorded, interictal EEG abnormalities may be absent)
- Seizures may arise from various frontal as well as from extra-frontal areas

Diagnostic criteria

Witnessed (possible) SHE

- Presence of seizures consistent with HS, as provided by an eyewitness
- Video-documented (clinical) SHE
- Reliable audio-video documentation of at least 1 entire HS (preferably 2), confirmed to be typical by witness

Video-EEG documented (confirmed) SHE

- Video-EEG documentation of HS from sleep associated with clear-cut epileptic discharges and/or interictal epileptiform abnormalities

Comorbidities with ID/neuropsychiatric disorders DO NOT represent exclusion criteria

I.2. GENETICS OF SHE

SHE is the first inherited focal epilepsy syndrome and the first epilepsy channelopathy described, as it was initially related to mutations in genes coding for subunits of a ligand-gated cationic channel, the neuronal nicotinic acetylcholine receptor (nAChR) (Steinlein et al., 1995).

Currently SHE has been associated with mutations in several genes encoding proteins with different functions and involved in different biological pathways, such as *CHRNA4*, *CHRNA2*, *CHRNA2*, *CRH*, *KCNT1*, *DEPDC5* and *PRIMA1*. Familial SHE usually shows an autosomal dominant pattern of inheritance (ADSHE), except for a single reported family mutated in *PRIMA1* showing an autosomal recessive inheritance (Hildebrand et al., 2015).

I.2.1. *CHRNA4*, *CHRN2*, *CHRNA2*

The first gene for ADSHE, *CHRNA4* (Cholinergic Receptor Nicotinic Alpha 4 Subunit, MIM *118504), encodes the $\alpha 4$ subunit of the nAChR. It was identified in 1995 in the original, large Australian family described by Scheffer and coauthors (Scheffer et al., 1994) where all affected family members studied carried a S280F amino acid exchange (Steinlein et al., 1995).

Subsequently, two homologous genes have been implicated in ADSHE: *CHRN2* (Cholinergic Receptor Nicotinic Beta 2 Subunit, MIM *118507), and *CHRNA2* (Cholinergic Receptor Nicotinic Alpha 2 Subunit, MIM *118502) encoding the $\beta 2$ and $\alpha 2$ subunit of the nAChR, respectively (De Fusco et al., 2000; Aridon et al., 2006).

Neuronal nAChRs are widely distributed in the brain, mainly localized at the presynaptic level where they modulate the release of different neurotransmitters (including gamma-amino butyric acid, glutamate and dopamine) with variable effects on excitatory/inhibitory pathways. They have a pentameric structure, the most common configuration being ($\alpha 4$)₂($\beta 2$)₃ subunits. Each subunit is composed of the extracellular portion, the cytoplasmic part and four transmembrane regions (TM1-TM4). The second transmembrane domain of the receptor (TM2) shapes the ion channel pore and is the site of most of the pathogenic variants implicated in ADSHE (Kurahashi and Hirose, 2015).

Functional studies of different pathogenic variants provide conflicting results (Weiland et al., 1996; Kuryatov et al., 1997; Steinlein et al., 1997; Bertrand et al., 1998; De Fusco et al., 2000; Phillips et al., 2001; Bertrand et al., 2002): although an increase in acetylcholine (ACh) sensitivity in vitro is typical for most known ADSHE-associated pathogenic variants (De Fusco et al., 2000; Phillips et al., 2001; Bertrand et al., 2002; Leniger et al., 2003; Bertrand 2005; Hoda et al., 2008; Aridon et al., 2006), other variants, including the more recent *CHRNA2* mutation have been associated with a loss of function (Conti et al., 2015). Thus, the mechanism whereby the pathogenic variants cause ADSHE is poorly understood. Knock-in mice carrying two *CHRNA4* mutations (S252F and +L264) presented abnormal electroencephalography patterns but only some of them presented spontaneous seizures. The seizures could be blocked by picrotoxin, an open channel blocker of the GABA_A receptors, and it was therefore suggested that hypersynchronization was mediated by GABAergic neurons (Klaassen et al., 2006). In agreement with this hypothesis, it had been documented that in the hippocampus, nAChRs can modulate the release of the neurotransmitter GABA and could interact with interneurons (Klaassen et al., 2006). Since neuronal nAChRs regulate sleep and arousal oscillations at both the cortical and subcortical level (Lena et al., 2004), altered channels lead to unbalanced excitation/inhibition circuitry within the GABAergic reticular thalamic neurons, thus favoring seizures through the synchronizing effect of spontaneous thalamo-cortical oscillations. Introduction of the $\alpha 4$ mutation S252L in a transgenic rat model (referred as S248L) yielded, however, different conclusions: electrophysiological recordings carried out in brain slices revealed two major abnormalities with a reduced GABAergic synaptic and extrasynaptic transmission and abnormal glutamate release during slow-wave-sleep (Zhu et al., 2008).

By now, 14 different mutations in *CHRNA4*, *CHRNA2* and *CHRNA2* have been reported in 20 ADSHE pedigrees (Steinlein et al., 2012a; Nobili et al., 2014; Conti et al., 2015) and three sporadic cases (Nobili et al., 2014; Wang et al., 2014). Table 1 shows all the mutations reported so far. Overall, they are found in less than 20% of SHE/ADSHE, reflecting the genetic heterogeneity of the syndrome and the possible role of systems other than the cholinergic one, involved in its pathogenesis (Steinlein et al., 2012a).

I.2.2. *CRH*

CRH (corticotropin-releasing hormone, MIM *122560) was the first gene not belonging to the nAChR subunits family that has been implicated in SHE, even if molecular findings are still not fully convincing. Originally, two different variants were identified in the promoter of *CRH*: the g.1470G>A polymorphism recurred in three ADSHE pedigrees and two patients without family history and it was shown to increase CRH levels; the g.1166G>C mutation was found only in the proband of a non-compliant family (Combi et al., 2005) and later recognized as non-causative (Combi et al., 2008). In an additional family the g.1470 G>A was shared by the two affected siblings, inherited by the father who was homozygous for the change (Combi et al., 2008) although healthy. One of the affected patients was compound heterozygous for both the variants. Finally, in 2013, a novel heterozygous exonic missense mutation was detected in an additional ADSHE family; in vitro assay in this case showed decreased CRH concentrations (Sansoni et al., 2013). *CRH* encodes for a neurotransmitter/neuromodulator widely distributed throughout the central nervous system that acts in extrahypothalamic circuits to integrate a multisystem response to stress that controls numerous behaviors such as sleep and arousal (Combi et al., 2005). The authors suggested that altered (decreased/increased) CHR levels cause increased susceptibility of seizures through excessive sleep fragmentation and brain hyperexcitability (Combi et al., 2005).

I.2.3. *KCNT1*

Further insight into the biology of SHE come only starting from 2012, when combining genome-wide linkage analysis with novel Next Generation Sequencing (NGS) techniques, Heron and coauthors identified a novel gene for SHE, *KCNT1* (Potassium Sodium-Activated Channel Subfamily T Member 1, MIM *608167), encoding a subunit of the sodium-activated potassium channel (Heron et al., 2012). *KCNT1* is expressed in the neurons of the frontal cortex (Bhattacharjee et al., 2002) and assemble with *KCNT2* to form heterotetrameric channel complexes composed of a small amino-terminal domain, a transmembrane domain containing six TM segments and a large intracellular carboxy-terminal domain containing tandem regulators of K⁺ conductance (RCK) domains and an NAD⁺-binding domain. Its activity contributes to the slow hyperpolarization that follows repetitive firing, regulates the rate of bursting and enhances the accuracy with which action potentials lock incoming stimuli (Bhattacharjee and Kaczmarek, 2005; Brown et al., 2008).

Mutations in *KCNT1* were detected in three ADSHE families with 100% of penetrance and a sporadic case, all with early-onset refractory seizures, possible ID and psychiatric or behavioral problems including psychosis, catatonia and aggression (Heron et al., 2012).

Simultaneously, de novo gain-of-function mutations in this gene were identified in six out of 12 unrelated individuals with Malignant Migrating Focal Seizures of Infancy (MMFSI) (Barcia et al., 2012), a rare early onset epileptic encephalopathy characterized by refractory, polymorphous focal seizures and arrest of psychomotor development within the first six months of life (Coppola et al., 1995). All the mutations initially described in both ADSHE and MMFSI were clustered around the RCK and NAD⁺-binding domains of the large C-terminal cytoplasmic region, which also interacts with a protein network, including fragile X mental retardation protein (FMRP). Functional study documented that *KCNT1* mutations cause a constitutive hyperactivation of the channel that impairs its gating and suppress its subconductance states with effect on ion currents; moreover, they may also alter the conformation of the C-terminal region and its ability to interact with developmentally relevant proteins (Barcia et al., 2012). Differences in the increase in current amplitude caused by the different mutations seemed to explain the different phenotypes associated with *KCNT1* mutations (Milligan et al., 2014).

In the last few years, *KCNT1* has been implicated in wide spectrum of focal/multifocal epilepsy and early onset epileptic encephalopathies, in addition to the “classical” ADSHE and MMFSI phenotypes (Shimada et al., 2014; Møller et al., 2015; Ohba et al., 2015; Rizzo et al., 2016).

Some of the variants recurred in several patients, suggesting the presence of mutational “hot spots” in *KCNT1* (Møller et al., 2015). Specific mutations (p.G288S and p.R398Q) can lead to either ADSHE or MMFSI, even within the same family, indicating that genotype–phenotype correlations are not straightforward (Kim et al., 2014; Møller et al., 2015).

1.2.4. *DEPDC5*

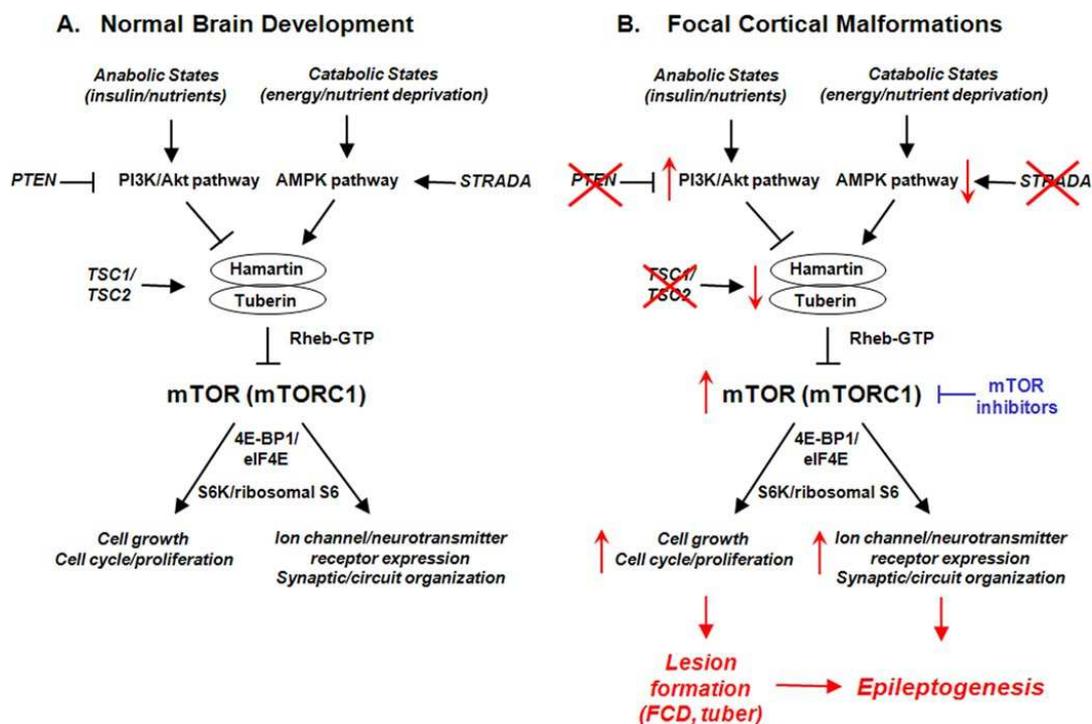
In 2013, mutations in *DEPDC5* (DEP Domain Containing 5, MIM *614191) were implicated in familial focal epilepsy with variable foci (FFEVF) (Dibbens et al., 2013), as well as and in a variable percentage (12.5%-37%) of heterogenous familial FEs, including ADSHE (Ishida et al., 2013; Picard et al., 2014). In particular, *DEPDC5* loss-of-function mutations were found in the 13% of a series of 30 families with ADSHE presentation (Picard et al., 2014). Electro-Clinical assessment reveal features comparable to those families from the literature (onset in childhood, clusters of brief hypermotor seizures with rare secondarily generalization, breathless feeling experienced by some of the patients, few abnormalities on interictal and ictal EEG, co-occurrence of intellectual disability (ID) and/or psychiatric features), except for a higher rate of drug resistance and of daytime seizures compared to classical phenotype (Picard et al., 2014). However, given the limited number of the affected members, it could be argued whether these pedigrees represent in fact small FFEVF families (Picard et al., 2014).

DEPDC5 encodes a 1603 amino acids protein which bears a DUF domain of unknown function and a DEP domain that participate in G-protein signaling (Chen and Hamm, 2006). *DEPDC5*, along with *NPRL2* (NPR2-Like, GATOR1 Complex Subunit, MIM *607072) and *NPRL3* (NPR3-Like, GATOR1 Complex Subunit, MIM *600928), is a component of the GATOR1 complex (Gap Activity Toward Rags 1), a negative regulator of the mammalian target of rapamycin (mTOR) complex1 (mTORC1) (Bar-Peled et al., 2013). Most of the *DEPCD5* variants described, including those described in SHE

patients, are loss of function mutations (LOF: nonsense, splice site and frameshift indels), with impact on the protein product and consequent hyperactivation of mTORC1 pathway (van Kranenburg et al., 2015). The hypothesized pathogenic mechanism in these cases is a reduction of the GAP activity of GATOR1 complex towards its targets RAG GTPases proteins, causing a loss of inhibition of the RAG complex, which in turn leads to the recruitment of mTORC1 complex at the lysosomal membrane and its activation (Bar-Peled et al., 2013; van Kranenburg et al., 2015).

In neurons, mTOR covers a wide range of cellular function ranking from the regulation of the neuronal soma size to axon pathfinding and regeneration, dendrite arborization, dendritic spine morphology and synaptic (Tang et al., 2002; Swiech et al., 2008; Takei and Nawa, 2014; Lasarge and Danzer, 2014). Dysregulation of mTOR pathway causes a number of pathological conditions (grouped under the term “mTORopathies”), including a range of malformation of cortical development (MCD) and neurodevelopmental diseases associated with severe mental retardation and epilepsy such as tuberous sclerosis (Curatolo and Moavero, 2013; Lipton and Sahin, 2014), as shown in Figure 1.

Fig. 1 Role of the mTOR pathway in pathogenesis and epileptogenesis of cortical malformations



From Wang M, *Exp Neurol* 2013, 244:22-6.

A. Physiological functions regulated by the mTOR pathway (primarily by mTORC1) during normal brain development, via activation of protein synthesis mechanisms (i.e. S6K/ribosomal S6 protein, eukaryotic initiation factor eIF4E pathways). In turn, mTORC1 is regulated by upstream signaling pathways (i.e. PI3K/Akt or AMPK pathway) in response to different physiological conditions and stimuli.

B. Hyperactivation of the mTOR pathway due to mutations in upstream regulators (e.g. *TSC1* or *TSC2* in TSC; *STRADA* gene in PMSE syndrome) led to focal MCD and tubers by abnormally increased cell growth and proliferation. The gross structural lesions themselves, as well as non-structural molecular and cellular changes in ion channel expression and synaptic organization may promote epileptogenesis in these disorders. mTOR inhibitors may represent a rational therapy for FCD and TSC by reversing mTORC1 hyperactivation.

In line with this evidence, so far a wide number of germline and somatic (brain-only) mutations of *DEPDC5* leading to mTORC1 hyperactivation have been associated with a range of lesional and non-lesional FEs. The MCD detected in some family members include different types of FCDs, subtle band heterotopias and hemimegalencephaly, with the predominant pattern being FCD type IIb (Scheffer et al., 2014; Lal et al., 2014; Martin et al., 2014; Scerri et al., 2015; D'Gama et al., 2015; Baulac et al., 2015).

Additional insights into the role of *DEDCD5* in FCD-related FE derive from the rat model recently published (Marsan et al., 2016). The heterozygous rats exhibited an altered cortical neuron excitability and firing patterns and cortical cytomegalic dysmorphic neurons and balloon-like cells strongly expressing phosphorylated rpS6, indicative of mTORC1 upregulation. These neuropathological abnormalities are reminiscent of the hallmark brain pathology of human FCD.

I.2.5. *PRIMA1*

In a two-generation Australian family of Italian origin affected with SHE and ID, Hildebrand and coauthors identified by WES analysis a homozygous mutation in *PRIMA1* (Hildebrand et al., 2015). This gene encodes a transmembrane protein that anchors acetylcholinesterase (AChE), the enzyme hydrolyzing Ach to membrane rafts of neurons. The c.93+2T>C mutation identified leads to knockout of *PRIMA1*, with reduction of AChE and accumulation of acetylcholine at the synapse, as shown in *PRIMA1* knockout mice. The authors concluded that, similarly to the gain of function mutations of the genes coding for nAChR subunits, the enhanced cholinergic responses are the likely cause the severe SHE and ID in this family. However, apart from this single pedigree, this findings has not been replicated yet, as no other mutations were identified in a confirmation cohort of hundreds of SHE probands (Hildebrand et al., 2015).

In summary, ADSHE is a heterogeneous genetic syndrome. Overall, mutations in the genes identified so far cumulatively explain 20% of families and fewer than 5% of sporadic cases (Kurahashi and Hirose, 2015), clearly pointing to additional genetic factors. The penetrance of the mutations identified is incomplete, except for mutations in *KCNT1*.

By now, the small number of SHE families suitable for traditional approaches (i.e. linkage studies) prevented the fast discovery of novel genes. However, in the last few years, the advent of Next Generation Sequencing (NGS) technologies, such as Whole Exome Sequencing (WES) has completely revolutionized gene hunting, allowing the application of this technology and the discovery of novel epilepsy genes in nuclear pedigrees or even in sporadic patients.

The high intrafamilial variability and the overlapping features of the clinical manifestations, together with the rarity of ADSHE and the differences in study designs have hampered the definition of a genotype–phenotype correlation.

I.3. NEUROPSYCHOLOGICAL FEATURES OF SHE

Intellect is usually preserved in SHE patients, as briefly alluded by some large series of both SHE and ADSHE cases (Ryvlin et al., 2006a). However, no systematic studies have evaluated the neuropsychological profile of representative cohorts of SHE patients yet. Data available are scant and contradictory and derive mainly from selected populations with ADSHE (Ryvlin et al., 2006a).

ADSHE was originally proposed as paradigm of a benign FE, occurring in patients with normal intelligence (Scheffer et al., 1995), as emphasized by the majority of reports (Nakken et al., 1999; Saenz et al., 1999). However, only few years later, several studies reported cognitive deficit, behavioral and psychiatric problems in some members of ADSHE pedigrees without specific molecular diagnosis (Khatami et al., 1998; Picard et al., 2000) or turned negative for mutations in nAChRs genes (Derry et al., 2008).

In some families found mutated in *CHRNA4*, in-depth and long-term clinical/psychiatric assessment revealed psychiatric comorbidity and ID. Firstly, in the Japanese pedigree with the S252L mutation Hirose and Ito described the occurrence of atypical features, namely mild ID and hyperactivity in two members of the third generation (Hirose et al., 1999; Ito et al., 2000). Moreover, long-term follow-up of the two siblings later revealed autistic disorder with profound ID (Miyajima et al., 2012). In 2003, Magnusson and colleagues described schizophrenia, recurrent psychosis and unspecified psychiatric disturbances in six out of the 11 members of the family carrying the 776ins3 mutation (Steinlein et al., 1997; Magnusson et al., 2003), previously reported as of normal intelligence (Nakken et al., 1999).

Following these reports, a number of studies described cognitive disabilities in ADSHE families carrying different mutations of the known genes. Specific mutations of *CHRNA4* (S248F, S252L, 776ins3), *CHRNA2* (I297F) and *CHRNA4/CHRNA2* (V287M, I312M) have been associated with ID, psychological or behavioral problems, including impulsivity, aggression, and hyperactivity, as shown in Table 1 (Steinlein et al., 2012a; Conti et al., 2015). The important effort of providing a genotype-phenotype correlation, revealed that the same molecular change could be associated with extremely variable endophenotypes, from severe ID to normal cognitive performance. This highlighted the need to extend in-depth neuropsychological assessments also in patients without apparent deficits.

A couple of small case-series systematically assessed the frequency and degree of neurocognitive disorders in *CHRNA4/CHRNA2*-mutated patients using a comprehensive battery of neuropsychological tests. Picard and coauthors studied 11 patients from four unrelated ADSHE pedigrees carrying different mutations of *CHRNA4* (S248F, S252L, T265I) and *CHRNA2* (V287L) (Steinlein et al., 2000; De Fusco et al., 2000; Rozycka et al., 2003; Leniger et al., 2003) including three families where neuropsychological problems were not previously reported (De Fusco et al., 2000; Rozycka et al., 2003; Leniger et al., 2003). All patients, regardless of the type of mutations or overt seizures had some degree of cognitive dysfunction: (i) borderline IQ/ID in the 45% of them, (ii) decreased performances in inhibitory task and/or verbal fluency in all them and (iii) deficit in verbal/non-verbal memory in 91% (Picard et al., 2009). These findings proved that neuropsychological disorders in ADSHE were underestimated.

Tab. 1 Neuropsychological findings in families and sporadic cases with mutations in *CHRNA4*, *CHRNA2* and *CHRNA2*

GENE	MUTATIONS		N	FAMILIES REPORTED		
	AA CHANGE/DOMAIN	FUNCTIONAL		ORIGIN	NEUROPSYCHOLOGY	REFERENCES
<i>CHRNA4</i>	S248F / TM2	Gain ↑ sensitivity to Ach (Bertrand 2002) Loss faster desensitization, slower recovery (Weiland 1996; Kuryatov 1997) ↓ affinity to Ach, low currents (Bertrand 1998)	4	<u>British-AU#</u>	-	Steinlein 1995
				Spanish	-	Saenz 1999
				<u>Norwegian*</u>	ID, beh, psy	Steinlein 2000
				Scottish	Psych	McLellan 2003
	S252L / TM2	Gain ↑ R sensitivity to Ach (Bertrand 2002)	4	Japanese	ID, ASD	Hirose 1999; Ito 2000; Miyajima 2012
				Lebanese	Low average intellect	Phillips 2000
				Korean	ID, beh (hyperactivity)	Cho 2003
			1	<u>Polish*</u>	-	Rozycka 2003
			Italian (S)	-	Sansoni 2012	
776ins3 / TM2	Gain ↑ R sensitivity to Ach (Steinlein 1997; Bertrand 1998, Bertrand 2002)	1	Norwegian	Psy: Schizophrenia, recurrent psychosis	Steinlein 1997, Nakken 1999, Magnusson 2003	
T265I/ TM2	Gain ↑ R sensitivity to Ach	1	<u>German*</u>	-	Leniger 2003	
R336H/ Intracel loop 2	NA	1	Chinese	-	Chen 2008	
c.823A>T	NA	1	Chinese (S)	-	Wang, 2014	
<i>CHRNA2</i>	V287L / TM2	Gain ↑ ACh-evoked current, retarded desens,	1	<u>Italian*</u>	-	De Fusco 2000, Gambardella 2000
	V287M / TM2	Gain ↑ sensitivity to Ach (Phillips 2001, Bertrand 2002)	2	Scottish	Psych	Phillips 2001, McLellan 2003
				Spanish	-	Diaz-Otero 2008
	L301V/ TM3	Gain ↑ R sensitivity to Ach	1	TurkishCypriot		Hoda, 2008
	V308A/ TM3	Gain ↑ R sensitivity to Ach	2	Scottish		Hoda, 2008
				English		
	I312M / TM3	Gain ↑ R sensitivity to Ach (Bertrand 2005)	2	English	ID, memory	Bertrand 2005
			Korean	Memory (v and nv)	Cho 2008	
V337G/ TM3-intrac loop	NA	1	Chinese (S)	-	Liu 2011	
<i>CHRNA2</i>	I279N /TM1	Gain ↑ R sensitivity to Ach	1	Italian	-	Aridon 2006
	I297F /TM2	Loss ↓ current density	1	Italian	ASD Attention deficit hyperactivity disorder	Conti 2015

Abbreviations AA aminoacid; ID: Intellectual Dysability; Psy: psychiatric disorders; Psych: psychological disturbances; Beh: behavioral problems; Memory: memory deficit; Memory v: verbal memory; Memory nv: non-verbal (visual) memory; ASD: autism spectrum disorder; S: sporadic case.
Grey lines reported cases with neuropsychological/psychiatric disturbances. Families studied by extensive neuropsychological assessment are underlined.

The impairment of executive functions as well as learning and memory, suggested a fronto-temporal dysfunction, differing from previous studies on frontal lobe epilepsies where memory seemed preserved (Picard et al., 2009). The authors hypothesized that neuropsychological deficits in ADSHE may be a consequence of seizures/interictal EEG abnormalities (despite the rarity of scalp anomalies and the focal seizures that remain localized to the frontal lobe) or fragmentation of NREM sleep, whose role in memory consolidation is well known (Tucker et al., 2006). However, they pointed to a contribution of nAChR subunits genes mutations, given the role of these receptors and nicotine in sustained and selective attention, automatic response inhibition and working memory (Poltavski and Petros, 2006; Meinke et al., 2006; Bacciottini et al., 2000; Levin et al., 2002).

The second case series evaluated a well-defined group of nine ADSHE members from the original Australian pedigree with the S248F mutation of *CHRNA4* and normal intelligence. The mutation group was compared to an age-, gender-, and education-matched control group. ADSHE patients showed significantly worse scores in task requiring flexible adaptation and verbal fluency (stroop test, trail making test B, controlled oral word association) while intellectual abilities were preserved. Deficits in verbal memory correlated with disease-related factors, or medications (Wood et al., 2010).

Identification of additional mutations and novel genes for ADSHE enriched genotype-phenotype correlations and at present, neuropsychological and psychiatric comorbidities have been definitively related also to mutations in *KCNT1* (Heron et al., 2012; Derry et al., 2008) and *DEPDC5* (Picard et al., 2014; Picard et al., 2000).

Conversely, the majority of sporadic cases affected by SHE do not seem to present with gross cognitive disturbance, even though many of these patients complain of chronically disrupted sleep and daytime sleepiness (Ryvlin et al., 2006a). No more specific data on this population are available, although it represents the largest part of SHE patients. Several neuropsychological studies evaluated the impact of frontal lobe epilepsy on cognition and behavior, but they may include other epilepsy syndromes, with different types of frontal seizures occurring in wakefulness (Helmstaedter et al., 1996; Upton and Thompson 1997 (a,b); Exner et al., 2002; McDonald et al., 2005; Riva et al., 2005; Risse, 2006; Centeno et al., 2010; Braakman et al., 2011; O'Muirheartaigh and Richardson, 2012; Rayner et al., 2015; Patrikelis et al., 2016). However, these studies cannot be representative of SHE, where typical ictal manifestations are mostly exclusively sleep-related and may originate from extra-frontal areas with secondary involvement of frontal structures.

II.FAMILIAL FOCAL EPILEPSY WITH VARIABLE FOCI (FFEVF)

FFEVF is an unusual epilepsy syndrome characterized by focal seizures originating from different cortical regions in different affected family members and multifocal EEG abnormalities.

FFEVF was originally described in a large Australian family (Scheffer et al., 1998); seven further pedigrees were later reported: three were French-Canadian (Xiong et al., 1999; Berkovic et al., 2004), two from Spain (Berkovic et al., 2004; Morales Corraliz et al., 2010), two Dutch (Callenbach et al., 2003; Klein et al., 2012). All these families showed an autosomal dominant inheritance with penetrance between 50% and 80% and

marked intrafamilial variation in severity (Xiong et al., 1999; Callenbach et al., 2003; Berkovic et al., 2004; Morales Corraliz et al., 2010; Klein et al., 2012). Structural MRI studies were reported as unremarkable. Patients usually have normal intellect but ID, psychiatric disorders or autism spectrum disorder have been reported (Xiong et al., 1999; Callenbach et al., 2003; Klein et al., 2012).

Affected family members can present with frontal, temporal (mesial and lateral) occipital, parietal or multifocal seizures starting by the third decade (Klein et al., 2012). Although the heterogeneous seizure pattern within the members of the same pedigree, seizure semiology is constant in individual subjects (Berkovic et al., 2004). In some of the original FFEVF pedigrees, nocturnal, sleep-related seizures with a frontal lobe semiology are the most common phenotypes, leading to consideration of the diagnosis of ADSHE (Berkovic et al., 2004). The clinical overlap between FFEVF and ADSHE may lead to misdiagnosis, in particular in smaller pedigrees where the wide intrafamilial phenotypic variability of FFEVF might not be appreciated.

Linkage studies confirmed chromosome 22q12 as the solitary locus for all the eight FFEVF pedigrees originally reported (Klein et al., 2012).

In 2013 Dibbens and colleagues performed WES analysis in one Australian (A1) and one Dutch (D1) FFEVF family (Callenbach et al., 2003, Klein et al., 2012), identifying in each family a novel heterozygous nonsense mutation in the *DEPDC5* gene, including c.21C>G (p.Tyr7*) in family A1 and c.1663C>T (p.Arg555*) in family D1. Mutations in the same gene were subsequently identified in additional five out of the six FFEVF families previously linked to chromosome 22q (S1, S2, F1-3), confirming *DEPDC5* as the major gene for FFEVF (Dibbens et al., 2013). Of the eight large families with FFEVF, only one (family A in Klein et al., 2012) did not have a *DEPDC5* mutation. The three French-Canadian families had the same deletion mutation (c.488_490delTGT; p.F164del), suggesting a shared ancestor.

This disorder is difficult to recognize in small families due to the low number of affected individuals and because the pedigrees are too small to demonstrate a clear autosomal dominant inheritance. The sequencing of the gene by high-resolution melt curve analysis (HRM) in other 82 unrelated probands of families with at least two affected members, identified *DEPDC5* mutations in ten (12.2%), confirming a major role of this gene in familial FE (Dibbens et al., 2013). Later, the evidence that some affected members had structural cortical alterations (i.e. bottom of the sulcus dysplasia, a form of FCD type II B), implying mutations in this gene in MCD (Scheffer et al., 2014).

AIMS

We conducted an accurate clinical, neuropsychological and genetic characterization of a large cohort of patients with SHE, negative for mutations in the genes coding for the nAChRs, in order to:

- (i) identify new genetic determinants for ADSHE/FFEVF by WES analysis;
- (ii) estimate the frequency of mutations in *KCNT1* and *DEPDC5*, recently implicated in ADSHE;
- (iii) assess the impact of SHE on neuropsychological functioning, characterizing a possible profile of impairment;
- (iv) correlate genetic findings with clinical and neuropsychological data.

The application of innovative tools for gene discovery (Whole Exome Sequencing – WES) allowed us to include in the genetic analysis small pedigree and sporadic patients studied by a trio approach.

METHODS

I. PATIENTS RECRUITMENT

I.1. SETTING AND PERIOD

The study was carried out over 2012-2016 at the IRCCS, Institute of Neurological Sciences of Bologna and the Medical Genetics of Sant'Orsola Hospital, following the approval by the Human Research Ethics Committee of Bellaria Hospital, Bologna (Prot. N 945/CE; cod CE: 13084).

The study was been supported by a no-profit association (Telethon foundation, GGP13200).

I.2. POPULATION AND INCLUSION CRITERIA

We included individuals of any age and gender, diagnosed with video/video-EEG documented SHE according to reliable diagnostic criteria, with/without a positive family history for FE.

The study population derives mainly from a larger cohort of SHE patients that, referred to the Epilepsy and Sleep centers of our Institute since 1980 for sleep-related motor events, were finally diagnosed with SHE.

All patients still followed up in our clinic and consecutively attending the Epilepsy center for a control visit between September 2012 and April 2016 were asked to participate in the study. Consenting patients negative for mutations in the AChRs genes were prospectively enrolled after obtaining specific written consent.

We included also newly-diagnosed cases referred and diagnosed in our Institute since 2012. Additional cases were referred by other Italian epilepsy Centers thanks to the collaboration with the Italian League against Epilepsy (Lega Italiana Contro l'Epilessia, LICE), patron of 47 Epilepsy Centers located in 15 Italian regions. We selected patients fulfilling the following inclusion criteria:

- (i) personal history of sleep-related paroxysmal motor events suggestive of hypermotor seizures;
- (ii) video-polysomnographic (VPSG) recording of at least one major event (asymmetric tonic seizures/hyperkinetic seizures/epileptic nocturnal wandering) or of two stereotyped minor events (paroxysmal arousals).

For all cases the diagnosis of SHE was confirmed by three experts in epileptology and sleep disorders (P. Tinuper, F. Bisulli and F. Provini) and conformed to the new diagnostic criteria, based on level of certainty (Tinuper et al., 2016).

We enrolled both sporadic and familial cases; the latter were defined as patients having at least one relative within three degrees of relatedness affected with SHE or other FE, including both ADSHE and possible FFEVF families. Biological samples (blood or saliva samples) were collected for DNA extraction and analysis from each index case and all the affected and unaffected consenting relatives available.

II. CLINICAL STUDY

All probands underwent a comprehensive evaluation including videopolygraphic monitoring for recording of at least one hypermotor sleep-related seizure. For each patient we reviewed the clinical history and the

neurophysiological and neuroradiological documentation. All data were collected in an *ad hoc* database, collecting:

- demographic data (age, gender, educational level);
- family history of intellectual deficit and psychiatric disorders;
- age at seizures onset, seizures semiology, presence of seizures on wakefulness, specific auras, bilateral convulsive seizures and status epilepticus;
- seizures frequency at the onset and during the last year; past and current antiepileptic treatment, compliance and response to therapy;
- interictal/ictal EEG abnormalities (specifying the distribution);
- presence of brain MRI abnormalities.

Clinical and neurophysiological assessment was extended to all the available affected relatives, to define the individual clinical phenotype and then, the familial epilepsy syndrome (ADSHE/FFEVF).

All patients were studied by a detailed and direct clinical interview, routine EEG (if necessary, video-EEG recording) and targeted brain 3-T MRI acquisitions following a specific “epilepsy protocol”.

Following the pedigree reconstruction, we drew each pedigree.

III. GENETIC STUDY

III.1. PRE-SCREENING OF KNOWN GENES

Since 1995, available patients from the historical cohort of SHE patients followed up in our Institute had been screened for mutations in *CHRNA4*, *CHRNA2* and *CHRNA2*. This analysis, conducted over the years at the Genetic Unit of the Meyer Hospital in Florence was in part performed by denaturing High Performance Liquid Chromatography (dHPLC) assay.

Additionally, in 2012 a subpopulation of patients underwent a preliminary screening for mutations in *KCNT1* and *DEPDC5* by high resolution melt curve analysis (HRM) at the Epilepsy Research Centre, University of Melbourne.

III.2 WES ANALYSIS

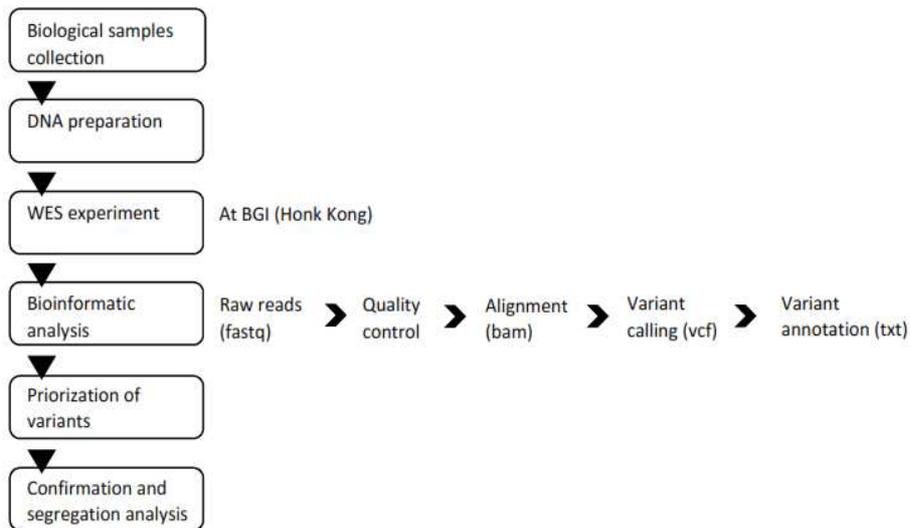
Genetic study was conducted by Dr. T. Pippucci and S. Baldassari, at the Genetic Unit, Policlinico Sant'Orsola Malpighi, University of Bologna.

WES analysis was performed in

- (i) familial cases, including at least one affected relative (when available);
- (ii) sporadic cases, in part analyzed by a trio approach (affected proband and healthy parents) in order to discover possible *de novo* mutations (DNMs).

Figure 2 summarize the experimental and analysis workflow.

Fig. 2 WES experiment workflow



III.2.1. DNA SAMPLE COLLECTION

DNA samples for WES analysis were extracted from peripheral blood collected specimens using the commercial kit QIAamp DNA Blood Mini Kit (QIAGEN), which is based on the usage of columns containing membranes that enhance a selective DNA adsorption and a final purified DNA elution.

III.2.2. WES EXPERIMENT

WES experiments were performed at Beijing Genomics Institute (BGI Tech Solutions, Hong Kong, www.bgi.com). Two different enrichment methods were used: NimbleGen SeqCap EZ (Roche), a BGI exome capture (BGI). Both kits cover more than 20,000 genes in the human genome and are based on oligonucleotide DNA probes that capture the target exome. The generated libraries have been sequenced as 91 bp (base pair) paired-end reads on the Illumina HiSeq2000 platform (Illumina) at BGI, requiring a mean coverage per sample of 50X for the exomes enriched with NimbleGen SeqCap EZ and 100X for the exomes enriched with BGI exome capture.

III.2.3. BIOINFORMATIC ANALYSIS

Bioinformatic analysis workflow is shown in Fig. 2. FASTQ format sequencing reads were received from BGI and processed for quality control following a pipeline developed at the Medical Genetics Unit of the Policlinic Sant'Orsola-Malpighi in Bologna. The reads were aligned against the hg19 reference genome with BWA. Single nucleotide variants (SNVs) and small indels were called using GATK Unified Genotyper, and GATK VariantFiltrationWalker was used to filter out variants by quality, according to specific parameters. Multi-sample variant calling for SNVs and indels has also been performed, to improve variant calling, using the GATK package utility HaplotypeCaller (www.broadinstitute.org/gatk/): variants flagged as “PASS”, meaning that they are reliable calls, were considered in the downstream steps of the analysis workflow. These were then annotated against the NCBI RefSeq (www.ncbi.nlm.nih.gov/RefSeq) and UCSC KnownGene (www.genome.ucsc.edu) databases with ANNOVAR (Wang et al., 2010), or against the Ensembl database (www.ensembl.org) using Variant Effect Predictor (www.ensembl.org/info/docs/tools/vep/index.html) and

organized in a structured query language-based database by Gemini (www.gemini.readthedocs.org/en/latest/) (Paila et al., 2013).

Once annotated, the variants were filtered. We retained all rare variants affecting coding sequences of the targeted exome by filtering out all SNVs and InDels observed in dbSNP137(www.ncbi.nlm.nih.gov/SNP/), 1000genomes (www.broser.1000genomes.org/), Exome Variant Server(www.evs.gs.washington.edu/EVS/) and CNVs overlapping with those observed in Database of Genomic Variants(projects.tcag.ca/variation).

The whole filtering procedure retained only rare coding variants with functional effect on the protein coding sequence and CNVs.

III.2.4. VARIANTS PRIORITIZATION

The variants prioritization was performed considering the following criteria: presence or absence of the variant in public databases, pathogenicity prediction by in-silico tools, conservation of the mutated site in other vertebrates, expression and function of the mutated gene in the brain, association of the mutated gene with other neurological diseases. The analysis was divided into 4 steps: the first including variants with highest pathogenicity scores, the last including variants with the lowest pathogenicity scores.

- (iii) LOF mutations, including nonsense or splicing affecting single nucleotide variants (SNVs) and frameshift indels, not described in public databases.
- (iv) Missense mutations, not reported in public databases, predicted to be damaging by Polyphen2 (www.genetics.bwh.harvard.edu/pph2), SIFT (www.sift.jcvi.org) and CADD (>15, www.cadd.gs.washington.edu) scores and affecting conserved sites (GERP++ score >4, www.mendel.stanford.edu/SidowLab/downloads/gerp) and rare LOF mutations with a frequency lower than 1% in an internal exome database.
- (v) Missense mutations, not reported in public databases, and predicted to be damaging by at least one in-silico predictor among Polyphen2, SIFT and CADD, and affecting conserved sites (GERP++ score >2).
- (vi) Missense mutations, reported with a low frequency in public databases, and predicted to be damaging by at least one in-silico tool among Polyphen2, SIFT and CADD, with a GERP++ score between 0 and 2.

In each mutational class, the most candidate variants were selected according to the available information on the expression or function of the mutated gene: to this purpose, different databases have been queried, including Pubmed (www.ncbi.nlm.nih.gov/pubmed), Braineac (www.braineac.org), Omim (www.omim.org) and STRING (www.string-db.org). Pubmed and Omim were used to assess the function of the gene of interest and its possible associated diseases. Braineac is a database of brain expressed genes, distinguishing for brain regions, and was interrogated to evaluate the expression pattern of the gene of interest in the brain. Finally, the protein-protein interactions involving the protein encoded by a mutated gene were analyzed using the STRING database.

Sanger sequencing was subsequently applied to validate the prioritized variants in the proband and to determine the segregation in the family.

IV. NEUROPSYCHOLOGICAL STUDY

This was a cross-sectional study carried out over 2013 and 2016 at the Neurological Clinic Unit of IRCCS, Institute of Neurological Sciences of Bologna.

All the patients recruited underwent an assessment of intellectual functioning and cognitive status by:

- Wechsler Adult Intellectual Scale (WAIS) or Stanford-Binet Intelligence Scale (SB, for patients <16 years);
- Raven's progressive Matrices;
- Mini-Mental State Examination (MMSE).

ID was defined when IQ score was <70; MMSE was considered pathological when corrected scores were <23.8.

Patients aged >16 years with normal cognitive functioning (IQ scores >70; MMSEc scores >23.8) carried on with an extensive, standardized neuropsychological battery. These additional neuropsychological measures were selected in order to explore a range of frontal and extra-frontal functions, schematically sampled in the following cognitive domains:

- language: semantic and phonemic fluency;
- verbal and non-verbal memory: Rey Auditory Verbal Learning Test (RAVLT), forward verbal span, verbal supra span + 2, paired-associated words learning (for verbal memory); Rey-Osterrieth complex figure (ROCF) immediate recall; Visual-Spatial supra span + 2, corsi-Block-Test (for visual memory);
- visuospatial ability (ROCF copy);
- attention and executive functioning: Trail Making test A, Trail Making test B (for attention, shifting and flexibility); backwards Verbal Span (for working memory); Wisconsin Card Sorting Test (WCST) (executive function); Stroop test (executive function, response inhibition).

The final score was calculated after adjustment for age and education.

Neuropsychological testing was conducted by a single expert neuropsychologist (R. Poda) at the neuropsychological Service of our Institute.

All the tests were administered to each patient in a standardized order, over a single session held in the morning and lasting between 1 and 2 hours.

A paired clinical assessment, performed on the same day as neuropsychological testing, was focused on seizure frequency and antiepileptic drugs taken at that stage, in addition to the other variables collected in the clinical database. For patients hospitalized at the time of the study, a VPSG recording and questionnaire evaluating daytime somnolence in the days immediately close were also available.

All neuropsychological data were collected in an ad hoc database.

Statistical analysis was performed using statistical package Stata SE, version 14.0.

For descriptive statistics, continuous variables were presented as mean \pm standard deviation, while categorical variables as absolute and relative frequency (%). Performances of SHE patients were evaluated with respect to age adjusted normative data of healthy controls.

Fisher's exact test was used to highlight possible associations between each neuropsychological test with clinical features, comparing variables among groups. All p-values were based on 2-sided tests; $p < 0.05$ was considered significant.

To further investigate the impact of disease severity on cognitive functioning, we used the non-parametric Wilcoxon Rank-Sum test, comparing the distribution of the scoring for each neuropsychological test between groups categorized according clinical variables.

V. GENOTYPE-PHENOTYPE CORRELATION

All anatomo-electro-clinical data were correlated with genetic findings in order to highlight differences on epilepsy phenotype related to mutations in a specific gene. In particular, we paid particular attention on particular clinical features (such as age at onset, presence of MCD or other cerebral structural lesions, subjective symptoms preceding the seizure onset, presence of seizure during wakefulness, ID and psychiatric comorbidity) in order to disclose possible key elements to direct genetic diagnostic tests. Binomial Exact test was used to calculate 95% confidence intervals (95%CI).

Similarly, the results of the systematic neuropsychological study were correlated with genetic findings in order to disclose possible differences in mutated and not-mutated cases.

RESULTS

I. STUDY POPULATION

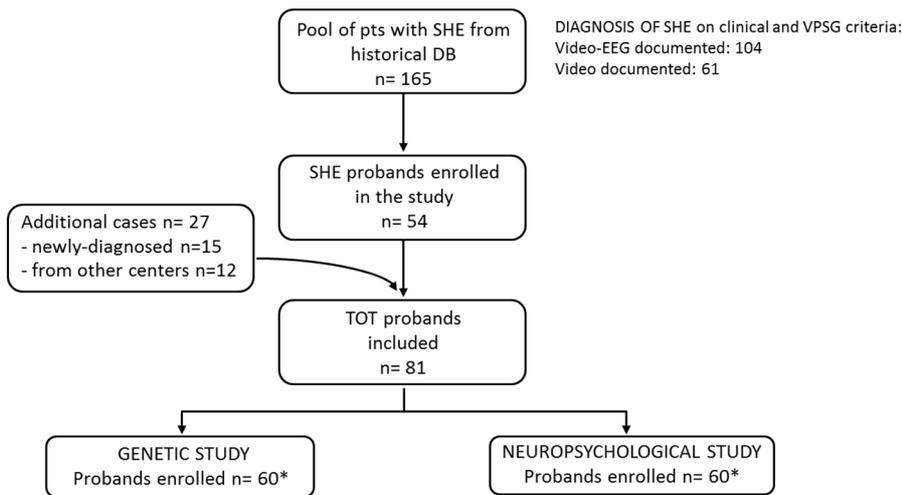
A total of 81 patients (M/F=45/36; mean age at enrollment available in 75 cases: 35.4 ± 14.6 , range: 2-69 years) were enrolled in the genetic and/or the neuropsychological study.

The flow-chart in Figure 3 provides details on patient recruitment. Briefly:

- 54 probands belonged to the historical cohort of 165 SHE patients diagnosed and followed up at our Institute since 1980;
- 15 were new-diagnosed cases, referred to our Institute since 2012;
- 12 patients were referred from other Italian Epilepsy Centers, by means the LICE.

Thirty-nine probands underwent both WES analysis and neuropsychological assessment, while 21 had only genetic study (in total, 60 probands) and another 21 had only neuropsychological assessment (in total, 60 patients).

Fig. 3 General recruitment flow-chart



*Consider overlapping of 39 patients who underwent both genetic and neuropsychological study.

II. GENETIC STUDY

II.1. PRE-SCREENING FOR MUTATIONS IN KNOWN GENES

Overall, 76 probands from the original cohort underwent preliminary genetic analysis. Details are reported in Table 2.

Seventy patients were analyzed for mutations in at least one of the genes coding for the nAChR subunits by dHPLC (27 cases) or by Sanger sequencing (43 cases): no mutations of these genes were detected.

A subsample of 43 probands underwent molecular analysis of *KCNT1* and/or *DEPDC5* performed at the Epilepsy Research center of Melbourne University. This study led to the identification of a mutation in *KCNT1*

and three in *DEPDC5* reported in the original publications on these genes (Heron et al., 2012; Dibbens et al., 2013; Scheffer et al., 2014; Ricos et al., 2016).

The pedigrees (indicated as P) and more detailed clinical data on these families (F) and sporadic cases (S) are provided as Supplementary materials, part A.

Tab. 2 Preliminary screening for mutations in SHE known genes

GENES	N# Pts	TYPE OF ASSAY			MUTATION	FAM/ SPO	REFERENCES
		Sanger	dHPLC	HRM			
<i>CHRNA4</i>	62	36	26	-	-		
<i>CHRNA2</i>	60	34	26	-	-		
<i>CHRNB2</i>	65	38	27	-	-		
<i>KCNT1</i>	43	-	-	43	p.Y796H	Fam	Heron 2012 (fam B); Phillips 1998
<i>DEPDC5</i>	37	-	-	37	c.279+1 G>A p.T329Lfs*7 p.R165Yfs*14	Fam Fam Spo	Dibbens 2013 (fam I), Scheffer 2014 (fam C) Ricos 2016 (fam 28) Ricos 2016

Abbreviations dHPLC: high performance liquid chromatography; HRM: high resolution melt curve analysis; fam: familial; spo: sporadic case.

KCNT1 - F1 The p.Y796H mutation of *KCNT1* (NM_020822.2) segregated in four affected members of an ADSHE pedigree (P1) with 100% of penetrance and a more severe phenotype: earlier age at onset (mean 5.5±2.1 years) compared with classical ADSHE, refractory seizures in two patients, recurrence of ID and psychiatric/behavioral problems (Heron et al., 2012, family B). The family was also reported in a previous publication (Phillips et al., 1998, as family C).

DEPDC5 - F2 Of the three mutations of *DEPDC5* (NM_001242896.1), the c.279+1 G>A insertion was first identified in the proband of a FE family (P2, IV.2) but not in her affected mother (III.2), suggesting gonadic mosaicism in this individual (Dibbens et al., 2013, Family I). A few months later an additional individual (V.1) developed frontal lobe seizures at 4 years of age. Interictal EEG showed frequent epileptiform abnormalities over the left frontal field (Supplementary Figure 1). He carried the familial c.27911G>A mutation and a left frontal subtle band heterotopia adjacent to the dysplastic cortex at brain MRI (Supplementary Figure 2). The other affected relatives had normal MRI. This was one of the first pieces of evidence implying mutations of *DEPDC5* in MCD (Scheffer et al., 2014, family C).

DEPDC5 - F3 The p.T329Lfs*7 *DEPDC5* frameshift mutation was identified in nine individuals of a four-generation FFEVF pedigree with five affected members available for genetic testing (penetrance 55%). SHE was the most common phenotype running in the family (P3, individuals III.6, IV.1, IV.2, IV.4). Affected members showed a wide range of disease severity: the proband (IV.2) had sleep-related seizures well controlled by low doses of carbamazepine, while several individuals (III.6, III.8, IV.1, IV.4) had seizures refractory to pharmacological treatment. Two of them (III.6, III.8) underwent pre-surgical work up but were excluded from surgery because of lack of a clear epileptogenic focus (III.6) or documentation of independent seizures arising from both left and right fronto-temporal regions (III.8). Brain MRI disclosed MCD in

individuals IV.1 (who showed FCD, Supplementary Figure 3) and IV.4 (who had a suspected gyral abnormality of the right middle frontal gyrus; not shown). Moreover, severe psychiatric and behavioral problems recurred in affected individuals II.1, III.8, IV.2, IV.4.

DEPDC5 - S1 An additional frameshift mutation p.R165Yfs*14 was identified in a sporadic case who underwent invasive pre-surgical assessment because of frequent seizures refractory to AEDs (P4, II.1).

Despite a bilateral limbic Stereo-EEG (SEEG) exploration (Supplementary Figure 4), no definitive epileptogenic focus was identified in this patient. The SEEG electrical pattern was suggestive of focal cortical dysplasia (Supplementary Figure 5) but tailored brain MRI (1.5 T) was unrevealing. The mutation was inherited by her healthy mother (I.2).

II.2 WES ANALYSIS

WES was performed in 60 probands with SHE (M/F:34/26) and 76 affected/not affected relatives, for a total of 136 individuals (Figure 4).

The proband sample includes:

- ten familial cases: five belonging to ADSHE pedigrees and five with a family history for other epilepsy, in some cases compatible with FFEVF;
- 50 sporadic cases, 31 of whom were studied as trios (the healthy parents were also sequenced to evaluate the potential impact of *de novo* mutations in the pathogenesis of SHE).

Thirty-nine probands were recruited from the historical pool, nine have been diagnosed and enrolled since the beginning of the study (2012) while 12 were referred by other colleagues for WES analysis.

Mean age at epilepsy onset (available on 53 probands) was 11.79±8.77 years (range: 0-42 years). Fourteen probands had a positive brain MRI: ten with MCD (FCD in eight, dysplastic hemimegalencephaly in one, abnormal cortical gyration in one), one patient had mesial temporal sclerosis, three with gross brain abnormalities.

WES analysis identified:

- a number of mutations in known genes and a novel gene responsible for SHE, *NPRL2*;
- 16 novel candidate genes, mainly identified as DNMs by trio analysis;
- several variants of unknown significance (VUS), eight in genes already implicated in SHE.

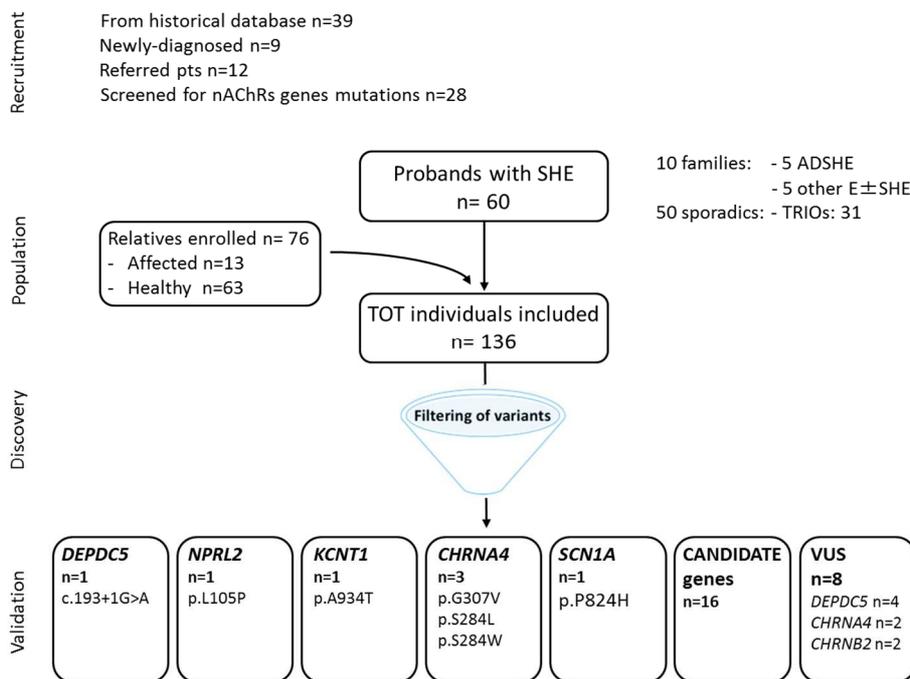
Supplementary Table 1 lists the DNMs identified in the trio cohort.

Figure 4 summarizes all WES findings that are detailed below.

II.2.1 MUTATIONS IN KNOWN/NOVEL GENES

DEPDC5 - S2 One novel splice donor site variant (c.193+1 G>A, NM_001136029.2) was identified in the male proband of pedigree 5 (P5). The patient had SHE with rare seizures in wakefulness controlled by medication. There was a history of sleep-related epileptic events in the paternal branch, but no definite clinical information was available. The trio study in this case highlighted that the variant was inherited from the healthy mother. The RNA sample of the proband was not available to confirm the effect of the variant on the *DEPDC5* transcript, but the variant was considered to be likely pathogenic.

Fig. 4 Summary of WES study results



NPRL2 - F4 WES analysis performed in two out of the four affected members of a FFEVF family (P6) allowed the identification of a heterozygous missense change (p.L105P) of *NPRL2* (NM_006545.4) coding for one interactor of *DEPDC5*. This variant is predicted to be deleterious by all *in silico* prediction tools used, affects a conserved aminoacid residue and has never been reported in public databases. The proband (II:3) and her son (III:3), were both affected with typical SHE, experiencing clusters of hypermotor seizures exclusively from sleep. Accurate phenotyping suggested an extra-frontal (insular) onset of the seizures in the proband who reported a painful sensation of the left arm, sometimes followed by contraction of facial muscles or auditory hallucinations preceding the motor events.

Segregation analysis by Sanger sequencing confirmed that the variant was present in four other family members, two of whom definitely affected (penetrance of 66%): the proband's father (I:1) who suffered from focal (possible temporal) seizures both from sleep and wakefulness; one healthy sister (II:2); another sister with unconfirmed seizures during childhood (II.1) and her son (III:1) who recently experienced a few sleep-related events described as bilateral convulsive seizures.

This family was published in a collaborative study (Ricos et al., 2016) describing mutations of *NPRL2* and *NPRL3* in FE for the first time. The study confirmed the role of GATOR1 components in the pathogenesis of FE and established that mutations in this complex account for about 9.5% of all genetic FEs (Ricos et al., 2016).

KCNT1 - S3 In a female patient (P7) studied as a trio we identified a *de novo* missense change of *KCNT1* (NM_020822.2), p.A934T, which affects a highly conserved residue in the cytoplasmic C-terminal domain of the protein.

This mutation has been already reported as pathogenic in a French patient affected with MMFSI (Barcia et al., 2012) and is published in public databases ClinVar and dbSNP with the code rs397515403 (<http://www.ncbi.nlm.nih.gov/clinvar/>; <http://www.ncbi.nlm.nih.gov/SNP/>).

The proband of our trio had a different phenotype, characterized by bilateral asymmetric tonic seizures occurring in clusters up to 40/night since the age of 9 years. Her epilepsy showed a spontaneous remitting-relapsing pattern of evolution, without a clear-cut pharmacoresistance. The patient also had a congenital profound sensorineural hearing loss and a cognitive delay with predominant language impairment.

Genetic analysis in this patient also highlighted the *CHRNA4* p.D104N change, inherited by her father who had a history of arousal parasomnias during childhood and “agitated” sleep-related events of uncertain etiology between 30 and 40 years of age. This variant has been considered of unknown significance (see later).

Finally, given the comorbidity with congenital hypoacusia (unlikely to be ascribable to the phenotypic spectrum of this gene), we conducted an additional analysis aimed at discovering possible causes of this specific disorder. The patient proved to be compound heterozygous for two mutations in *TMPRSS3* (MIM 605511), involved in an autosomal recessive form of non-syndromic congenital hearing loss. Both mutations were inherited by the healthy parents. In particular, the first mutation (p.P277L, NM_032404), reported as pathogenic in the public databases Omim and ClinVar (rs28939084), was inherited by the mother. The second is a deletion p.H70Tfs*X19 (NM_001256317) reported in ExAc database with a frequency of 5/10000 and was inherited by her father.

CHRNA4 - F5 WES analysis identified three mutations in *CHRNA4* the first of which had been missed by the previous dHPLC analysis.

The novel heterozygous variant p.G307V (NM_000744.6) was found in two sisters with typical SHE of family 5 (P8). The variant was confirmed by Sanger sequencing and the segregation analysis revealed that it was inherited from the healthy father (penetrance of 66%). This change is predicted to be damaging by all the *in silico* tools (Table 3) and affects a conserved aminoacid located close to the third transmembrane helix (Figure 5). The change in the aminoacid sequence in this region may alter the protein structure.

Tab. 3 Features of the novel mutation in *CHRNA4*

hg19_coordinate	transcript	N_change	AA_change	zyg	N_PhyloP	predictions	freq_ExAC
chr20: 61981843	NM_000744.6	c.G920T	p.G307V	het	0.998	D,D	-

Abbreviations N_change: nucleotidic change; AA_change: aminoacidic change; zyg: zygotes; N_PhyloP: normalized PhyloPhen*; freq_Exac: allele frequency from Exac (Exome Aggregation Consortium) database; het: heterozygous; D: damaging.

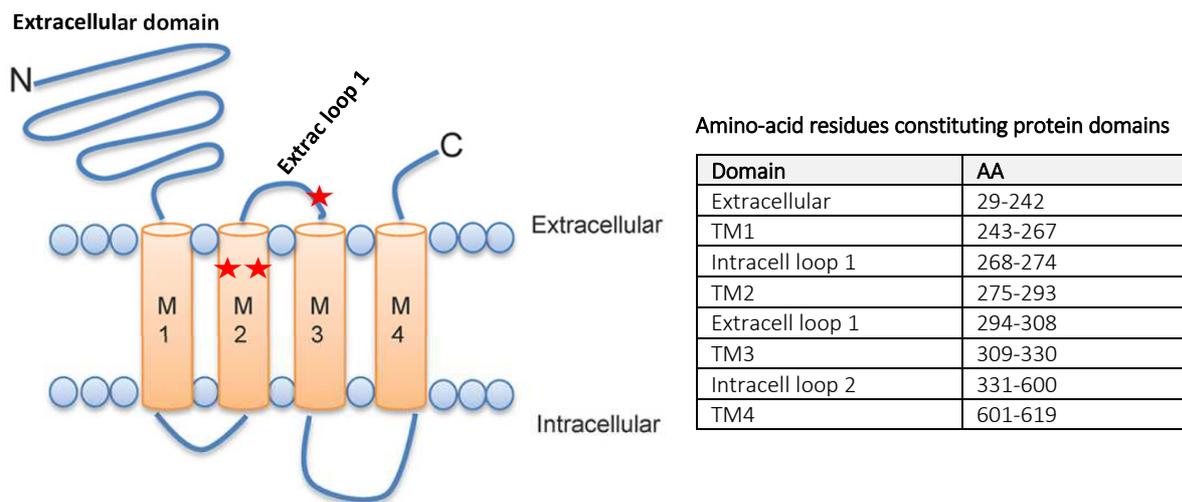
*PhyloPhen (evaluates conservation among different species);

Predictive tools included SIFT (whose prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences) and Polyphen (predicts the possible impact of amino acid substitutions on the stability and function of human proteins using structural and comparative evolutionary considerations).

CHRNA4 - S4 The missense variant p.S284L (rs28931591) of *CHRNA4* is a hotspot mutation associated with a CpG hypermutable site in the TM2 domain of the protein. It corresponds to the well-known *CHRNA4* p.S252L mutation, reported since 1999 in four ADSHE families and one sporadic case (Hirose et al., 1999; Phillips et al., 2000; Cho et al., 2003; Rozycka et al., 2003; Sansoni et al., 2012). Cognitive deficit has been reported in some affected family members (see Table 1). Similarly, our patient (P9) showed a particularly severe SHE phenotype with early onset refractory seizures and ID. There was a positive family history for NREM parasomnias in both parents and his sister, however the mutation was *de novo*. This patient carried also a *de novo* mutation in a candidate gene, *MIOS*, as detailed below.

CHRNA4 - S5 Finally, a novel missense change p.S284W was identified in the female proband of pedigree 10 (P10) affected with refractory SHE. The variant is predicted to be damaging for protein function, and affects the same aminoacidic residue that has been found mutated in different SHE patients, including our sporadic case of pedigree 9 (P9). The segregation analysis revealed that the variant is not present in the patient's healthy father and healthy brother, reinforcing the pathogenic role of the mutation, although the segregation in the healthy mother could not be assessed.

Fig. 5 Schematic representation of *CHRNA4* (NM_000744.5). The mutations found are indicated by red stars.



SCN1A - S6 A missense change p.P824H of *SCN1A* (NM_001165963) was identified in a sporadic case referred to us as affected by SHE for genetic study (P11). The mutation has never been reported so far, especially in association with the SHE phenotype, but evaluation of the patient's whole clinical history suggests that this variant could underlie the pathogenesis of his epilepsy. In fact, the patient had a history of recurrent, prolonged febrile/afebrile seizures and status epilepticus starting from age nine months, slow regression of cognition after seizure onset, and polymorphic seizures (hemiclonic seizures, temporal seizure with loss of awareness, atypical absences) refractory to antiepileptic medications (of note, the clinical worsening induced by lamotrigine and other sodium blockers and the efficacy of valproate and topiramate treatment). However, the sleep-related paroxysmal motor events from age 13 years are not commonly reported in *SCN1A*-related epilepsy phenotype.

II.2.2 MUTATIONS IN CANDIDATE GENES

PIK3R3 Three out of the five affected individuals of an ADSHE family were sequenced. The analysis, focused on shared heterozygous variants according to an AD inheritance model, led to the identification of a shared, novel candidate variant p.N110S in *PIK3R3* (NM_001303428.1) that affects a conserved aminoacid residue and is predicted to be damaging by Polyphen2, Sift and Cadd scores.

Moreover, an additional missense change p.R16M in this gene was identified in a male patient of a trio, inherited from the healthy father. The variant is predicted to be damaging by Polyphen2 and Cadd scores, and is not reported in public databases. *PIK3R3* encodes a regulating subunit of the phosphoinositide-3 kinase (PI3K), which is highly expressed in the brain cortex and interacts with PIK3R1 and PIK3CA to regulate mTOR, whose deregulation has already been described in FE. To date, pathogenic germline and mosaic mutations in multiple PI3K/AKT pathway genes have been associated with focal MCD manifesting with FE (Lee et al., 2012; Jansen et al., 2015; D’Gama et al., 2015). None of the reported patients showed structural lesions at conventional 1.5 T brain MRI, but a targeted high-resolution MRI was not available.

PIK3CA A missense variant p.R770Q (NM_006218.3) of *PIK3CA* was identified in a male proband of a trio, inherited by his mother. The patient had refractory SHE of unknown etiology. Given the persistence of highly frequent hypermotor seizures from sleep, following invasive SEEG recordings he underwent a left orbito-frontal lesionectomy-cortectomy. Histological analysis of the resected brain specimen disclosed a FCD type IIb.

The aminoacidic change found is predicted to be damaging by Sift and Cadd scores and affects a conserved residue. However, it has been reported in four alleles in ExAC database, and was therefore classified as a VUS. *PIK3CA* encodes a catalytic subunit of PI3K, which interacts with the regulatory subunit encoded by *PIK3R3* and *PIK3R1*. Interestingly, somatic mutations in this gene have been associated with abnormalities of brain development including FCD, as reported above.

PIK3C2B A *de novo* missense change in *PIK3C2B* (p.E1294Q; NM_002646.3) has been identified in the male proband of a trio with unconfirmed family history for sleep-related paroxysmal attacks which were reported in his father. The proband had documented hypermotor seizures arising exclusively from sleep, well controlled by antiepileptic medication. Brain MRI did not show structural lesions.

The protein encoded by this gene belongs to the PI3K family and consecutively considered a strong candidate gene that has not yet been reported.

The same patient carried also a novel nonsense mutation of **AMBRA1** p.Q276* (NM_001300731.1) that was inherited from the father. *AMBRA1* is a gene intolerant to missense and LOF mutations according to ExAC database. The encoded protein regulates autophagy and the development of the nervous system, controlling the protein turnover (Fimia et al., 2007); it is expressed ubiquitously and has a high expression in neuronal cells of the brain cortex. Impaired autophagy has already been implicated in the pathogenesis of neurological diseases, including epilepsy, emphasizing the possible causative role of the identified mutation.

MTOR A novel missense change in *MTOR*, p.L2354M (NM_004958.3) was identified in the male proband of a trio, inherited from the healthy mother. The variant is predicted to be damaging by Polyphen2, Sift and Cadd

scores, and affects a conserved residue. It could affect the function of mTOR protein but a functional study of mTOR activation is necessary to assess whether this change could underlie epilepsy in this patient.

An additional novel splicing mutation in **LMTK3** (NM_001080434.1), c.448+1G>A was identified *de novo* in the same individual. This gene encodes a protein kinase, predominantly expressed in the brain, whose physiological functions are unknown. Lmtk3 knockout mice exhibit abnormal behaviors, and in vitro assays have shown that LMTK3 is involved in the endocytic trafficking of N-methyl-d-aspartate (NMDA) receptors, a type of ionotropic glutamate receptor whose expression is altered in epilepsy animal models (Ghasemi and Schachter, 2011).

MIOS In the sporadic case carrying the pathogenic mutation in **CHRNA4** (S4, P9) we also found a *de novo* p.A138G change in **MIOS** (NM_019005.3). This gene encodes a component of the GATOR2 complex, which regulates the mTOR pathway. This implies a possible role of **MIOS** in epileptogenesis and MCD, although it has not been confirmed by recent publications (Weckhuysen et al., 2016).

GSE1 A novel missense variant of **GSE1** (p.S917C, NM_014615.3) segregated in the two affected sisters of a family also sharing a missense VUS in **DEPDC5** (p.M1217I, see below). The identified variant is predicted to have deleterious consequences on protein function. GSE1 is a proline-rich protein highly expressed in brain cortex and cerebellum (<http://www.braineac.org/>), but its function is largely unknown. Despite this poor information, two additional variants have been found in this gene: the p.R425W is predicted as damaging by *in silico* tools and identified *de novo* in a female proband of a trio. The p.P1003L change, identified in a sporadic case, is predicted to have a functional effect on the encoded proteins. However it is reported in 16 control alleles and, moreover, it was not possible to analyze its segregation in the healthy parents, therefore it was classified as VUS.

VPS13D In the sporadic patient, we also identified a heterozygous missense variant in **VPS13D** (p.N2397S, NM_018156.3), reported in five control alleles and considered VUS for the same reasons. Furthermore, the p.E2073* mutation was identified *de novo* in an affected female analyzed by a trio approach. **VPS13D** encodes a protein belonging to the vacuolar-protein-sorting-13 gene family, highly expressed in brain, and has been found *de novo* mutated in autistic and schizophrenic patients (Iossifov et al., 2014; McCarthy et al., 2014), suggesting a possible pathogenic role also in epilepsy.

PDE2A We identified the heterozygous, *de novo* p.S663F change (NM_002599.4) in a female proband affected by SHE and FCD. This gene codes for a phosphodiesterase with a dual-specificity for the second messengers cAMP and cGMP.

GPRI62 encodes an orphan receptor (the associated ligand and signaling pathways are unknown) with a very high expression in brain, especially in the frontal cortex (<http://www.braineac.it>). We found a *de novo* variant (p.S95F, NM_019858) in a patient with symptomatic SHE.

KCNT2 A missense change p.C484Y (NM_198503.4) in the **KCNT2** gene was found in a proband of a trio, inherited from the healthy mother. The variant is predicted to be damaging by all the *in silico* tools used and affects a conserved aminoacid residue, but it has been reported in three alleles in ExAC. **KCNT2** encodes a protein that interacts with **KCNT1** to form a functional sodium-activated potassium channel. As **KCNT1** has

already been found mutated in SHE patients, this makes *KCNT2* a very good candidate for the epilepsy phenotype seen in the patient. The same individual also carried a missense variant of *RYR3* (p.G4670S NM_001243996.2) inherited from the healthy mother. Another change in this gene was identified in an affected male of a trio (p.R1333H), inherited from the healthy father. Both these variants have already been reported in healthy subjects, despite being predicted to be deleterious for protein function and therefore classified as VUS. *KDM5C* A novel missense change p.C1190W (NM_004187.3) *KDM5C* was identified in one male proband in hemizygous state, inherited from the healthy mother, who is heterozygous for the variant, predicted to be damaging by Polyphen2 and Sift. This gene encodes a lysine demethylase, whose mutations have been associated with X-linked recessive mental retardation and epilepsy in some patients. Our proband showed a mild ID, mild dysmorphisms with low forehead, short stature and microcephaly in addition to SHE.

UBN2 We found a *de novo* frameshift mutation (p.K1201Nfs*18, NM_173569.3) in a sporadic patient, also carrying a VUS on *CHRNA2* (see below). *UBN2* codes for Ubinuclein 2, a protein expressed in brain cortex whose function is largely unknown: it interacts with SUMO2 and may be involved in transcription regulation. The gene is highly intolerant to genetic LOF mutations, and DNMs have been identified in patients with autism, suggesting its possible implication in neurological conditions (Iossifov et al., 2014).

CNTNAP5 This gene was found *de novo* mutated in a trio (p.Q889R; NM_130773.3). Moreover, two missense variants were identified in two sporadic patients: p.R461C and p.P552S. Both these variants are predicted to be damaging and affect conserved residues. However, they have been reported in ExAC database in five and one alleles respectively, and finally considered VUS, since the segregation analysis could not be performed. *CNTNAP5* encode proteins named contactins belonging to the neurexin family, whose members are highly expressed in brain cortex and have a role in the vertebrate nervous system, acting as cell adhesion molecules and receptors.

II.2.3 VARIANTS OF UNKNOWN SIGNIFICANCE (VUS) IN KNOWN SHE GENES

DEPDC5 Four heterozygous missense changes in *DEPDC5* (p.F1321L, p.M1217I, p.R509C, p.V272I) were classified as VUS; a functional assessment is needed to confirm their causative role.

- (i) p.F1321L, identified in the male proband of another trio, inherited from the healthy mother. This variant is novel, predicted to be damaging by Polyphen2 and Cadd scores (29.8) and affects a conserved aminoacid. However, it was predicted as Tolerated by SIFT and, more importantly, we could not perform a functional characterization to confirm its causative role.
- (ii) the heterozygous aminoacidic change p.M1217I recurred in two affected sisters of a family including six affected individuals over two generations (pedigree not shown). The 39-year-old proband had mild psychomotor delay and typical SHE phenotype, with sleep-related events from the age of 19 years controlled by oxcarbazepine. Interictal EEG showed left fronto-temporal paroxysmal activity during sleep. Prolonged video-EEG monitoring captured three of her typical attacks from sleep associated with a left anterior-temporal discharge. Brain MRI revealed mild atrophy of the left hemisphere. Both her sisters, now aged 48 and 45 years, had brief episodes in wakefulness at three years of age characterized diffuse rigidity, breathing difficulty and impaired awareness. Another three affected relatives had a history of

epileptic seizures (described as GTCS in two) and were not available for an accurate phenotyping or for genetic testing. The variant is not predicted to have important consequences on protein function or structure, although affecting a conserved residue, and is reported in six alleles in ExAC database. No additional affected/unaffected relatives were available for familial segregation study.

(iii) p.R509C was found in the proband of a trio and his healthy father; this variant is reported in ExAC in 52 alleles and is predicted to be tolerated, albeit affecting a conserved aminoacid residue.

(iv) the heterozygous missense p.V272I was identified in a trio as carried by the female proband and her healthy mother. The change showed a Cadd score of 27.4, but was present in 1000 genome database (rs187334123).

CHRNA4 A missense variant p.A71T was identified in the female proband with SHE. However, this variant was considered to be of unknown significance, as it is reported in public databases (six alleles in ExAC), the pathogenicity predictions are discordant and the segregation in the healthy parents could not be evaluated.

Moreover, as reported above, the novel missense variant p.D104N was identified in the female proband carrying the *de novo* mutation of KCNT1 (S3). Although the change is predicted to be damaging by all the *in silico* tools used, it resulted to be inherited from the father who had a history of parasomnias. The identified variant could be the cause of these episodes in this trio.

CHRN2 The missense heterozygous change Q397H of *CHRN2* (NM_000748.2) was found in the female proband also carrying the DEPDC5 VUS p.V272I. The *CHRN2* change, inherited by her healthy mother and predicted as benign by SIFT and Polyphen and with a Cadd score of 5.23, is reported as VUS by Clinvar.

An additional missense variant of *CHRN2* (p.L376F) was identified in the male sporadic case also carrying the *de novo* frameshift mutation of *UBN2*.

III. NEUROPSYCHOLOGICAL STUDY

We recruited 60 patients (M/F=28/32, mean age 38.23±12.43 years, range 14-69). Forty-five (75%) had a video-EEG documented (confirmed) diagnosis of SHE and 15 (25%) a video-documented (clinical) SHE, according to the novel criteria (Tinuper et al., 2016). The clinical features are detailed in Table 4.

The mean age at epilepsy onset was 12.63±8.15 years (range 3-42). Forty-nine (81.7%) were sporadic cases while 11 patients (18.33%) had a positive family history for SHE (three cases) or other focal epilepsy (eight). Most patients had unknown etiology (63.33%), 11 showed abnormalities on neuroimaging (18.33%) and 11 were genetic (18.33%). Genetic cases include four *CHRNA4*-mutated patients (two belonging to F5, II.1 and II.2), four with mutations in *DEPDC5* (including two members of F2, III.2 and IV.2), one patient with *KCNT1* mutation (S3) and two members of the family carrying the *NPRL2* change (F4-II.3, III.3).

Tab.4 Clinical features of 60 patients diagnosed with SHE included in the neuropsychological study.

		Tot 60	Valid %	Missing (%)	
SEIZURE FREQUENCY	Daily/multi-daily	26	47.27	5	
	Weekly	16	29.09	(8.33)	
AT ONSET	Monthly	5	9.09		
	Yearly	8	14.55		
SEIZURES IN WAKEFULNESS		34	56.67	-	
AURA		33	55.00	-	
BILATERAL T-C SEIZURES		24	40.00	-	
STATUS EPILEPTICUS		6	10.00	-	
EPILEPTIFORM INTERICTAL EEG		38	63.33	-	
EEG ICTAL PAROXYSMAL CHANGES		37	61.67	-	
PATHOLOGICAL NE		5	8.33	-	
ABNORMAL BRAIN MRI		11	18.33	-	
ANY UNDERLYING BRAIN DISORDER		14	23.33	-	
PERSONAL HISTORY	FS	3	5.00	-	
	Perinatal insult	4	6.67	-	
	Psychomotor delay	4	6.67	-	
	Psychiatric disorders	15	25.00	-	
FAMILY HISTORY	FS	3	5.00	-	
	Epilepsy	Total	11	18.33	-
		SHE	3	5.00	-
		Other±SHE	8	13.33	-
	ID	5	8.77	3 (5.0)	
Psychiatric disorders	9	15.79	3 (5.0)		

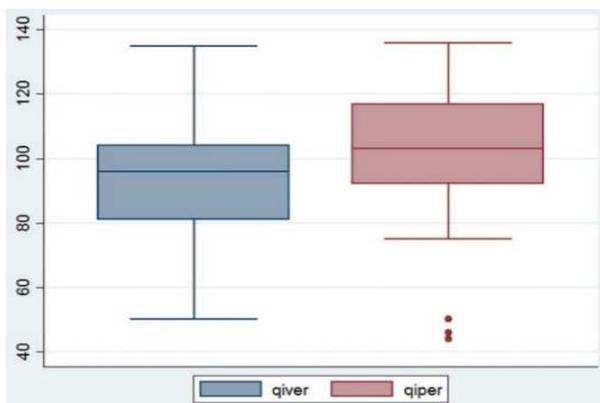
Abbreviations: NE: neurological examination; FS: febrile seizures; ID: intellectual disability.

Thirty-four patients (56.67%) experienced at least one seizure in wakefulness lifetime, 33 (55%) reported subjective sensation before seizures.

At the time of neuropsychological assessment, 18 patients were seizure-free (30%) whereas the remaining 42 (70%) continue to experience seizures with variable frequency (15 daily, 16 weekly/monthly, 11 yearly/sporadic). Eleven patients were off medications, 26 on monotherapy (20 with carbamazepine, four on oxcarbazepine, one on topiramate and one on lamotrigine) and 23 were taking a combination of two or three antiepileptic drugs (AED). All patients were right-handed except for two (one ambidextrous and one left-handed corrected).

The neuropsychological findings are reported in Table 5. The total IQ score ranged from 45 to 138 (mean total IQ: 96.96 ± 21.50) with significant differences between verbal IQ (mean: 93.38 ± 19.50) and performance IQ (mean: 101.35 ± 21.10), $p < 0.0001$ (Figure 6).

Fig. 6 Verbal versus performance IQ.

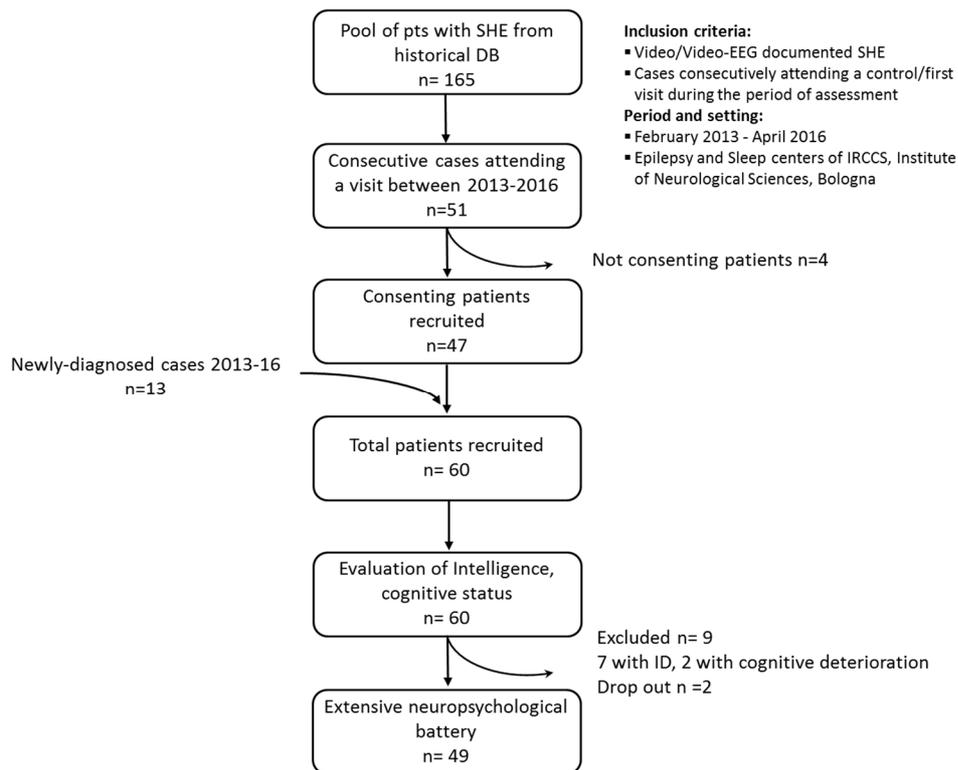


Wilcoxon signed-rank test highlighted significant lower mean scores in verbal IQ.

Six patients with ID (median total IQ score 52.2; range 45-64), two with pathologic MMSE corrected score (MMSEc scores: 18 and 21) and one patient untestable at WAIS and with MMSEc score of 9, were not included in the extensive neuropsychological study. Two additional patients with normal intellectual functioning did not complete the assessment. The remaining 49 patients underwent the full neuropsychological battery evaluating language, memory, visual-spatial abilities and executive functions. Figure 7 represents the flowchart of patients recruitment.

Twenty-three patients out of the 49 tested (46.9%) showed deficits in at least one test, with multiple impaired tasks in 13. Twelve patients (24.5%) showed deficit in language, with selective impairment of phonemic fluency. Memory was impaired in 12 cases (24.5%); in particular, five showed deficits in verbal memory, four in visuo-spatial memory and three in both. Among tests evaluating the executive functions more selectively, the Stroop test (assessing inhibitory control and selective attention) was the most impaired, showing pathological scores in 11 cases (22.4%); five patients showed impaired working memory (10.2%), whereas performance on shifting and cognitive flexibility (WCST) were normal in all the patients (Table 5).

Fig. 7 Recruitment flowchart of neuropsychological study



At univariate analysis, patients with pathological Neurological Examination (NE) showed more deficits at WAIS (66.67% vs 7.69%, $p = 0.029$) and significantly worse scores at MMSE (24.52 ± 5.7 vs 28.03 ± 1.45 , $p = 0.010$) compared to patients with normal NE. Similarly, a higher seizure frequency at last visit correlated with worse performances in cognitive tests (WAIS: 90.96 ± 20.13 vs 103.17 ± 21.45 , $p = 0.030$; MMSE: 27.39 ± 2.68 vs 28.28 ± 1.11 , $p = 0.044$) and in nonverbal memory (13.90 ± 6 vs 17.32 ± 5.16 , $p = 0.038$). Overall, a significantly worse scoring in tests exploring non verbal memory and visuo-spatial abilities was attained in all the patients with a poor prognosis (failure to achieve remission in the last 5 years) (Rey fig memory: 15.42 ± 5.75 vs 20.34 ± 3.12 , $p = 0.040$; Rey fig copy: 33.25 ± 1.76 vs 35.02 ± 1.05).

The variable “any underlying brain disorder” (at least one among: pathological NE and abnormal brain MRI) was more frequently associated with deficits in verbal long-term memory (30% vs 2.63%, $p = 0.025$) and attention/inhibitory control (Stroop test; 50% vs 15.38%, $p = 0.033$), with significantly worse performances in task evaluating shifting abilities (TMTB test: 99.9 ± 30.9 vs 73.38 vs 32.95 , $p = 0.027$). This finding was also seen in patients with a personal history of status epilepticus (TMTB: 65.4 ± 18.51 vs 46.18 ± 23.15 , $p = 0.035$) and poor prognosis (TMTBA: 50.12 ± 23.47 vs 30.83 ± 8.9 , $p = 0.020$), while bilateral convulsive seizures correlated with worse scores in working memory (Verbal span backward: 3.56 ± 1.04 vs 4.27 ± 1.06 , $p = 0.049$).

Tab. 5 Neuropsychological findings

Pts	Domain	Test	Mean \pm SD (nv)	# Pts with impaired tests (%), score	Impaired pts/domain	
60	Intelligence and cognitive status	Raven Matrices	29.75 \pm 2.76 (>18,96)	0/49	9 (15%)	
		WAIS-R IQ t	96.96 \pm 21.50 (>70)	6/57 (10.53%), range 45-64		
		IQ v	93.38 \pm 19.50	range 50-62		
		IQ p	101.35 \pm 21.10	range 44-81		
		MMSE	27.82 \pm 2.11 (>23.8)	3/58 (5.17%), range 16-23.59		
49	Language	Phonemic fluency	25.88 \pm 11.37 (>17.35)	12/49 (24.49%), range 6.1-17.3	12 (24.49%)	
		Semantic fluency	36.77 \pm 6.87 (>24)	0/49		
	Memory	Verbal	Rey short-term memory	42.39 \pm 8.87 (>28.53)	3/48 (6.25%), range 17.05-26.8	8 (16.32%)
			Rey long-term memory	8.39 \pm 2.77 (>4.69)	4/48 (8.33%), range 1.85-4.63	
			Verbal span (forward)	5.87 \pm 1.13 (>4.26)	2/49 (4.08%), range 3.92-4	
			Verbal supraspan + 2	4.36 \pm 2.61 (<11)	2/49 (4.08%), range 13- >15	
			Associated words learning	13.79 \pm 4.01 (>8.73)	5/48 (10.42%), range 4.49-7.73	
	Visuospatial	Rey Figure-memory	15.61 \pm 5.80 (>4.69)	1/48 (2.08%), 3.75	7 (14.3%)	
		Corsi Block-Test	5.30 \pm 1.56 (>3.46)	5/49 (10.2%), range 2.37-3.39		
		Visual-spatial supraspan + 2	20.21 \pm 6.88 (>5.5)	2/48 (4.17%) –		
	Visuo-spatial abilities		Rey complex figure-copy	33.49 \pm 1.72 (>28.88)	1/48 (2.08%), 28.25	1 (2.08%)
	Executive functions	Attention/ inhibitory control	Trail making test A	35.44 \pm 11.83 (93)	0/48	11 (22.45%)
			Stroop (time)	23.92 \pm 8.71 (<27.5)	11/49 (22.45%), range 27.62-48.88	
			(errors)	1.12 \pm 0.90 (<7.5)	0/49	
Shifting		Trail making test B	78.88 \pm 34.00 (<262)	0/48	-	
		Trail making test BA	48.18 \pm 23.30 (<186)	0/48		
Working memory		Verbal span (backwards)	4.00 \pm 1.10 (> 2.65)	5/49 (10.2%), range 1.52-2.58	5 (10.2%)	
Pianification	WCST	26.41 \pm 11.98 (<90.6)	0/49	-		

Abbreviations Pts: patients; SD: standard deviation; nv: normal value; ID: intellectual disability; IQ t: total IQ; IQ v: verbal IQ; IQ p: performance IQ.

IV. GENOTYPE-PHENOTYPE CORRELATIONS

A total of 106 probands underwent genetic testing by different techniques other than WES analysis, including those used in the preliminary screening (Sanger sequencing, dHPLC and HRM). In particular, *KCNT1* and *DEPDC5* were analyzed in 87 and 84 probands by WES and/or HRM, respectively; *CHRNA4*, *CHRNA2* and *CHRNA2* were screened in 97, 97 and 100 patients, respectively, by means of WES, Sanger or dHPLC. However, the low sensitivity of dHPLC should be considered, since this assay gave false negative results in F5, where the causative p.G307V missense change was identified only later by WES analysis.

Genetic analysis allowed us to solve 11 cases in our whole cohort (10.4%), of whom five familial and six sporadic.

The mutation frequencies were 2.3% (CI: 0.3-8.1%) for *KCNT1*, 5.9% (CI: 2.0-13.3%) for GATOR1-complex genes, 3.1% (CI: 0.6-8.8%) for *CHRNA4*, 1.7% (CI 0-8.9%) for *SCN1A*.

Table 6 summarizes the overall genetic findings in our cohort and reports the key clinical features for each family and sporadic cases that we found mutated in SHE genes.

In familial cases, except for *KCNT1*, mutations in the other genes showed incomplete penetrance: 55% for *DEPDC5* (for which the segregation in F2 was compatible with mosaicism) and 66% for both, *NPRL2* and *CHRNA4*.

Although the small sample size precluded a reliable statistical analysis, a comparison of clinical data related to each gene allowed us to make some useful considerations.

The mean age at epilepsy onset is lower in patients carrying *KCNT1* variants (Table 6). Among *CHRNA4*-mutated patients, the well-known *CHRNA4* mutation p.S284L is associated with the earliest age at onset. As the mutation is *de novo* and is located in the domain forming the channel pore (TM2), we assume that the intrinsic molecular features of mutations in this gene may influence the phenotype severity. Conversely, in *DEPDC5*-mutated families F2 and F3, the variability of age at onset even in affected members sharing the same mutation (range: 4 -15 years and three months-12 years, respectively) suggests additional genetic determinants, epigenetic modulators or, environmental factors co-acting to determine the phenotype. Indeed, the patients with earlier age at onset in these families (V.1 in F2; IV.1 and IV.3 in F3), also had refractory epilepsy and were those showing structural brain lesions, supporting the hypothesis of a “double hit” mechanism, namely the occurrence of a second-hit brain somatic mutation.

DEPDC5-related epilepsies also showed a higher frequency of seizures in wakefulness and subjective symptoms preceding hypermotor seizures. This could be considered predictable, considering that the families included in our population represent FFEVF pedigrees. However these features also apply to sporadic cases and FFEVF-affected members with the SHE phenotype.

Surprisingly, psychiatric and behavioral disorders were observed with a similar frequency among *KCNT1* and GATOR1 gene-mutated cases, while the rate of drug resistance was comparable among the three genes.

Tab. 6 Correlations between genetic results and key clinical features

Gene	N# total screened	code	Mutation	Phenotype	Penetrance/ occurrence	N# affected	Age at onset (y)	Aura*	Seizures on wakefulness*	MRI lesion	ID	Psy	DR
<i>KCNT1</i>	87	F1	p.Y796H	ADSHE	100%	4	5.5±2.1	-	-	-	3/4	2/4	2/4
		S3	p.A934T	SHE	na/ <i>de novo</i>	1	9	-	-	-	1	-	rr
Tot <i>KCNT1</i>					100%	5	6.2 ±1.56	0%	0%	0%	80%	40%	40%
<i>DEPDC5</i>	84	F2	c.279+1 G>A	FFEVF	na/ mosaicism	3	11.0±6.08	2 (1)	3 (2)	1	-	-	1
		F3	p.T329Lfs*7	FFEVF	55%	7	5.15±4.52	4 (3)	4 (3)	2	-	3	4
		S1	p.R165Yfs*14	SHE	na/inherited	1	10	1	1	-	-	-	1
		S2	c.193+1 G>A	SHE	na/inherited	1	Na	-	1	-	-	-	-
<i>NPRL2</i>	60	F4	p.L105P	FFEVF	66%	4	11.5±5.97	1 (1)	1 (0)	-	-	2	1
Tot <i>GATOR1</i> genes					55-66%	16	8.82±5.58	50% (37.5%)	62.5% (43.7%)	18.7%	0%	31.2%	43.7%
<i>CHRNA4</i>	97	F5	p.G307V	ADSHE	66%	2	7.5± 0.7	-	1 (rare)	-	-	-	rr
		S4	p.S284L	SHE	na/ <i>de novo</i>	1	3	-	-	-	1	-	1
		S5	p.S284W	SHE	nav	1	12	1	1 (rare)	-	1	-	1
Tot <i>CHRNA4</i>					66%	4	7.5±3.69	25%	0%	0%	50%	0%	50%

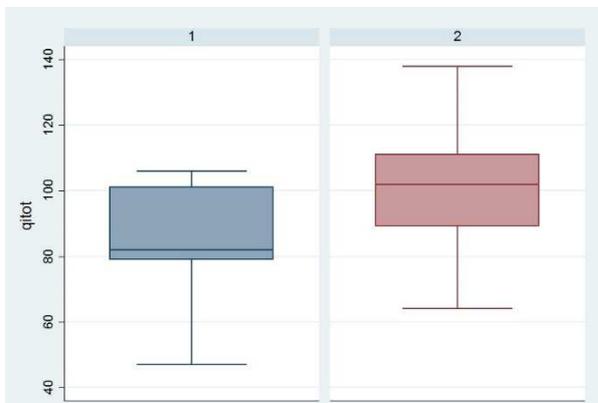
Abbreviations ID: intellectual disability; Psy: psychiatric disorders; DR: drug-resistance; rr: relapsing remitting; na: not assessable; nav: not available

*For FFEVF pedigrees is also indicated the N# of affected members with SHE experiencing aura and seizures in wakefulness (in brackets).

As is known, ID recurred more frequently in *KCNT1*-mutated patients (80%) but was also related to the *CHRNA4* hotspot mutations, confirming literature data (Steinlein et al., 2012a). Conversely, there were no significant limitations in intellectual functioning among individuals with GATOR1 gene mutations, despite the evidence of ID in several FFEVF family members (Dibbens et al., 2013).

In order to highlight a possible influence of genetics on cognition, we performed an additional statistical analysis in the 60 patients who underwent the standardized neuropsychological assessment. Mutated cases scored lower in total IQ than non-mutated cases (84.91 ± 18.54 vs 99.53 ± 21.47 ; $p: 0.0176$, Figure 8), without statistically significant differences between the two groups in other tests (Supplementary table 2).

Fig. 8 Total IQ in mutated and non-mutated cases



Wilcoxon signed-rank test highlighted significant lower mean scores in total IQ in mutated patients (blue) with respect to non-mutated cases (red).

DISCUSSION

Recent insights into the genetics of FE revealed several novel genes implicated in ADSHE (*CHRNA4*, *CHRNA2*, *CHRNA2*, *KCNT1*, *DEPDC5* and *PRIMA1*). Differences in penetrance, epilepsy phenotype and even endophenotype have been related to mutations in specific genes, such as *KCNT1* (Heron et al., 2012). Neuropsychological deficits have also been associated with specific mutations in nAChRs genes (Steinlein et al., 2012a).

However, the genes identified so far cumulatively account for less than 25% among familial and sporadic cases, suggesting further genetic heterogeneity. Systematic neuropsychological investigations on comprehensive SHE cohorts are lacking.

This study provides an in-depth clinical, genetic and neuropsychological characterization of a large cohort of patients diagnosed with SHE according to the novel, reliable diagnostic criteria (Tinuper et al., 2016). Using a WES strategy, we analyzed both sporadic and familial SHE cases. The familial cases belonged to ADSHE and FFEVF pedigrees, as SHE phenotype frequently runs also among FFEVF family members. A subgroup of patients from the historical pool of our Institute had been previously screened for mutations in the known genes, with interesting findings in *DEPDC5* and *KCNT1* but no mutations in nAChRs genes. This preliminary analysis would have enhanced the chance of identifying novel genetic determinants for SHE by the application of advanced NGS technologies.

Using a number of strategies, overall we identified causative mutations in 10.4% of our cases. WES analysis allowed the identification of a novel epilepsy gene, *NPRL2*, coding for one of the three components of GATOR1, an inhibitor of the master regulator of cell growth and metabolism, mTOR-signaling pathway. Altogether, mutations in the GATOR1 complex genes *DEPDC5* and *NPRL2* account for the majority of our cases (6%). This percentage is in line with genetic and statistical data from the literature implicating GATOR1 genes in 6.9% (28/404) of probands affected with heterogeneous forms of FE (Ricos et al., 2016). The similarity on GATOR1 mutation rates was actually unexpected, taking into account that familial cases represent a minority of our population compared with those of Ricos et al. where families represent 67.3% (272/404) of the whole. However, it should be considered that we included a homogeneous cohort of well-selected cases of SHE, one of the most common phenotypes among GATOR1 gene-related epilepsies.

DEPDC5 in particular showed the highest mutation rate (4.8%), strengthening its role in FE. This frequency would rise to 9.5% if the additional four changes classified as VUS were considered. VUS changes include missense variants inherited from healthy parents (p.F1321L, p.R509C and p.V272I) or the SNV shared by two affected family members (p.M1217I) but without available familial segregation analysis and with poor pathogenicity predictions. This family, in particular, would match the definition of FFEVF since it comprises affected members with variable epilepsy phenotypes (from epileptic spasms to isolated bilateral convulsive seizures) and a variable degree of ID, in addition to the index-case with typical SHE. The functional consequences of missense SNVs in *DEPDC5* are difficult to

predict: a recent study on 12 selected *DEPDC5* variants described in patients with FE has shown that only a portion have an effect on *DEPDC5* signaling in terms of mTORC1 activation, *DEPDC5* expression, GATOR1 complex formation and functional interaction with active RagA/B-RagC/D heterodimers in vitro (van Kranenburg et al., 2015). These findings may suggest that the identified variants could have distinct consequences on GATOR1 function, which could explain the phenotypic variability observed (van Kranenburg et al., 2015).

We did not detect mutations in the third component of GATOR1 complex, *NPRL3*. This gene has been implicated so far in a single family with early childhood onset ADSHE with 50% of penetrance (Korenke et al., 2016), in addition to the five FFEVF families published in Ricos et al., 2016.

Genotype-phenotype correlations in our cohort confirmed that mutations in GATOR1-complex genes *DEPDC5* and *NPRL2* are mainly implicated in those forms of FE associated with MCD, mainly FCD. A higher rate of drug resistance, aura and seizures in wakefulness may also suggest an involvement of these genes in patients with SHE phenotype, with remarkable implications in terms of diagnostic and therapeutic strategies. On the one hand these clinical features may prove useful to drive molecular diagnosis, even if NGS techniques have been increasingly employed to allow the simultaneous analysis of multiple genes. On the other, detection of mutations in these genes lead clinicians to search for focal MCD (namely FCD), especially in refractory cases. FCDs represent the most common, potentially treatable architectural disorder underlying FE, responsible for up to 42% of drug-resistant cases (Harvey et al., 2008). In general, epilepsy surgery is a highly effective curative option in these patients, affording the opportunity to achieve seizure freedom and potential medication withdrawal also with improvements in quality of life, employment rates and school attendance (Wiebe et al., 2001). As mentioned, a range of mosaic and somatic mutations in *DEPDC5* and in other mTOR-pathway genes have been identified in several MCDs and in up to 46% of FCD type IIb (Nakashima et al., 2015). Although mutated patients who have undergone epilepsy surgery are anecdotal, surgery has proved to be curative in these cases (Baulac et al., 2015), suggesting that epileptogenesis is underpinned by a genetically determined, focal cerebral structural lesion, even in the presence of germline mutations.

Of the seven patients with refractory seizures and mutations in GATOR1 genes, only three had MCD; none of them have undergone surgery yet. In the remaining four patients, no brain lesions were captured by conventional neuroimaging techniques. In the female patient carrying the *DEPDC5* truncating mutation p.R165Yfs*14 (S1), even SEEG study failed to identify a definitive epileptogenic zone, and tailored brain MRI was unrevealing despite a SEEG electrical pattern suggestive for FCD. Interestingly, two members of our largest FFEVF family carrying the *DEPDC5* p.T329Lfs*7 frameshift mutation (F3) were also excluded from intervention after a presurgical workup revealing bilateral temporal epileptiform activity. This evidence suggests *DEPDC5* germline mutations may also play a role in non-lesional, refractory FE with multiple independent epileptogenic foci or widespread epileptogenic networks. Otherwise, our inconclusive s-EEG results could in part be explained by a limited s-EEG exploration that did not fully cover the anterior part of mesial frontal lobe and dorso-lateral frontal lobes. Moreover,

given the association of *DEPDC5* mutations also with FCD type I lesions, we cannot exclude the presence of multiple, diffuse subtle dysplastic areas missed by conventional MRI in our patients.

In any case, the identification of pathogenic or possibly pathogenic mutations in GATOR genes, as well as in genes coding proteins acting upstream in the mTOR pathway (e.g. *PIK3CA* and *PIK3R3*) could have important future therapeutic implications even in mutated patients considered not eligible for surgery. In fact, the development of a novel class of therapies based on mTOR inhibitors, whose prototype is rapamycin, will improve the treatment and prognosis of these patients. Both preclinical and clinical trials using mTOR inhibitors to treat epilepsy, and possibly prevent it, are currently underway (Citraro et al., 2016).

Although dysregulation of the mTOR-pathway has been regarded as a root cause of several neurodevelopmental diseases with cognitive impairment (megalencephaly, microcephaly, MCD, autism spectrum disorders, schizophrenia, epilepsy, ID), we did not find a specific association between GATOR1 gene mutations and ID, whereas endophenotyping of our patients highlighted a number of cases with variable degrees of psychiatric disorders. Instead, we confirm the association of ID and psychiatric/behavioral disturbances with *KCNT1*-related ADSHE, as they are present in both F1 and S3. Several pieces of evidence document the emerging role of Slack channels in intellectual impairment. To date, multiple literature reports have implied mutations in this gene in severe childhood epileptic disorders ranging from MMFSI to Ohtahara syndrome. Moreover, the notions that Slack channels interact directly with the fragile X mental retardation protein (FMRP) and that I_{KNa} current (outward K^+ current with dependence on $[Na^+]_i$ current) is reduced in animal models of Fragile X syndrome lacking FMRP provide a molecular link between this gene and developmental disorders (Kim and Kaczmarek, 2014).

Finally, our data on *CHRNA4* support the evidence that cognitive deficits may also result from mutations tampering with the functional properties of neuronal nAChRs that are known to have an important role in shaping synaptic connections and determining plasticity in brain areas involved in fundamental aspects of cognition. All this evidence suggests that the mechanisms underlying learning and memory processes involve the recruitment of multiple signaling pathways and gene expression (Ménard et al., 2015). From this standpoint, the finding of significantly lower mean IQ scores in mutated patients of our cohort (independently by the specific gene) confirms the important role of genetics in neurodevelopmental disorders and suggests that ID in SHE patients may result from defects in genes with several biological functions and involved in different pathogenic mechanisms.

Except for ID, our sporadic case with the *KCNT1* mutation (S3) did not show other clinical features of disease severity, such as early onset refractory epilepsy or psychiatric disorders. This is surprising as she carried the same *de novo* missense mutation (p.A934T) as a patient with MMFSI (Barcia et al., 2012). According to literature data, two other *KCNT1* mutations, p.G288S and p.R398Q, give rise to either SHE or MMFSI, suggesting that variable phenotypic expression of this gene cannot be attributed

solely to differences in the effects stemming from the different aminoacid alterations, as previously supposed (Kim et al., 2014).

Unexpectedly, WES analysis revealed three pathogenic missense mutations in *CHRNA4*, the major nAChRs gene associated with the disease. Two of these patients had been previously screened for mutations in this gene by dHPLC, which did not reveal any pathogenic mutation. This could be due to the presence of SNPs in the DNA of the patient corresponding to the primers used for the PCR amplification. Of these mutations, two were novel. The p.S284W found in the sporadic female (S5) fall into the mutational hotspot of the gene (differing only for the aminoacid residue change), therefore clustering in the second TM. TM2 represents the major pore-forming part of each nAChR subunit, mostly affecting aminoacids belonging to the aminoacid residue axis that rotates when agonists such as acetylcholine attach to the binding site, opening the ion channel. These mutations are therefore likely to interfere with the channel's kinetics (Steinlein et al., 2012b). The second novel *CHRNA4* variant (p.G307V) locates in the first extracellular loop between TM2 and TM3, a structure of unknown significance for receptor function. Although this mutation has not been functionally characterized yet, it segregated in two affected sisters of F5 and is therefore considered causative.

The search for DNMs by the analysis of the 31 trios also highlighted an increased rate of missense SNVs (especially deleterious ones) and an increased rate of LOF mutations. The same finding was reported in previously published trio studies on different neuropsychiatric disorders (Iossifov et al., 2014). Some the *de novo* mutated genes identified were found to be more interesting depending on their biological function and expression level in the brain, intolerance to genetic variations and the prediction of pathogenicity of the identified mutations. Of particular interest are *MIOS* and *PIK3C2B* that respectively encode a component of the GATOR2 complex and a phosphoinositide 3-kinase which are involved in the regulation of mTOR pathway, with possible implications in epileptogenesis. Similarly, inherited variants with a strong prediction of pathogenicity were selected in other potential candidate genes acting in the mTOR-pathway, namely *PIK3R3*, *PIK3CA* and *MTOR*.

Overall, our the genetic results demonstrate that SHE originates from alterations in a heterogeneous group of genes implicated in a broad range of biological functions, and that both inherited as well as *de novo* mutations can play a role in its pathogenesis. In particular, DNMs mainly seem to affect genes involved in synaptic functions (like ion channel activity) while inherited mutations, both in familial and sporadic cases, seem to affect genes involved in a wider range of biological mechanisms, such as behavior, dendrite morphogenesis, mTOR pathway and cation channel function.

The systematic neuropsychological assessment was performed using a comprehensive battery of tests exploring frontal as well as extra-frontal functions. The main original feature of this study is that it evaluates a representative cohort of patients affected by SHE without focusing on specific etiology, thereby providing a comprehensive overview of the syndrome's neuropsychological features.

Our data showed that neurocognitive deficits are not uncommon in SHE, but the high frequency of ID and cognitive decline (15%) may be due to a referral bias as our Institute is a tertiary-care center. As

mentioned above, our data support a crucial role of genetics in the cognitive deficits of SHE patients involving different biological mechanisms. Statistical analysis also disclosed an association of pathological NE and a higher seizure frequency with worse performances in cognitive tasks.

Even among patients with normal intelligence the extensive battery of neuropsychological tests revealed some degree of cognitive dysfunction in 46.9% of cases regardless of etiology. The neuropsychological impairment is characterized by significantly worse scores in verbal IQ, deficits in memory and selected executive functions (phonemic fluency, inhibitory control and working memory), with preserved shifting abilities and cognitive flexibility. Overlapping results derived from the two genetically well-defined case-series including patients with nAChRs gene mutations (Picard et al., 2009; Wood et al., 2010) that reported impaired inhibitory task, verbal fluency and verbal/non-verbal memory, ascribable to a pattern of fronto-temporal dysfunction. Although the authors obviously hypothesized a role of nAChRs, the similarities with our findings (in patients with different etiologies) indicate that neuropsychological deficits may not be attributable to disruption of nAChRs mutated-channels alone.

In addition to alterations in executive functions, several other neuropsychological studies on cohorts of patients with surgical/non-surgical frontal lobe epilepsy (FLE) showed poor long-term memory with impaired encoding, free recall and retrieval, failing to differentiate FLE patients from those with temporal lobe epilepsy (Exner et al., 2002; Nolan et al., 2004; Patrikelis et al., 2016). Some of these studies offered several reasons for the limited differences between FLE and TLE, including rapid propagation of the seizures and the interictal spread of epileptic activity among reciprocally interacting fronto-temporal networks (Patrikelis et al., 2016).

More recently, the role of the frontal lobe during memory process has gained attention: several studies showed that specific areas within the frontal cortex are involved in memory encoding and retrieving, contributing to longer term memories, contrary to the traditional view that the frontal lobe role is limited to working memory (Centeno et al., 2010). Given all this evidence, the finding of memory deficits in our SHE cohort is not surprising and can be readily explained by both, the possible origin of hypermotor seizures from extra-frontal (temporal) networks, as proved by SEEG studies, and the main involvement, whether primary or secondary, of frontal areas that represent the merging point of epileptic discharges. We found a significant correlation of seizure-related variables (seizures frequency and poor prognosis) with worse performance on nonverbal memory, but we did not assess, and therefore cannot exclude, the role of NREM sleep fragmentation in memory deficits.

Finally, we found that pathological brain MRI and NE, together with variables of clinical severity, significantly correlated with worse performances in executive functioning, as reported in other studies (Upton and Thompson 1997 a,b; Exner, et al., 2002). None of these studies mentioned the discrepancy between verbal and non verbal IQ which strongly emerged from our analysis. The lower scores in verbal abilities may reflect impaired executive functioning (in particular verbal fluency and working memory), since there is a correlation between intelligence test scores and frontal executive function measures (Ardila et al., 2000).

In conclusion, this study contributed to widen the current knowledge on genetic and neuropsychological aspects of SHE. The identification of a new causative gene reinforced the role of mTOR-signaling pathway in this epilepsy syndrome and other FE, allowing the development of personalized therapeutic targets.

For many families and sporadic cases affected with SHE, the underlying molecular cause still remains unknown, implying the relevance of the candidate genes identified in this study. Larger replication cohorts are needed to confirm the role of these genes and their signaling pathways in the pathogenesis of SHE. Other unaddressed issues concern the impact of genetics on surgical outcome and the role of sleep in memory deficits. New insights in these aspects will have important implications in patient management.

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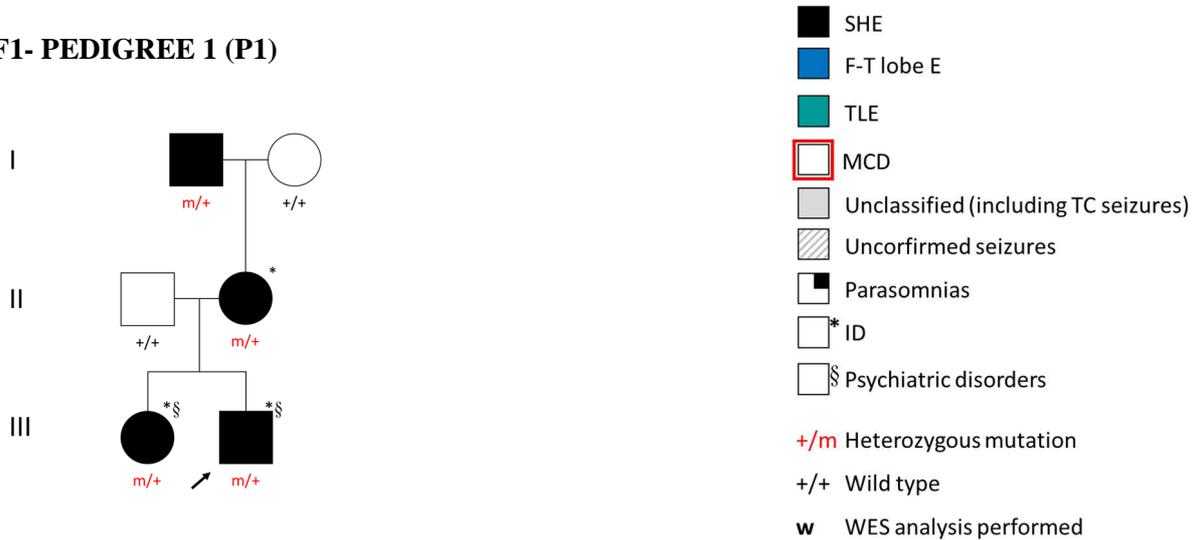
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SUPPLEMENTARY MATERIALS

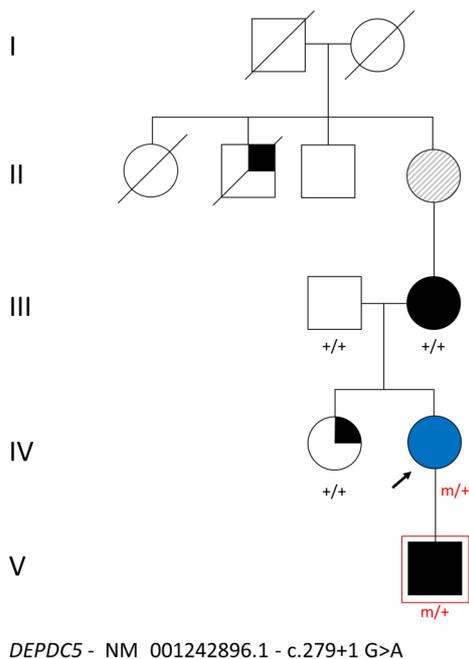
Part A – Mutations identified by the preliminary genetic screening

F1- PEDIGREE 1 (P1)



Extensively described in two papers (Heron et al., 2012; Phillips et al., 1998).

F2 - PEDIGREE 2 (P2)



Family history positive for parasomnias, such as sleepwalking (II.2) or sleeptalking (IV.1); several members belonging to the first/second generation had also sleep-related events of uncertain etiology (II.IV) or were on carbamazepine without clear-cut reasons (I.2, II.1).

III.2 IR, F, 59 yo, right-handed

The patient had the first seizure, at the age of 14 years: she was found by her parents on the floor, near the bed, with limb jerking. She reported that during the event there was no loss of awareness, or incontinence or tongue biting. Before the event she had been dreaming of falling slowly. After this seizure she was hospitalized elsewhere and underwent some investigations, reported as normal; then she was discharged without any diagnosis or medication.

She continued having nocturnal sleep-related events with variable frequency; at 15 years she was started on carbamazepine with seizure control for four years. At the age of 19 years she started having sleep-related events characterized by stiffness of the right limbs which started as a painful rigidity of the foot. Her husband, who witnessed the events, described abrupt arousal with stiffness and mild jerking of the right side lasting a few seconds. Rare seizures occurred during wakefulness. At the age of 41 years she was first admitted to our Institute. NE was unremarkable. Interictal EEG showed epileptic abnormalities over the left frontal field and rare complexes of slow spikes-slow wave of high amplitude, bilateral and prevalent over the anterior regions during stage 2NREM.

During videopolysomnographic (VPSG) recording we captured five seizures arising out of NREM sleep: four minor events were characterized by a slow intra-rotation of the right leg, lasting 3-5 seconds, not associated with an awaking; these events were not associated with clear-cut epileptiform abnormalities. In the major event, the intra-rotation of the right leg was followed by right arm flexion and moaning. This asymmetric dystonic posture was maintained for 8 seconds, then the seizure evolved into a bilateral convulsive seizure. Ictal EEG showed a focal attenuation of the background activity with superimposed a low amplitude activity over the left frontal region and the vertex, then a diffuse and rhythmic spike-wave discharge.

Brain MRI was unremarkable (focal area localized in the left paramedian frontal gyrus at a cortical-subcortical junction, possible ischaemic). The patient has been seizure-free since age 41 on carbamazepine 400 mg/die.

IV.2 HF, F, 36 yo, right-handed

Seizures started at 15 years of age with brief episodes in wakefulness characterized by a sudden feeling of “living in a movie with some characters who wanted to hurt her”. During the seizures she did not seem to recognize her surroundings, but if called she could answer. The events lasted a few seconds and recurred 2-3/week. One month later the patient started experiencing sleep-related events (usually 30 minutes after falling asleep) characterized by a similar aura (sensation of living in a dream) and followed by hyperkinetic motor activity sometimes evolving into a bilateral convulsive seizure.

The seizures progressively reduced in frequency, then they stopped at age 24 years, when she was started on CBZ 400 mg/die.

NE was negative. Interictal EEG showed frequent sharply contoured theta slowing over the left temporal and anterior regions, in particular during drowsiness. VPSG monitoring captured five stereotyped seizures from stage 2 NREM, during which she suddenly woke up, opened her eyes and initially seemed

aware and was able to reply to the technician. A few seconds later, she stared, changed facial expression (“she smiles”) and was unable to answer questions properly. On two occasions this phase was followed by involuntary, “choreic” movements of the left limbs, dysphasia and manual automatisms. The seizures lasted between 40-150 seconds. Ictal EEG before seizure onset showed a diffuse attenuation of the background activity, immediately masked by muscle artifacts. This was followed by a diffuse delta slowing over the right anterior and temporal regions and over the vertex, spreading to both the two hemispheres. Her brain MRI was unremarkable.

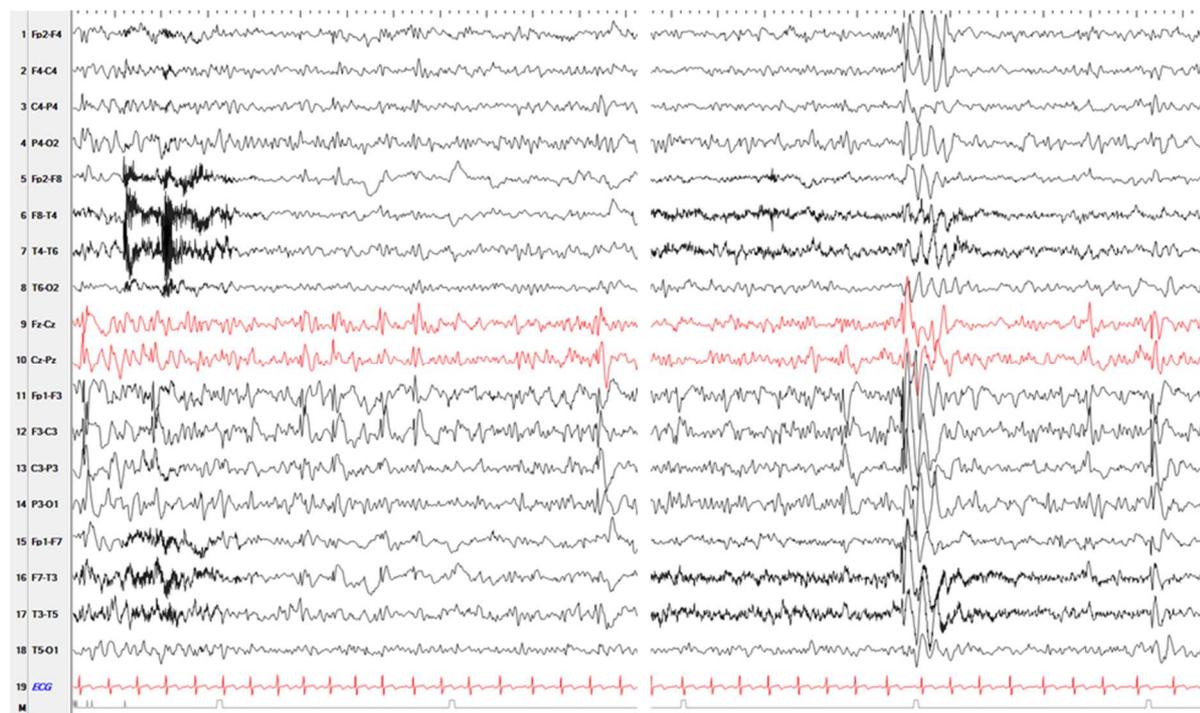
V.1 FV, M, 9 yo, right-handed

The patient was born preterm (at eight months) by cesarean delivery. At age four years he had three febrile convulsions from sleep; valproate was started as prophylaxis, given the tendency to have recurrent tonsillitis. At age five he experienced an episode of loss of consciousness on wakefulness with some “jerks” whilst standing, preceded by a “feeling of tiredness” and followed by fast recovery without no post ictal confusion. This event was first interpreted as convulsive syncope.

However, at age six years, he had several sleep-related episodes characterized by head deviation to the right with stiffness and jerks on the right side. Seizures were initially controlled by carbamazepine, then relapsed.

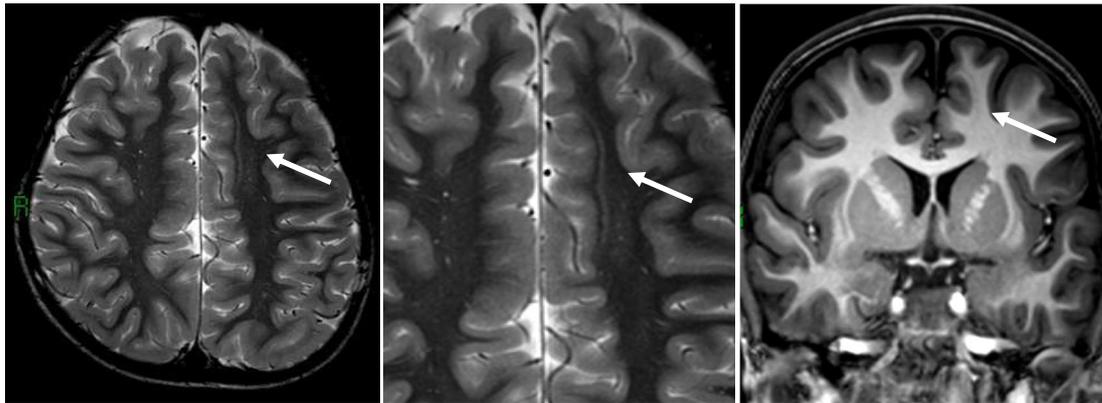
Since age seven years he has experienced seizures also in wakefulness characterized by staring, eyelid myoclonias and loss of consciousness with possible fall to the ground. Seizure are refractory to a combination of valproate and lamotrigine.

Supplementary Fig. 1 Interictal EEG of individual V.1



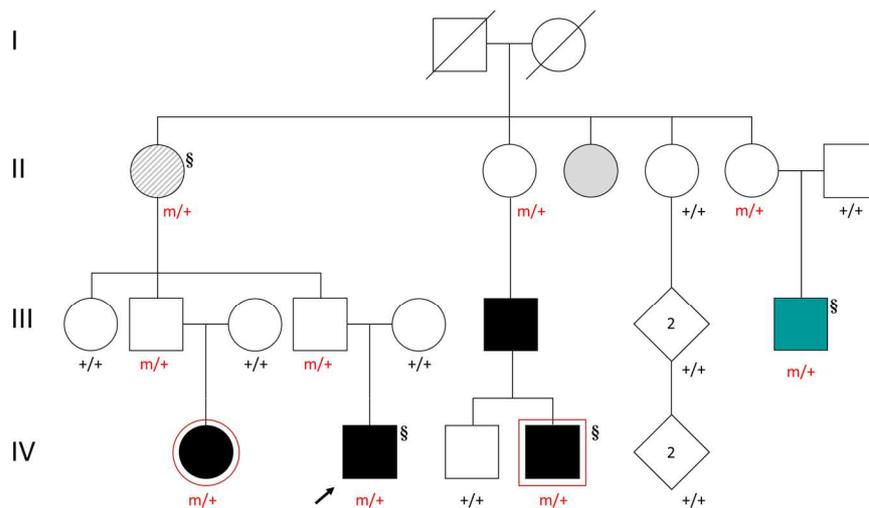
Frequent spike-wave discharges over the left frontal region (sometimes with phase opposition on F3) enhanced by drowsiness and light sleep, spreading to the ipsilateral and contralateral hemispheres.

Supplementary Fig. 2 Brain MRI of the same individual (P2, V.1)



The white arrows point to the unilateral subtle band heterotopia within the white matter adjacent to dysplastic cortex in the left frontal lobe. Blurring of the gray–white matter junction involving part of the cingulate cortex and left frontal cortex was seen.

F3 - PEDIGREE 3 (P3)



DEPDC5 - NM_001242896.1 - p. T329Lfs*7

II.1 LM, F, 79 yo

The patient had rare episodes of uncertain etiology that started at the age of 30 years. The events were characterized by a sensation of anguish and oppression, followed by loss of consciousness. For 20 years she took antiepileptic drugs (phenytoin, carbamazepine and phenobarbital) then stopped without any relapse. She also suffers from generalized anxiety and mood disorders, for which she was on citalopram, lorazepam, and trazodone. Tics (like repetitive blinking and throat-clearing) have been present since adulthood.

II.3 LM, F

Affected by epilepsy –clinical details not available.

III.6 EF, M, 50 yo, right-handed

The patient started having seizures at the age of 12 years. Seizures were refractory from the beginning and were characterized by a sudden arousal from sleep, intense fear with terrified gaze, inability to speak, bilateral gestural automatisms. He experienced rare episodes on wakefulness with the same semiology, where he could fall to the floor. Seizures were multiple per month, despite adequate polytherapy (lamotrigine, carbamazepine, levetiracetam).

The patient underwent a prolonged video-EEG monitoring: interictal EEG showed bitemporal asynchronous spikes; ictal events showed an early bitemporal desynchronization. A clear epileptogenic focus was not identified, therefore he was excluded from a pre-surgical workup. Brain MRI (1.5 Tesla) was unremarkable. At neuropsychological evaluation mild impairment in language, visual orientation and attention emerged, alongside a tendency to deflated mood.

III.8 FDR, M, 34 yo, right-handed

Severe obesity (126 kg).

Since three-four years of age the patient has experienced frequent sleep-related episodes characterized by epigastric ascending aura associated with intense fear and panic, need to escape, embracing his mother and assuming a prone posture. He also had episodes on wakefulness of pleasant or unpleasant feelings associated with forced thinking (which the patient could not recall nor describe), without loss of awareness. Duration was 5-10"; at that stage seizures were multiple per day and occurred mainly during sleep. At five years of age he started therapy, without seizure control despite several AEDs in different combinations. Since age 15 years seizures have become predominant during wakefulness, maintaining the same semiology.

The patient underwent a prolonged video-EEG monitoring with the recording of independent seizures arising from both left and right fronto-temporal regions. Brain MRI (1.5 Tesla) was unremarkable. Therefore he was excluded from a pre-surgical workup. Neuropsychological evaluation showed normal IQ (total IQ: 99, verbal IQ: 102, performance IQ: 96).

Since the age of 28 years he has developed a severe psychiatric disorder (depression) that worsened at 30 years of age, when he was hospitalized for psychotic symptoms (auditory, visual hallucination and paranoid behavior). His medication for epilepsy and psychiatric symptoms included high doses of carbamazepine, valproate, lacosamide, paliperidone, clonazepam, and clotiapine.

IV.1 JDA, F, 13 yo, right-handed

Obesity and hypothyroidism in therapy.

The patient was born by dystocic delivery for acute fetal distress (Apgar: 6 at first minute, 8 at 10th minute) followed by mild motor delay.

She presented at three months of age with episodes during drowsiness characterized by a stereotyped "smile" sometimes associated with trismus lasting a few seconds with vomiting at the end. The frequency increased progressively and an EEG documented an episode characterized by "face muscles contraction to the left" associated with a "recruiting theta activity over the fronto-temporal field, then generalized". She was diagnosed with "focal spasms" and started on vigabatrin, with partial control.

The drug was stopped 6 months later. However, during sleep she continued experiencing episodes consisting of arousal, eye opening, trismus and right limbs rigidity.

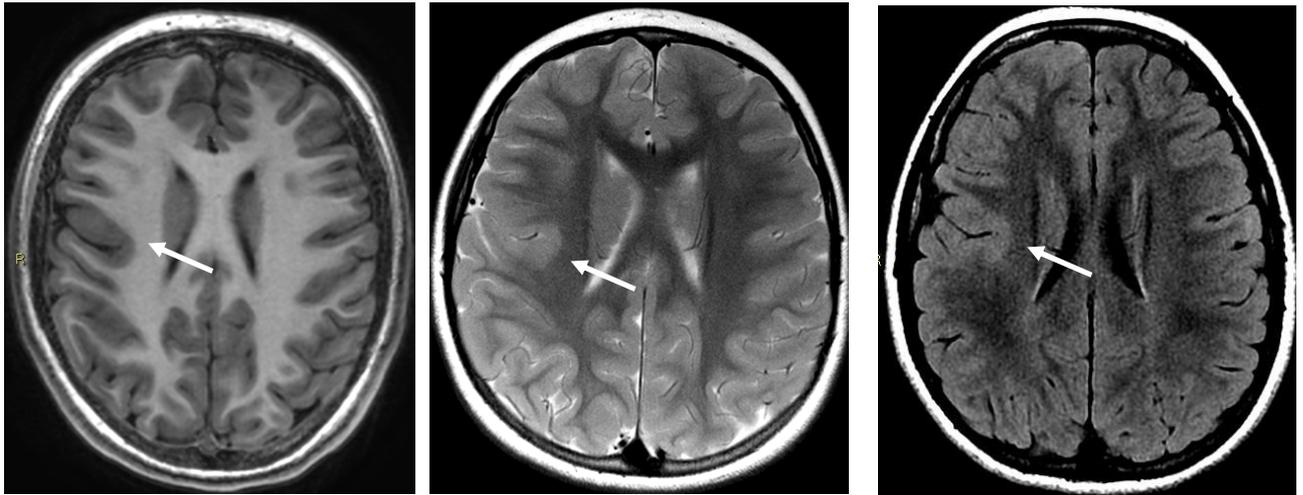
At the age of 4 years she experienced seizures in wakefulness characterized by stiffness and jerks on the right hemibody with possible falls. On one occasion she had a clusters of repetitive seizures stopped by BDZ, followed by hyposthenia of the left hemibody for three days. She was started on topiramate, later associated with valproate for poor seizure control. At that stage (five years) she experienced brief seizures occurring several times per night with different semiology: she felt a sensation of “abdominal tremor” and woke up crying, with open arms, sometimes associated with apnoea and cyanosis. Later the sleep-related events started with a respiratory sound, asymmetric tonic posturing with abduction and open widen limbs, eyes deviation, open month with drooling, jerks of the right side, sometimes spreading to the left. Possible post-ictal left hyposthenia. These events occurred several times per night, exclusively from sleep.

NE at time of her first hospitalization at our Institute (six years) was normal. Interictal EEG showed a rhythmic theta-delta activity over the right fronto-central region and the anterior vertex. During Video-EEG monitoring, an asymmetric tonic seizure with right side stiffness was recorded. Several brain MR scans were reported as unremarkable and neuropsychological assessment showed a normal IQ. She was started on carbamazepine with initial reduction of frequency (to two-three seizures/month) and intensity (deep inspiration with apnoea and diffuse stiffness lasting a few seconds, exclusively from sleep) of the seizures. Interictal EEG showed paroxysmal activity over the left fronto-central field and the vertex.

Two years later (eight years) the seizures returned multiple per night, despite further therapeutic changes. On several occasions she had seizures with moaning, apnea, upper limbs extension and stiffness, open month, staring gaze, lasting 15 seconds and recurring several times every 4-5 minutes. Following one of these episodes that required administration of BDZ, she was hospitalized.

Interictal EEG showed a diffuse slowing of background activity; during sleep, epileptiform abnormalities over the left centro-(fronto) temporal field diffuse to the vertex and the right fronto-central region. VPSG monitoring documented an asymmetric tonic seizure prevalent on the left and lasting 20 seconds followed by four-five bilateral jerks, lasting 50 seconds. Ictal EEG showed an initial diffuse attenuation of the EEG tracing prevalent on the left then slow waves and rhythmic activity prevalent on the left (during the tonic phase) and diffuse slow waves (during the clonic phase) associated with severe tachycardia and tachypnea, then apnea. Post-ictally, the EEG trace was characterized by a diffuse slowing, prevalent on the right, with interictal slow spikes on the left hemisphere. One further minor event with brief upper limbs hyperextension was captured, associated with a rhythmic theta activity on the vertex and central fields, followed by a diffuse slowing prevalent on the right. Brain MRI showed a focal thickening of the right medium temporal gyrus, compatible with FCD (Supplementary Figure 3). At last assessment (13 years of age) she continued experiencing multiple weekly sleep-related seizures despite a combination of levetiracetam, carbamazepine and clobazam.

Supplementary Fig 3 Brain MRI of individual IV.1



IV.2 MDA, M, 25 yo, left-handed

The proband was born by caesarean section; at birth, umbilical cord wrapped around the fetus' neck with cyanosis. The subsequent developmental milestones were normal. Obesity (current weight 129 kg) and OSA syndrome have been present since adolescence and he underwent adenoidectomy at the age of 11 years. He also has a history of gastro-esophageal reflux.

At the age of 7 years he had a single episode from sleep: he suddenly woke up with a sensation of breathlessness, sat up in bed and then lost consciousness. Two EEG performed at that stage were normal. At 17 years sleep-related seizures of variable duration and intensity re-appeared: (i) brief abrupt arousal with breathlessness sensation, multiple/week (ii) episodes starting with the same aura followed by flailing movements with legs, sleepwalking (he woke up and went to his parents room), inability to speak, possible repetitive movements of punching with left hand while the right one is carried to the tongue, head deviation to the right and loss of consciousness with limbs stiffness and jerks. During the post-ictal period the parents reported confusion, language deficit for 6 minutes and possible left hyposthenia. The latter events lasted 3 minutes and occurred about once every 2 months.

Alongside the epileptic disorder, the patient had a psychiatric comorbidity (generalized anxiety disorder) and multiform tics with repetitive blinking, throat clearing, shoulder jerking since early adulthood. The tic disorder persists despite polytherapy with pimozide, delorazepam, clonazepam, and pregabalin. Moreover, between 7 and 17 years he experienced episodes on wakefulness of unclear etiology, characterized by objective vertigo and a cephalic sensation of confusion, without loss of consciousness, lasting 1-2 minutes with a frequency of two-three per day, temporarily controlled by music therapy.

At 18 years of age he was first admitted at our Institute. NE was normal. Interictal EEG showed some bursts of rhythmic, sharply-contoured theta activity over the right fronto-polar region during drowsiness and light sleep and groaning. Another video-EEG monitoring, performed after a focal seizure with secondary generalization, showed a diffuse theta-delta slowing, predominant over the right anterior regions, with intermixed diffuse fast activities and slow spikes over the right fronto-polar and central

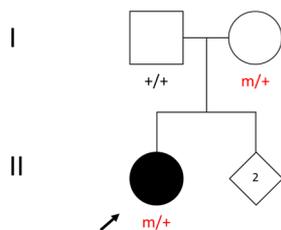
fields. An electric seizure characterized by a fast activity at 10 Hz over the right fronto-centro-parietal fields was also recorded. During VPSG recording we captured a seizure from stage 3 NREM sleep characterized by sudden arousal sitting on the bed, extension of the right arm, tachypnea and dyspnea, repetitive fast movements of hyperextension of the right leg, nodding and rhythmic swinging of the left leg. The event lasted 20 seconds and was associated with tachycardia. Ictal EEG was uninformative because of muscle artefacts. Brain MRI (1.5 T) was normal. A cognitive behavioral assessment showed aspects of phobic anxiety with obsessive thoughts. He was diagnosed with SHE and started on small doses of carbamazepine, 200 mg/die. Since then he has been seizure-free.

IV:4 MF, M, 19 yo, right-handed

Seizure onset was at age three years. Seizures were mainly sleep-related and characterized by arousal, terrified gaze, yelling and a tendency to turn on the right side, sometimes with pedaling movements. He also had rare diurnal seizures, with intense fear sensation associated with epigastric ascending aura, need to escaping and embracing persons around him, sometimes associated with oral automatisms (chewing). His current anti-epileptic therapy includes oxcarbazepine, valproate, clobazam and lacosamide.

Interictal EEG showed bilateral frontal spikes and slow waves. A prolonged video-EEG monitoring documented several seizures from sleep arising from the right frontal region. Brain MRI (1.5 Tesla) showed a suspected gyral abnormality of the right middle frontal gyrus. Neuropsychological testing showed mild visual-spatial memory deficit with preserved intelligence (total IQ: 83; performance IQ: 99; Verbal IQ: 73). The patient developed a substance abuse disorder (heroin).

S1 - PEDIGREE 4 (P4)



DEPDC5 - NM_001242896.1 - p.R165Yfs*14

II.1 MB F, 53 yo, ambidextrous

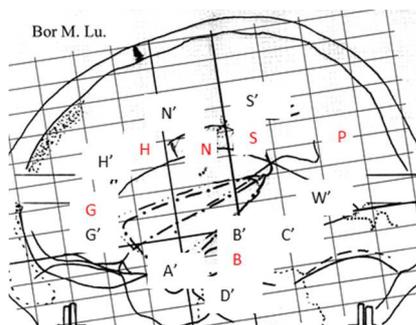
The patient was born by dystocic delivery (use of forceps), followed by normal developmental milestones. She presented at the age of 10 years with seizures in wakefulness: she turned right round with a sensation of depersonalization, a funny sensation of dizziness that made her laugh. Interictal EEG at that time showed right fronto-temporal abnormalities and she was started on carbamazepine and phenytoin. Six months later she had episodes during which she could fall down slowly from a standing position or suddenly drop her head, extending her arms. Both these events lasted a few seconds and were followed by rapid recovery and complete recall.

Since 15 years of age she has experienced sleep-related hypermotor seizures, sometimes heralded by the same sensation as the daytime events, and episodic nocturnal wanderings. Seizures in wakefulness persisted, and were described as a sensation of instability, of “being physically on the edge of an abyss opening on the right side” associated with fear and tachycardia and sometimes followed by grasping or repetitive movements of the left arm. Throughout adult life, nocturnal attacks persisted at higher frequency (up to several times per night) despite trials with different antiepileptic drugs. Daytime seizures occurred occasionally, during changes in therapy.

At time of our first evaluation (37 years) NE was normal. Interictal and ictal EEG showed epileptiform abnormalities over the fronto-central-parietal regions, occasionally with a left emphasis. During nocturnal VPSG monitoring we recorded eight seizures from stage 2NREM/REM sleep and one during wakefulness. The events were characterized by hyperkinetic movements mainly involving the lower limbs (in particular the right one), flexion of the right arm behind the head (while the left one was maintained in the initial position and only in one episode it appeared stiff), sometimes associated with head/ body orientation to the right. The single episode that occurred in wakefulness was preceded by a warm sensation in the whole body and grasping. Ictal EEG tracing was first masked by muscular artefacts, then showed a fast activity in the vertex and frontal regions without a clear lateralization. Brain MRI was unrevealing.

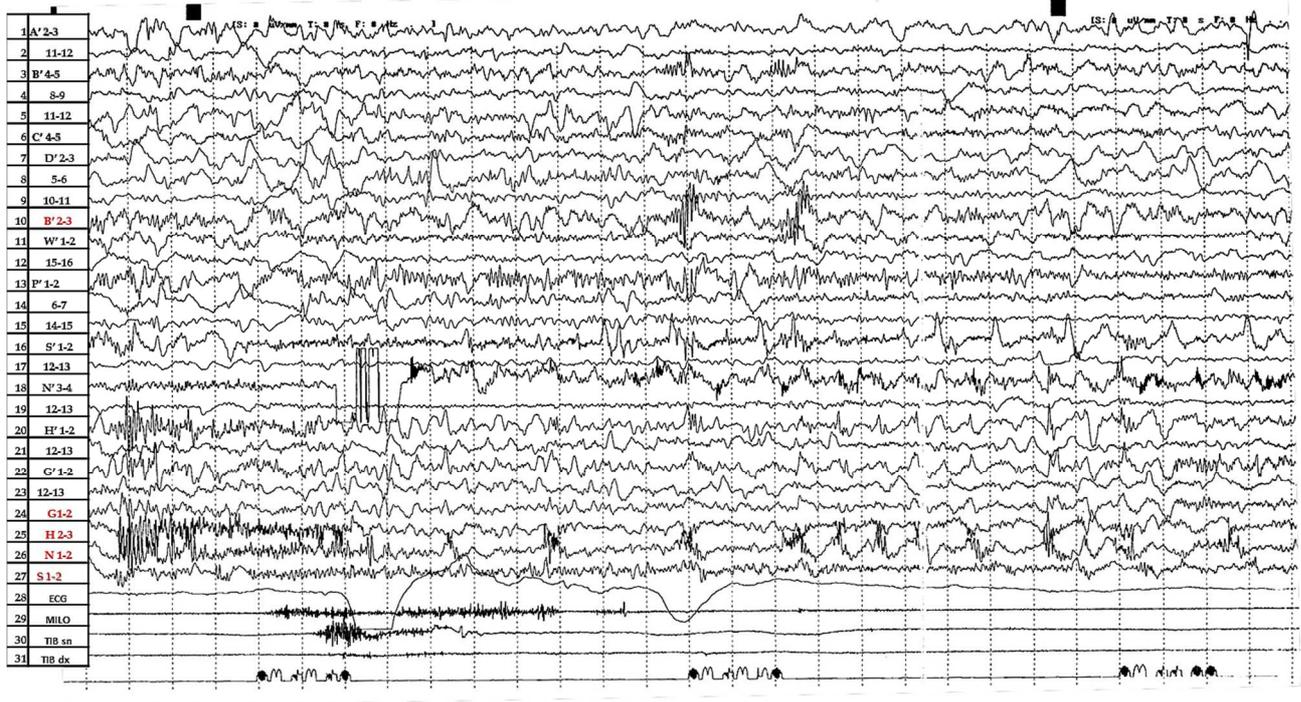
At the age of 43 years the patient underwent stereo-EEG study with bilateral limbic exploration extended to the inferior parietal lobe (Supplementary Figure 4). The study showed bilateral ictal and inter-ictal activity, prevailing on right central-anterior cingulate cortex (H electrode); the ictal activity could be synchronous over anterior cingulate cortex or arising from both anterior mesial cortex. The electrical pattern was suggestive for focal cortical dysplasia (Supplementary Figure 5). However, tailored brain MRI (1.5 T) was unrevealing.

Supplementary Fig. 4 Stereotactic scheme of the patient



The stereo-EEG exploration shown in this figure (lateral view) included 15 intra-cerebral electrodes implanted mainly on the left. The EEG focus area was mainly explored by electrode H. Black letters with the accent (A', B', C', D', G', H', N', S', W') indicates left side; red letters (B, G, H, N, S, P) indicated the right.

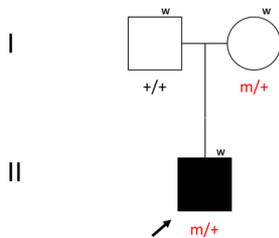
Supplementary Fig. 5 SEEG ictal recording



Fast polyspike activity over bilateral anterior-mid cingulate gyrus preceding typical nocturnal hypermotor seizure. Note that interictal activity is recorded also in electrodes remote from ictal onset zone

Part B –WES analysis findings

S2- PEDIGREE 5 (P5)



DEPDC5 - NM_001136029.2 - c.193+1G>A

II.1 RP, M, 9 yo, right-handed

The patient is followed up by a colleague from the “Civili Hospital” of Brescia who reported a positive family history for epilepsy in the paternal branch (two first-degree cousins with seizures from sleep but no more information was available).

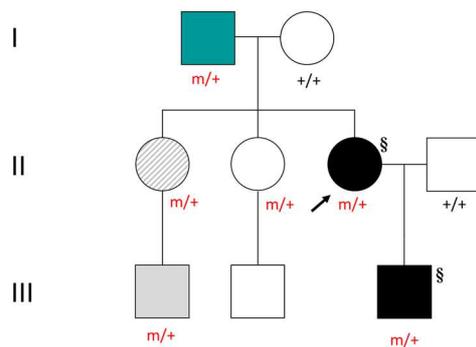
The patient had a history of hypermotor seizure occurring predominantly during sleep (only two events on wakefulness). Interictal EEG showed epileptic abnormalities (sharply waves, sharply-contoured slow waves or slow sharps) over the right frontal and fronto-temporal fields.

VPSG at 6 years of age (15/12/2010), during which an episode from sleep was captured: he moved and raised the right arm, then had a body rotation followed by a fall out of bed. Immediately after the seizure

he appeared confused and aphasic for 10 seconds. Ictal EEG showed an arousal followed a fast activity over the right anterior region, intermixed by a rhythmic theta activity lasting about 10 seconds. The post-ictal phase is characterized by focal monomorphic theta activity followed by slow waves with the same topography.

He was started on carbamazepine 400 mg, with immediate seizure control. In September 2013 (age 9 years) the drug was stopped and he was seizure-free for 2 years, then seizures relapsed. His NE was normal. Brain MRI was unremarkable.

F4 - PEDIGREE 6 (P6)



NPRL2 - NM_006545.4 - p.L105P

I.1 UL, M, 74 yo

At age nine years the patient started experiencing two kinds of events: (i) sleep-related seizures characterized by head deviations to the right/left, moaning, staring with “emotionless” facial expression, hyperextension and raising of the upper limbs and lower limbs stiffness, then diffuse jerking, trismus, and loss of consciousness, drooling and sometimes incontinence. In the post-ictal period, stertorous breathing and confusion for 1-2 minutes, then he went back to sleep. The following day he was amnesic for the event but he felt dull and weak; (ii) rare episodes in wakefulness, characterized by a change in facial expression and staring, diffuse stiffness and limb jerking with possible falls.

At 25 years of age, on phenobarbital 200 mg and dintoine 400 mg, seizures occurred two-three times per month, mainly in sleep. At 28 years he took dintoine 400 mg/die with a reduction of seizure frequency (two-three every 2 months). The AED was stopped at age 62 years without relapse.

II.2 EL, F, 47 yo

This individual experienced brief episodes of “staring” at three years of age. She was hospitalized and underwent several investigations, including a lumbar puncture; she was discharged without any antiepileptic medication. No additional medical information related to that episode was available. The patient has never had further paroxysmal events lifelong.

II.3 PL, F, 48 yo, right-handed

The proband was born at term by eutocic delivery and had normal developmental milestones. She suffered from anxiety with previous panic attacks. Since the age of 5 years she has experienced sleep-

related seizures of variable intensity and duration: (i) in the minor events she woke up due to a sensation of discomfort, she felt a heaviness/painful sensation of the left arm and a sensation of freshness coming out from the head, then the event might stop; (ii) in the middle episodes, the arousal with the left arm paresthesia was followed by contraction of facial muscles, raising of the left arm (sometimes also the right one) and hyperextension of the legs. During the events, lasting about 5 seconds, she is aware, although unable to reply.

(iii) the major events are characterized by the same aura (paresthesia at the left arm and whole head) and auditory hallucination (sound increasing of intensity) followed by bizarre disorganized limb and pelvic movements for 10-20 seconds. In the post-ictal period she could experience hyposthenia of the left arm (sometimes, left leg). The events occurred exclusively from sleep.

At onset she had two-three seizures/week, with up to six events on the same night. Seizures were refractory to several AEDs (phenobarbital, carbamazepine, topiramate, clobazam): the frequency of minor events ranged from monthly to weekly, with possible periods of seizure freedom for months, while major events were rare (last one at about 20 years of age). Frequency at last assessment (48 years) was of multiple events per month, despite a combination of three AEDs.

Interictal EEG showed rare spike-wave discharges over the right frontal fields during sleep. During VPSG recording at age of 22, we captured a motor event from stage 3NREN characterized by abduction of the arms, upper limbs hyperextension and eye opening, hyperextension of the whole body arching of the trunk with deviation to the right, abduction and hyperextension of the lower limbs, raising of the left arm, limbs stiffness and jerks prevalent on both the upper limbs and on the left leg. The seizure lasted 43 seconds after which she could reply promptly. The EEG trace immediately before the seizure showed a single burst of diffuse delta waves, then an arousal for 30 seconds, followed by a diffuse spike/spike-wave discharge prevalent anteriorly and on the right lasting 20 seconds, associated with tachycardia and tachypnea. Brain MRI was unrevealing.

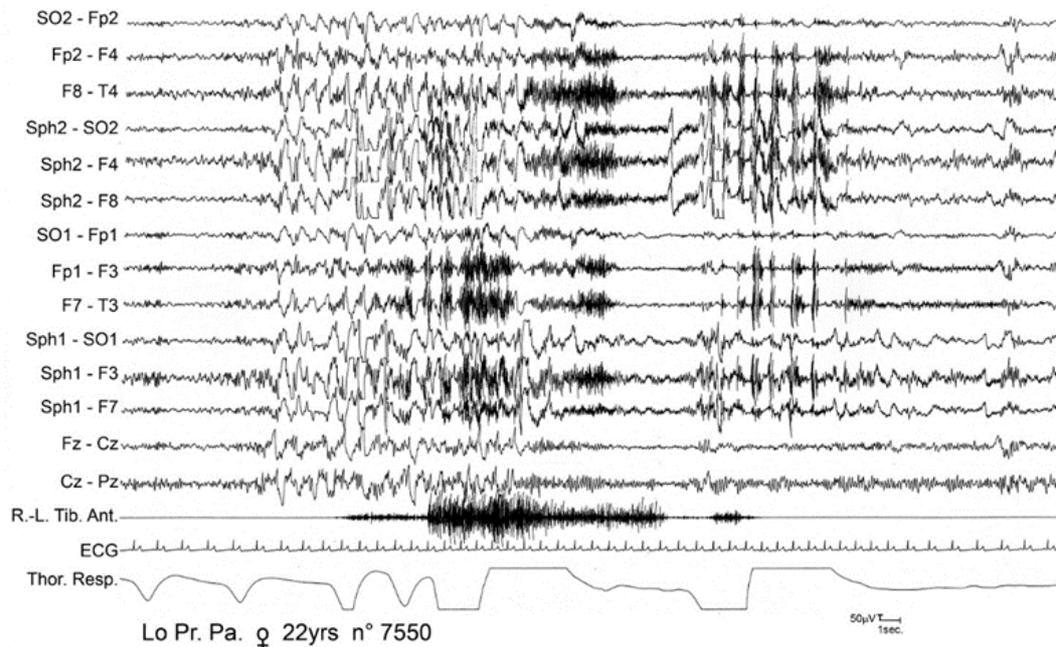
III.1 SC, M, 13 yo, right-handed

The patient had normal delivery and psychomotor development. He had a history of motor “tics” (like blinking and forceful, stereotypic movements of the shoulder). On specific questioning, the mother reported sleep-talking and, at age 12-13 years a few episodes from sleep characterized by sudden, brief arousal during which he sat on the bed and then went back to sleep. At 13 years of age he experienced a few sleep-related seizures reported as GTCS. During the first one his father, who was sleeping with him, was awakened by a squeaky bed sound: the patient had disorganized body movements and could not reply. Then he presented a loss of consciousness with ocular revulsion, stiffness and jerks, and drooling. In the ER an EEG and brain CT scan were negative.

A few months later he was hospitalized (elsewhere) and underwent an EEG after sleep deprivation that showed sharply-contoured abnormalities, asynchronous over the fronto-temporal fields and bursts of diffuse sharply slow waves. He was diagnosed with probable GGE and started on valproate 800

mg/die. Brain MRI was normal. At the following visit he had been seizure-free and EEG showed a clearcut reduction of interictal abnormalities.

Supplementary Fig. 6 Ictal EEG of the proband (II.3) during one of the minor episodes recorded.



Ictal EEG showing a high amplitude sharply contoured slow waves/spike and wave discharge, diffuse but prevalent over the right frontal field.

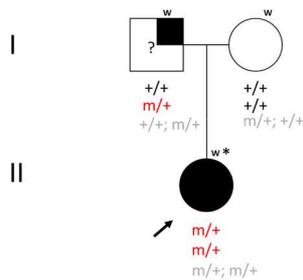
III.3 ML, M, 21 yo, right-handed

At the age 2-3 years, the patient had a few brief episodes of “blinking out” without automatisms followed by sleep. No medical investigations were done at that stage. On specific questioning, his parents reported sleep-talking and “movements” during sleep since the age of 13 years.

The patient first came to our attention at age 19 years, following the first convulsive seizure on sleep: the father, alerted by a noise, found him on the ground presenting bizarre movements of the upper limb, hyperextension and repetitive movements/jerks of the left leg and moaning for about one minute; then he was atonic and asleep and was reawakened by his father: this post-ictal phase was characterized by confusion, fluent aphasia and muscle pain with a possible strength defect of the right leg and agitation, lasting 10-15 minutes.

Following this seizure, a prolonged nocturnal home-made video recording captured several bizarre motor events from sleep, nearly every night, up to four in the same night. Interictal EEG was unremarkable. VPSG monitoring documented a hypermotor seizure from stage 3NREM, characterized by abrupt arousal and asymmetric tonic posturing prevalent on the right arm. Ictal EEG showed a rhythmic theta activity over the left frontal fields. He was started on low dose carbamazepine, with seizure control (documented also by prolonged nocturnal home-video recording). The patient had also a history of obsessive-compulsive disorder at age 18 years, successfully treated by cognitive-behavioural therapy.

S3 - PEDIGREE 7 (P7)



KCNT1 - NM_020822.2 - p.A934T

CHRNA4 - NM_000744 - p.D104N

TMPRSS3 - NM_032404 - p.P277L

TMPRSS3 - NM_001256317 - p.H70T fsX19

II.1 CS, F, 20 yo, right-handed

The 59-year-old father (I.1) had a history of sleeptalking and sleepwalking during childhood that started at 6 years of age and progressively stopped during his teens. However, between 30 and 40 years of age his wife reported “agitated sleep”, with events of different intensity and duration: (i) brief, abrupt arousal during which he sit up in the bed with a jump and then went back to sleep; (ii) longer events during which he screamed, terrified and could present motor behavior correlated to a congruous dream. The frequency at the beginning was of two-three per month, rarely two on the same night.

The proband was born at term by a eutocic delivery. At 18 months of age she was diagnosed with a congenital profound sensorineural hearing loss. Sequencing analysis of the gene *GJB2* (coding for connexin-26) and analysis for the deletion involving del*GJB6*-DS13S1830 of *GJB6*, coding for connexin 30, were negative; array-CGH was normal. Despite implantation of bilateral external prosthesis, her developmental milestones were characterized by a cognitive delay with predominant language impairment.

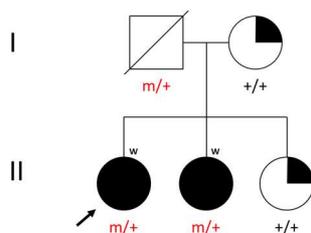
She began having seizures at the age of 9 years, when she experienced two events from sleep consisting of a sudden arousal, eye opening, diffuse rigidity and limbs jerks lasting less than 1 minute. A video-EEG monitoring captured eight episodes on the same night. She was diagnosed with focal epilepsy and after oxcarbazepine was started she was seizure-free for 2 years. At age 11 years seizures relapsed and she was treated with a combination of oxcarbazepine and clobazam, with seizure control for 16 months. In the following years, her epilepsy showed an intermitting pattern, with a relapsing period about every 2 years (at age 13 years and 6 months, 15 years, 17 years, 19 years) during which she could have multiple events per night (up to 40/night) for 2-6 months, followed by prolonged periods of seizure control obtained by small therapeutic changes.

The events were documented by several videopolysomnographic recordings performed elsewhere; in particular at the age of 17 and 19 years, more that 15 and 40 seizures, respectively were captured. The Ictal EEG did not showed clearcut epileptic abnormalities.

During VPSG performed at our first evaluation (21 years of age) we captured 20 motor events from stage 2-3 NREM sleep, mainly characterized by a slow and sustained asymmetric tonic contraction of the right limbs, with hyperextension and abduction of the leg and flexion of the arm lasting 20 seconds, associated with tachycardia and changes in respiratory rate. In some events (when she was lying on the right side) the tonic contraction involved both lower limbs but was prevalent on the left. On the EEG tracing, before the motor event there was a fast low amplitude activity over the anterior fields, followed by a run of sharply contoured slow waves of high amplitude, diffuse but prevalent over both the fronto-central regions; then the trace became diffusely flattened and masked by muscle artifacts.

The neuropsychological assessment (including Mini Mental State Evaluation and the Wechsler Revised (WAIS-R) test showed an ID with higher scores at the performances subtests compared to the verbal ones (total IQ: 62; performance IQ: 81; verbal IQ: 56). This difference might also be related to the impact of hearing loss on language performance. Brain MRI was normal.

F5- PEDIGREE 8 (P8)



CHRNA4 - NM_000744.6 - p.G307V

II.1 FB, F, 44 yo, right-handed

Family history for NREM parasomnias (sonnambulism) in the mother. The father experienced a single event of loss of consciousness when he was 13 year old. At the age of 8 years, she started experiencing motor events in sleep, characterized by moaning, twisting movements “like a contortionist” sometimes followed by perioral myoclonia, blinking or jerks of the left hemibody. Occasionally she would fall out of bed. The events lasted 1 minute, usually occurred during the first part of the night, every night, up to five-six/night. If awakened, she reported being afraid and did not recollect a dream. She was diagnosed with “pavor nocturnus”.

Between age 9 and 10 years the episodes disappeared spontaneously. Then, at the age of 10 years, she presented different events described as an arousal with a diffuse sensation of stiffness especially on the mouth muscles, throat noise, elevation of the back on the bed and limb jerking, without loss of awareness. The seizures lasted less than 1 minute and recurred multiple times per night, every night. After being on carbamazepine and valproate, seizures ceased for 4 years.

At the age of 14 she had a status epilepticus treated with diazepam. After a few months seizures stopped and she was seizure-free for 6 years. In the following years, she reported a long-lasting period of

remission, interrupted by seizure relapse without apparent causative factors. At the age of 25 years she was hospitalized in our Institute: at that time she had 15-20 seizures/night.

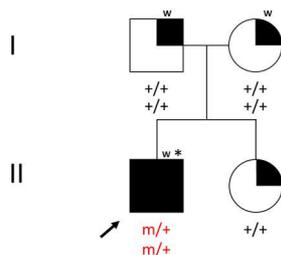
NE was normal. Interictal EEG showed theta activity over both temporal regions, prevalent on the left; during drowsiness, burst of sharply contoured theta activity on the anterior fields, sometimes with a right emphasis. VPSG recorded five seizures from NREM sleep consisting of eye opening raising the head, pedaling movements and back arching with vocalization. Ictal EEG was uninformative. Brain MRI showed two small subcortical areas of T2 hyperintensity on the right frontal area. Neuropsychological assessment was normal. At present she has monthly seizures despite a combination of carbamazepine 600 mg/die, topiramate 200 mg/die and clobazam 10 mg/die.

II.2 MB, F, 39 yo, right-handed

Seizure onset was at age 7 years. She had two-three events in wakefulness of brief loss of contact with staring. After one month, there were three additional episodes in a day characterized by sudden fall to the ground, floppy, with immediate recovery. She was started on carbamazepine and phenobarbital with seizure freedom for 13 years. At the age 20 years, following therapy withdrawal, she started experiencing episodes of “sleepwalking” and “sleeptalking”, multiple/week. At 25 years of age the nocturnal events became very frequent, nearly every night and sometimes several times/night: she woke up while standing near the door of her room, hand to neck due to a sensation of suffocation. On one occasion she fell out of bed. A home video recording at that time documented several episodes (four-five in 1 night) during which she sat on the bed, bringing her right hand to her forehead, she looked around for some seconds, then fell asleep. She was started on carbamazepine, up to 600 mg/die with seizure control.

NE was normal. Interictal EEG showed left fronto-temporal abnormalities. VPSG recorded three paroxysmal arousals from NREM sleep sometimes associated with pelvic thrusting or oral automatisms. The EEG trace was masked by muscle artefacts, then showed a diffuse, rhythmic delta activity prevalent anteriorly. Brain MRI was normal. Neuropsychological assessment was normal except for two mistakes in tests evaluating phonemic fluency and visuo-spatial abilities. At present she has two-three seizures/month during menses, on carbamazepine 600mg/die.

S4 - PEDIGREE 9 (P9)



CHRNA4 - NM_000744.6 - p.S284L
MIOS - NM_019005 - p.A138G

II.1 GDV M, 26 yo, right-handed

Family history positive for sleepwalking and sleeptalking (parents and sister). Preterm birth (34th week) by eutocic delivery. Perinatal respiratory disorder, with frequent apnea in the first days of life requiring pharmacological therapy and ventilatory support. On the third day of life, subependymal hemorrhage was documented by cranial ultrasound that disappeared at the following control at the age of 3 months. Two episodes of cardiocirculatory arrest occurred at the 40th and 50th day of age. Mild psychomotor delay (language); middle school diploma with teacher support. Adeno-tonsillectomy at 6 years, following a diagnosis of sleep apnea syndrome. Headache since the age of 8 years.

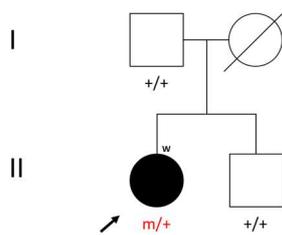
Seizure onset was at 3 years of age. Seizures consisted of a respiratory sound, diffuse stiffness prevalent on the right arm, head deviation to the right, right hand automatism, sometimes followed by ocular revulsion, cyanosis and snorting. The events could be triggered by sudden acoustic or tactile stimuli, occurring during nocturnal sleep and nap. Since the age of 8 years he has experienced events during which he sits up with crossed legs and beats his left/right hand repetitively on the bed, or manual automatisms. The events last 20-30 seconds and occur every night, up to ten/night. He had three episodes of agitated sleepwalking and intense fear. Occasional falls out of bed with secondary traumatism were also reported.

Seizures were refractory to several antiepileptic drugs (carbamazepine, valproate, vigabatrin, acetazolamide, clonazepam, lamotrigine, tiagabine, topiramate, levetiracetam, oxcarbazepine). At our last visit (26 years) he continued experiencing multiple seizures/night despite a combination of oxcarbazepine 1200 mg/die, topiramate 300mg/die and clobazam 20 mg/die.

Physical examination and NE showed stuttering speech, bilateral strabismus, flat feet and hyperlordosis. Interictal EEG showed nonspecific and epileptic abnormalities (sharp-wave complexes or bursts of sharply contoured slow waves) over the vertex, the anterior field and on the left, enhanced by sleep. Repeated VPSG documented many seizures from NREM sleep characterized by hyperextension of the right leg and asymmetric dystonic posturing prevalent on the right lasting 6-12 seconds associated with an apnea and tachycardia. The events were sometimes preceded by nose-rubbing, mainly with the right hand. In some seizures he presented stiffness of the right arm followed by agitated limbs movements. Ictal EEG showed (i) bursts of sharply-contoured theta activity preceding some of the events, then movement artifacts masking the EEG tracing; (ii) a recruiting fast rhythm on the anterior fields with a left emphasis in some episodes; or (iii) sharp-wave discharge on the frontal area followed by a fast activity on the anterior fields and the vertex, followed by a diffuse theta-delta slowing for 20-30 seconds. Brain MRI was normal.

Because of the early bilateral involvement of the ictal discharge (even though prevalent on the left) and the history of perinatal diffuse suffering, a surgical workup was excluded. Neuropsychological assessment at the age of our first assessment (15 years) was normal. However, WAIS was repeated at the age of 26 years (last assessment), showing a total IQ of 47.

S5- PEDIGREE 10 (P10)



CHRNA4 - NM_000744.6 - p.S284W

II.1 AM, F, 31 yo, right-handed

The patient was born by dystocic delivery, followed by normal developmental milestones. She had a history of anxiety-depression syndrome associated with eating disorder (bulimia), severe obesity with insulin resistance from 17 years of age, Hashimoto's thyroiditis on levothyroxine from age 18.

Between 12 and 15 years of age the patient experienced episodes in wakefulness characterized by a sensation of shortness of breath, warm feeling, tachycardia and headache, followed by limb stiffness, salivation with possible loss of consciousness. The duration was unknown; during the first months the events were multiple per day (4/die) then they progressively decreased until disappearing spontaneously. Since the age of 14 years she has also experienced stereotyped episodes from sleep: she suddenly raises her trunk up, crossing her arms across the chest in a stiff posture with moaning or echolalia and flushing. The seizures lasted 1-2 minutes and occurred six-seven times every night. She had several injuries related to the nocturnal episodes. At 25 years of age (2010) she was diagnosed with “left frontal epilepsy” and started on topiramate, later associated with carbamazepine, with poor seizure control. Seizure frequency remained high, despite several additional AEDs (levetiracetam, phenobarbital, clobazam).

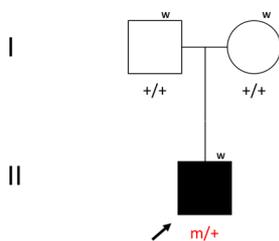
At the time of the first hospitalization in our Institute (27 years) she reported up to seven fits every night; the NE was negative. Interictal EEG was normal (single burst of rhythmic theta activity on the left temporal region). During video-EEG monitoring, we captured a seizure from NREM sleep: she opened her eyes and flexed her neck, raising her head from the pillow with concomitant moaning and diffuse stiffness prevalent on the lower limbs, bilateral grasping, dystonic posturing of the legs, with dorsal extension of the right foot and plantar extension of the left. During this phase, lasting 46 seconds, moaning, echolalia and sometimes swearing would occur. She did not reply to the technician. The seizures stopped abruptly: she had a prompt recovery and she could speak immediately after, although she was amnesic for what had happened during the event. Ictal EEG was immediately masked by muscle artifacts and did not show clearcut paroxysmal changes.

VPSG showed four additional stereotyped seizures from NREM sleep: she raised her head abruptly, widening her legs, then she grabbed the bedrail with the right arm, moaning and screaming, and presented some repetitive movements of pelvis; then she grabbed the bed with her left hand and extended the legs with the right foot in a tonic hyperextension while the left is flexed and intrarotated. She could

have oral automatisms, echolalia and swearing. The events lasted between 35 and 50 seconds and were associated with tachycardia. Afterwards she was immediately aware, despite being amnesic for the event. On EEG tracing, no ictal abnormalities were appreciated. Neuropsychological assessment showed a borderline IQ of 79. Brain CT scan and low-resolution MRI were normal.

Because of the persistence of seizures, up to 15 per night, the patient was recently re-assessed, at 31 years of age. MESAM excluded associated OSA syndrome. Further VPSG monitoring documented several additional stereotyped hypermotor seizures. It was not possible to perform an MRI study for technical limitations (due to the patient's size).

S6- PEDIGREE 11 (P11)



SCN1A - NM_001165963 - p.P824H

II.1 M, 15 yo, right-handed

Family history was positive for febrile seizures (a cousin in the maternal branch). The patient has a normal delivery and psychomotor development. His epilepsy started at 9 months of age with two prolonged generalized tonic-clonic seizures from sleep during hyperthermia (38.3°C). The first seizure lasted about 20 minutes; he was admitted in the ER and treated with diazepam. After 15 minutes he had a second seizure that required diazepam IV. NE was normal; the EEG at that stage was normal. During the second year of age he continued experiencing febrile/afebrile convulsion and was started on valproate, with transitory seizure control.

Since age 2-3 years the patient experienced episodes with different semiology: (i) seizures characterized by staring, oral automatisms, sometimes head deviation to the right, pallor and vomiting, lasting a few seconds; (ii) left hemiclonic seizures. VPSG (elsewhere) at this stage showed rare epileptiform abnormalities over the left temporal field during sleep. At 5 years (iii) seizures with hypotonia, loss of consciousness and fall to the ground, and (iv) frequent absences documented by video-EEG monitoring at age 6 years, recording spike-wave complexes at 1.5 Hz with a delay on patient reply. In the same year the patient experienced a convulsive SE with admission to the intensive care unit. On that occasion antiepileptic drug dosage showed levels of levetiracetam under the therapeutic range.

Between age 8 and 10 years he was seizure-free on valproate and topiramate, then brief seizures reappeared with a frequency of one/week, characterized by a possible sensation of abdominal discomfort, loss of awareness and diffuse atonia. Moreover, since the age of 11 years he had a worsening of school performances with learning difficulties that required a support teacher. Since the age of 13

years he has also experienced sleep-related events characterized by (i) diffuse stiffness, possible vocalization, loss of awareness with a frequency of one/week; (ii) abrupt arousal with repetitive oroalimentary automatisms, multiple/week. VPSG monitoring (elsewhere) documented several seizures arising from sleep; interictal EEG showed a disorganized background activity during both wakefulness and sleep. Previous therapies with lamotrigine, carbamazepine and oxcarbamazepine caused a worsening of seizures; levetiracetam, ethosuccimide, zonisamide, clabazam, lacosamide, and stiripentol were stopped because ineffective or because of adverse effects.

At age 15 years, when the patient was first admitted to our clinic, he presented multiple types of seizures: (i) weekly sleep-related seizures, as described above; (ii) episodes on wakefulness with sudden pallor, yawning, he tended to bring the hand to the abdomen, without loss of awareness; (iii) sporadic episodes with staring, loss of consciousness and possible fall to the ground, diffuse stiffness and possible morsus lasting less than 1 minute. Seizures were resistant to polytherapy with valproate and perampanel. Moreover in recent his parents reported a worsening of cognitive performances with ideomotor slowing and attention and memory deficits.

Ictal EEG showed a marked slowing of the background activity and epileptiform abnormalities, synchronous/asynchronous over both fronto-temporal regions, with a left emphasis, exacerbated by sleep. During VPSG no sleep-related events were captured. Brain MRI was unremarkable. Neuropsychological testing showed a moderate ID, with a significant worsening of cognitive performances compared to the previous assessment (age 10 years).

Supplementary Tab. 1: List of the DNMs identified in the SHE trio cohort.

Trio	Gene	Transcript	AA_change	ExAC	Polyphen2	Sift	Cadd	Classification	Segregation
T1	MAP2K3	ENST00000342679	p.Thr117Met	8,24E-06	Probably_damaging	Deleterious	4,21999979	VUS	De novo
T2	GSE1	ENST00000253458	p.Arg425Trp	None	Probably_damaging	Deleterious	4,659999847	Possibly pathogenic	De novo
T3	VPS13D	ENST00000613099	p.Glu2073*	None	NA	NA	5,03000021	Possibly pathogenic	De novo
	ZNF687	ENST00000336715	p.Phe572Val	1,66E-05	Probably_damaging	Deleterious	3,980000019	VUS	De novo
	ZDBF2	ENST00000374423	p.Ser41Gly	8,29E-06	Benign	Tolerated	-0,52700001	VUS	De novo
T4	HTR3D	ENST00000382489	p.Leu249His	None	Probably_damaging	Deleterious	3,289999962	VUS	De novo
	TDP2	ENST00000378198	p.Ala14Ala	None	NA	NA	1,899999976	VUS	De novo
	PHKA1	ENST00000339490	p.Arg688Trp	3,46E-05	Possibly_damaging	Deleterious	5,579999924	VUS	De novo
T5	WDR4	ENST00000398208	p.Leu314Arg fs*20	None	NA	NA	-6,760000229	VUS	De novo
T6	MIB1	ENST00000261537	p.Asp871Asp	None	NA	NA	6,03000021	VUS	De novo
T7	CHRNA4	ENST00000370263	p.Ser284Leu	None	Probably_damaging	Deleterious	5,059999943	Pathogenic	De novo
	MIOS	ENST00000340080	p.Ala138Gly	None	Possibly_damaging	Tolerated	5,849999905	Possibly pathogenic	De novo
T8	ZNF44	ENST00000356109	p.Cys529Arg	None	Probably_damaging	Deleterious	-0,070299998	VUS	De novo
T9	LAMA2	ENST00000421865	p.Asn358Asn	None	NA	NA	0,89200002	VUS	De novo
	PDE2A	ENST00000334456	p.Ser663Phe	None	Probably_damaging	Deleterious	5,889999866	Possibly pathogenic	De novo
	SH3KBP1	ENST00000397821	p.Ile302Ile	2,46E-05	NA	NA	-0,150999993	VUS	De novo
T10	PDE4DIP	ENST00000313382	p.Pro50Pro	7,33E-04	NA	NA	-9,619999886	VUS	De novo
	CUBN	ENST00000377833	p.Leu2050Leu	None	NA	NA	-4,380000114	VUS	De novo
	GPR162	ENST00000311268	p.Ser95Phe	None	Probably_damaging	Tolerated	4,130000114	Possibly pathogenic	De novo
T11	KIAA1462	ENST00000375377	p.Thr272Met	2,49E-05	Benign	Tolerated	-2,579999924	VUS	De novo
	S1PR4	ENST00000246115	p.Glu153Lys	8,11E-05	Benign	Tolerated	1,99000001	VUS	De novo
	KRTAP12-2	ENST00000360770	p.Cys105*	None	NA	NA	0,093000002	VUS	De novo
T12	PIK3C2B	ENST00000367187	p.Glu1294Gln	None	Benign	Tolerated	5,889999866	Possibly pathogenic	De novo
	SURF6	ENST00000372022	p.Arg120*	8,54E-06	NA	NA	3,710000038	VUS	De novo
T13	PPIL2	ENST00000412327	p.Thr383Met	8,24E-06	Probably_damaging	Deleterious	4,650000095	VUS	De novo
	LMTK3	ENST00000270238	c.448+1G>A	None	NA	NA	4,340000153	Possibly pathogenic	De novo
T14	ZNF576	ENST00000336564	p.Gly68Arg	None	Probably_damaging	Deleterious	3,930000067	VUS	De novo
T15	SCN1A	ENST00000303395	p.Pro824His	None	Probably_damaging	Deleterious	4,659999847	Pathogenic	De novo
	CDC73	ENST00000367435	p.Gly449Val	None	Probably_damaging	Deleterious	5,449999809	VUS	De novo
	DCAF12	ENST00000361264	p.Arg203Cys	8,39E-06	Probably_damaging	Deleterious	5,510000229	VUS	De novo

T16	UBN2	ENST00000473989	p.Lys1201Asn fs*18	None	NA	NA	4,309999943	Possibly pathogenic	De novo
	SIGLEC10	ENST00000353836	p.Pro262Ile	1,45E-03	Benign	Tolerated	2,109999895	VUS	De novo
T17	C12orf54	ENST00000314014	p.Phe50Leu	None	Possibly_damaging	Deleterious	5,059999943	VUS	De novo
	FRMPD2	ENST00000374201	p.Phe430Leu	None	Possibly_damaging	Deleterious	4,25	VUS	De novo
T18	CNTNAP5	ENST00000431078	p.Gln889Arg	None	Possibly_damaging	Tolerated	5,630000114	Possibly pathogenic	De novo
	TJAP1	ENST00000259751	p.Arg330Trp	5,77E-05	Probably_damaging	Deleterious	5,570000172	VUS	De novo
	JPH3	ENST00000284262	p.Asp180Asn	1,72E-05	Benign	Tolerated	4,070000172	VUS	De novo
T19	CNST	ENST00000366513	p.Asp572His	1,67E-05	Probably_damaging	Deleterious	5,389999866	VUS	De novo
	PRKAR2B	ENST00000265717	p.Gly47Asp	None	Benign	Tolerated	3,740000001	VUS	De novo
	PPFIA1	ENST00000389547	p.Ala623Ala	1,17E-04	NA	NA	-5,780000021	VUS	De novo
	RIPK4	ENST00000332512	p.Gln592Glu	None	Probably_damaging	Deleterious	4,889999866	VUS	De novo
	MXRA8	ENST00000309212	c.1105+1G>A	None	NA	NA	3,339999914	VUS	De novo
T20	KCNT1	ENST00000371757	p.Ala934Thr	None	Benign	Tolerated	3,769999981	Pathogenic	De novo
	MMP28	ENST00000250144	p.Arg119His	4,14E-05	Probably_damaging	Deleterious	4,239999771	VUS	De novo
T21	IQCE	ENST00000402050	p.Gly109Gly	8,29E-06	NA	NA	3,079999924	VUS	De novo
	TMPRSS9	ENST00000332578	p.Ile233Thr	None	Possibly_damaging	Deleterious	4,429999828	VUS	De novo
T22	CAPN12	ENST00000328867	p.Ala186Thr	8,30E-06	Probably_damaging	Deleterious	4,570000172	VUS	De novo
	PPFIA3	ENST00000334186	p.Ala384Ala	None	NA	NA	-7,829999924	VUS	De novo

The genes with pathogenic/ possibly pathogenic DNMs identified are indicated in bold.

Supplementary Tab. 2 Impaired test in mutated vs non-mutated patients

Domain		Test	Mutated pts	Not- mutated pts	P value
Intelligence and cognitive status		Raven Matrices	-	-	-
		WAIS-R IQ t	2 (18.18%)	4 (8.89%)	0.586
		MMSE	1 (9.1%)	2 (4.35%)	0.481
Language		Phonemic fluency	3 (33.33%)	9 (23.08%)	0.671
		Semantic fluency	-	-	-
Memory	verbal	Rey short-term memory	0 (0.0%)	3 (7.69%)	1.000
		Rey long-term memory	1 (12.50%)	3 (7.69%)	0.539
		Verbal span (forward)	1 (11.11%)	1 (2.56%)	0.343
		Verbal supraspan + 2	1 (11.11%)	1 (2.56%)	0.343
		Associated words learning	1 (12.50%)	4 (10.26%)	1.000
	Visuospatial	Rey Figure-memory	0 (0.0%)	1 (2.56%)	1.000
		Corsi Block-Test	2 (22.22%)	3 (7.69)	0.231
		Visual-spatial supraspan + 2	0 (0.0%)	2 (5.3%)	1.000
Visuo-spatial abilities		Rey complex figure-copy	1 (12.50%)	0 (0.0%)	0.170
Executive functions	Attention/ inhibitory control	Trail making test A	-	-	-
		Stroop (time)	4 (44.44%)	7 (17.95%)	0.180
		(errors)	-	-	-
	shifting	Trail making test B	-	-	-
		Trail making test BA	-	-	-
	working memory	Verbal span (backwards)	0 (0.0%)	5 (12.82%)	0.568
	planification	WCST	-	-	-