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GENETIC BIOMARKERS IN DRUG-RESISTANT FOCAL EPILEPSIES AND THEIR ROLE IN SURGICAL OUTCOME

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Summary

Background

Epilepsy accounts for a significant proportion of the world's disease burden, affecting around 50 million people worldwide. It is associated with stigma, psychiatric comorbidity and high economic costs [1].

Focal epilepsies (FEs), where seizures initiate in spatially limited cortical networks (unifocal or multifocal) [2], account for 2/3 of epilepsy cases [3]. More than 1/3 of FE patients have drug-resistant (DR) seizures [4], with significant morbidity and increased mortality rate [5].

DRFEs are often caused by structural brain lesions in both children and adults, particularly malformations of cortical development (MCDs), such as Focal Cortical Dysplasias (FCDs)[6] type I and II[7].

Corticogenesis is a complex sequential process that, in humans, leads to the formation of a layered cortex with consistent gyral patterns and axonal connections. Broadly, corticogenesis can be divided into three partially overlapping stages, consisting of cell proliferation, neuronal migration, and postmigrational cortical organization [8]. Disruption of any of the steps may result in MCDs, a large spectrum of disorders with varied cortical morphologies, genetic or extrinsic etiologies, related syndromes, and clinical manifestations [9,10]. A simplified classification of genetic MCDs is reported in Table 1 [11].

MCD group	MCD type	Morphologies	Related pathways
Disorders of proliferation, apoptosis, and/or differentiation	Microcephalies	Microcephaly, microlissencephaly Alobar, lobar, variant holoprosencephaly	Tubulinopathies, microtubule-associated proteins; Decreased RTK-PI3K-AKT-mTOR; Sonic hedgehog pathway; Midline differentiation
	Cortical overgrowth disorders (focal and diffuse)	Megalencephaly, hemimegalencephaly, polymicrogyria, FCD-II	Overactive RTK-PI3K-AKT-mTOR
Disorders of neuronal migration	Classic lissencephaly spectrum	Smooth lissencephaly, microlissencephaly, subcortical band heterotopia	Tubulinopathies, microtubule-associated proteins; Variant lissencephalies (non-

			cytoskeletal)
	Cobblestone malformations	Rough lissencephaly, polymicrogyria, leptomeningeal glioneuronal heterotopia	Dystroglycanopathies; Other basement membrane - glia limitans interaction disorders
	Periventricular heterotopia	Nodular or linear periventricular heterotopia	Microtubule-associated proteins
	Dyslamination without cytologic dysplasia or growth abnormality	FCD-I	Overactive RTK-PI3K-AKT-mTOR; Other rare forms (e.g., variant Rett syndrome)
Disorders of axon pathway formation	Isolated callosal defects	Agenesis, hypogenesis, dysgenesis of corpus callosum	Axon growth and guidance; Midline differentiation
	Other isolated axon defects (putative)	Unknown	Axon growth and guidance

Table 1: simplified classification of genetic malformations of cortical development (MCDs). FCD= focal cortical dysplasia. [11]

FCDs encompass a spectrum of lesions from the highly localized bottom of the sulcus dysplasias to hemispheric malformations. The current clinicopathological classification of FCDs includes (i) type I, characterized by isolated cortical dyslamination; (ii) type II, characterized by dyslamination and dysmorphic neurons (type IIa) or with balloon cells (type IIb); (iii) type III, encompassing cortical lamination abnormalities associated with other brain lesions [7]. Yet, the histopathological distinction between FCD Type IIa and IIb may be problematic, if non-representative or small surgical specimens are submitted for microscopic inspection.

The histopathological features of hemimegalencephaly (HME) resemble type II FCD but the lesion extends to unilateral hemispheric enlargement.

Also histopathological findings of tuberous sclerosis complex (TSC) resemble cortical dysplasias [12,13]. TSC brain specimens reveals three major lesions: subependymal nodules, subependymal giant cell tumors, and cortical tubers. Cortical tubers consist in areas of

cortical dyslamination that contain different cell types, including dysmorphic neurons, giant cells and reactive astrocytes [14].

About 16% of DRFE patients have no lesions detectable by Magnetic Resonance Imaging (MRI) and histology [15].

For both structural and non-structural cases, epilepsy surgery is the best option to achieve seizure freedom and potential medication withdrawal [16].

The only reliable predictor of postoperative seizure freedom, regardless of etiology, is the complete resection of the epileptogenic zone (EZ) [17], the cerebral area responsible for the onset and propagation of the epileptic discharge, whose removal is necessary to abolish seizures.

In refractory FE patients, the presurgical study to identify the EZ can require invasive recording by Stereo-Electro-encephalography (SEEG) [18] to map interictal and ictal discharges otherwise undetectable with surface EEG [19]. SEEG investigation is often required in more complex/negative MRI DRFEs [18], but it has a 0.8% risk of major haemorrhagic complications per electrodes [20].

Negative MRI cases and post-surgical lesion remnants are significant risk factors for seizure recurrence [17,18].

Regarding the role of etiology in predicting the outcome, several studies correlate histopathological phenotypes with surgical prognosis, especially for FCDs [7,21–24]. Type II FCD is highly epileptogenic and frequently associated with early-onset refractory FE, susceptible to surgery with an excellent outcome [25,26], whereas type I FCD has a poor surgical outcome because the lesion is not well demarcated and the surgical resection may be incomplete[27]. The clinical differentiation between FCD Type IIa and IIb is a yet unresolved issue [7]. A better surgical outcome in FCD IIb patients than in those with FCD IIa (91 % vs. 68 %) is reported [28], which may be partially explained by the more focal pattern of FCD IIb. In fact abnormal MRI results as well as the peculiar “funnel-shaped” transmantle sign are significantly more frequent in FCD IIb [28].

Despite continuous improvements in MRI techniques, 15% of FCD type II and most of FCD type I are not detected by diagnostic imaging [27,29], highlighting the importance of comprehensive presurgical study to suspect the presence of subtle lesions.

Also cortical tubers are targeted for surgical resection. Although surgical prognosis in selected TSC patients is often favourable, increasing evidence supports the importance of

the perituberal cortex [30,31] in propagating discharges. In fact focal seizures and interictal epileptiform discharges detected in the centre of epileptogenic tubers have been shown to propagate to the tuber rim, perituberal cortex and other epileptogenic tubers [32]. The detection of multiple extensive zones with high occurrence rate of interictal high-frequency oscillations also supports the presence of a complex and widespread epileptic network in TSC [33].

With the advent of next-generation sequencing (NGS), new genetic causes have been implicated in lesional and non lesional DRFEs, with an overlap between genetic and structural aetiologies [34–44].

The spectrum of germline and/or somatic variants, including channelopathies and disorders of synaptic transmission, glycosylation defects and dysregulation in the mammalian target of rapamycin (mTOR) pathway, points to differences in the underlying pathophysiology and is reflected by phenotypic and histopathological differences between genetic patients.

The mTOR pathway is a crucial regulator of the development of the cerebral cortex. mTOR is a kinase ubiquitously expressed which is part of two complexes: mTOR complex 1 (mTORC1) when associated to the protein raptor or mTOR complex 2 (mTORC2) when bound to the protein Rictor. These two complexes regulate fundamental cell physiological processes in response to distinct cellular inputs including growth factors and nutrients. While mTORC2 mainly controls cell proliferation and survival, mTORC1 governs protein and lipid synthesis, cell growth and proliferation as well as metabolism and autophagy [45].

mTORC1 hyperactivation, caused by mutations in the mTOR pathway genes, leads to abnormal neuronal migration and cell growth, in other words, epileptogenic MCDs [46].

Indeed, brain somatic and/or germline mTOR pathway variants are found in patients carrying circumscribed lesions (e.g. FCD type II, HME, glioneuronal tumors, cortical tubers)[37–39,41–43,47]. The most common germline and/or somatic mTOR pathway variants responsible for FCD type II/HME are shown in Figure 1[48].

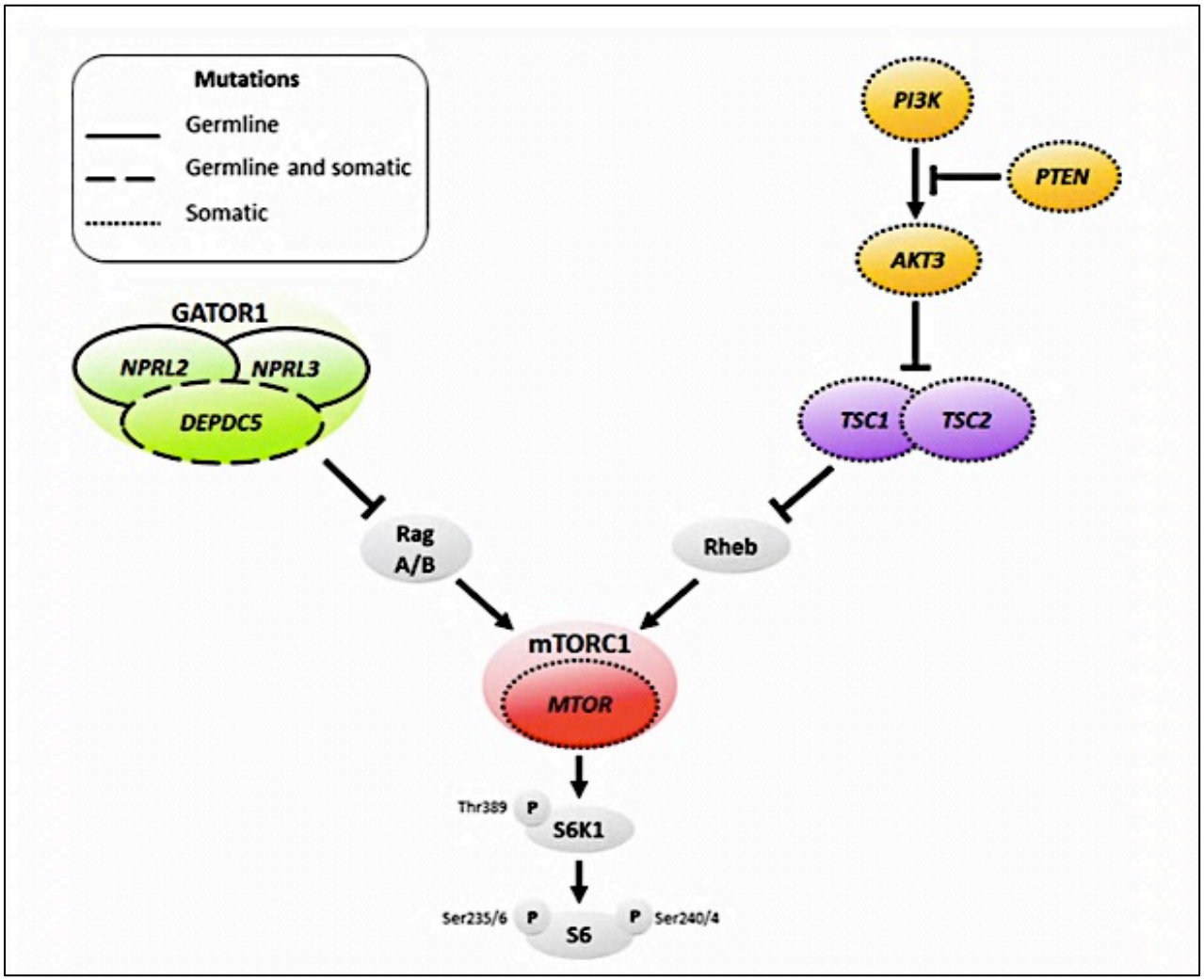


Figure 1: Mutations in genes of the mTOR pathway responsible for focal cortical dysplasia (FCD) and hemimegalencephaly (HME). Germline and somatic mutation in the mTOR complex 1 (mTORC1) pathway cause FCD and HME. Germline mutations in GATOR1 genes have been reported in FCD and HME, as well as a two-hit in DEPDC5 in an FCD patient. Moreover, brain somatic mutations have also been reported in PIK3CA, AKT3, PTEN and MTOR. TSC, tuberous sclerosis complex. [48]

FCDs are often sporadic conditions and only a few subsets of studies reported a family history with multiple FCD individuals within the same family. Indeed so far, six families have been reported with the co-occurrence of FCD, ganglioglioma, hemimegalencephaly (HME) and dysembryoplastic neuroepithelial tumours [49]. Two other pedigrees, with two first-degree relatives with a FCD in the context of familial focal epilepsy, are also reported [40].

Somatic *MTOR* variants account for 15.6–46% of FCD individuals across different cohorts and represent the most common genetic cause of FCD, so far [42,43,50]. *PIK3CA* genes are mostly encountered in HME accounting for 17–33% of patients [38,39,51,52]. In contrast, only few somatic variants in mTORC1 inhibitor genes (*TSC1*, *TSC2*, *DEPDC5*) have been

reported in FCDs [40,42,53]. Carrying somatic *TSC1/TSC2* variants only is not sufficient to cause TSC syndrome. On the contrary, a double hit somatic *TSC1/TSC2* inactivation can cause TSC, even if a recent study indicates that somatic variants are detectable in one-third of cortical tubers only [54]. The identification of either a *TSC1* or *TSC2* pathogenic germline variant (sporadic or inherited) is sufficient to make a definite diagnosis of TSC, regardless of the clinical findings [13].

Very recently, a study on a large cohort of 80 children subjected to surgery for the treatment of DRFEs with a neuropathological diagnosis of MCDs, has evaluated the contribution of each gene on MCDs. All together, authors identified germline, somatic, and somatic two-hit variants in 29% of mild MCD/FCDI cases (*SLC35A2* gene), 59% (32/54) of FCDII cases and 87.5% (7/8) of HME cases in genes of the mTOR pathway (*AKT3, DEPDC5, MTOR, PIK3CA, RHEB, TSC1, TSC2*). *MTOR* somatic variants are reported in 32% of the type II FCD/HME patients, while the contribution of somatic and germline *TSC1/TSC2* variants on the pathogenesis of FCDII/HME is lower (6.5% for somatic and 1.6% for germline variants) [42]. Finally, germline *DEPDC5* variants are found in 4.8% of FCDII/HME cases [42]. Variant allele fractions (VAFs) range from as low as 0.37% up to 8.9% among type II FCD, and up to 18.6% in large type IIa FCD, while in HME VAFs range from 7.5% up to 34%[42]. In other words, low-level somatic mutations have mainly been associated with type II FCD, intermediate-level somatic mutations affecting a single hemisphere with larger brain malformations such as HME, and high-level somatic mutations affecting multiple organs (systemic mosaicism) and constitutional mutations with megalencephaly (MEG)/dysplastic megalencephaly [38,55,56].

As already mentioned, mTOR pathway brain somatic mutations may occur as second-hit and are supposed to explain the phenotypic variability in familial affected individuals as well as in animal models, the presence of structural abnormalities and a higher risk of sudden unexpected death in epilepsy (SUDEP)[40,47]. Indeed a sizeable proportion of SUDEP cases have clinically relevant mutations in cardiac arrhythmia and epilepsy genes, including *DEPDC5* [47,57]. Recent studies have shown that spontaneous recurrent seizures can mediate cardiac dysfunction via mTOR pathway upregulation, suggesting the regulatory control of mTOR pathway as a potential target for SUDEP management [58].

Different genetic pathways seem to be related to other histological subtypes [42,59]. For example, brain somatic variants in *SLC35A2* related to glycosylation defects have been

documented in non-lesional and FCD type I cases [35,42], both related to a poor surgical outcome.

Accordingly to the above mentioned recent research findings, genetic screening before surgery is advised at least in patients with FCD, because germline variants may occur in genes that influence the epileptogenic potential of the brain beyond the visible dysplasia [60]. These include, for example, *TSC1* and *TSC2*, which may manifest with a single tuber but also genes that are not related to mTOR pathway, such as *SCN1A* [44] or *CNTNAP2* [61].

To date, few genetic surgical cases have been reported, with poor long-term follow-up data [40,42,62,63]. Epilepsy surgery seems rarely successful in patients with epilepsy due to germline mutations in genes involved in channelopathies or disorders of synaptic transmission, suggesting a contraindication for surgery, particularly in MRI-negative patients [62]. However, the low number of genetic surgical cases reported, the selection bias, the variability in data collection, including follow-up duration, genetic and histopathological findings of the reviewed studies [62], represent some limitations.

Very recently, Baldassari *et al*. observed an overall good post-operative outcome in a cohort of MCDs children, without finding any predictive correlation between a given mTOR pathway mutated gene and surgery outcome after a mean postsurgical follow-up duration of 2.5 ± 1.0 years [42].

Thus, the real impact of FE-related genes variants on surgical outcome and presurgical workup is still controversial.

Hyphotesis, significance and aims

Genetic etiology of DRFE can be linked to specific histopathological findings and might influence the efficacy of surgical treatment. Germline and/or somatic variants have been implicated in structural (particularly MCDs) and nonstructural DRFEs. In particular, the role of somatic variants seems crucial to determine the severity of phenotype, histology and surgical outcome.

A recent genetic study on a selected cohort of surgical cases has evaluated the contribution of each gene on MCDs. Somatic gain-of-function variants in *MTOR* are the most frequent. In addition, some genotype-histotype associations have been performed: type II FCD/HME are mosaic mTORopathies, while ~30% of the "mild" MCD/type I FCD cases are related to glycosylation defects [42].

To date, the role of genetic mutations in predicting the surgical outcome is not clearly understood because genetic diagnostics are still not routinely performed in surgical series and exhaustive reports on genetic cases undergoing epilepsy surgery are limited, sometimes without follow-up data [62].

Identifying pathogenic mutations will allow stratifying patients based on genetic data. Correlations between surgical outcome, histotypes and genetic results could help in routine screening for such mutations in presurgical evaluation and can prevent invasive diagnostic and risky therapeutic approaches (SEEG and surgery), particularly in MRI-negative cases. On the other hand, finding mTOR pathway mutations can increase the likelihood of identifying underlying MCDs suggesting surgical candidacy or leading to a targeted medical treatment (i.e. mTOR pathway inhibitors)[64]in cases ineligible for surgery.

Thus, the main goal of this study is to determine the incidence of mTOR pathway germline/somatic variants in DRFE patients undergoing epilepsy surgery with MCDs and negative histology.

Second goal is to perform correlations between genetic features, phenotypes and histopathological findings and to identify genetic biomarkers of surgical outcome.

In order to achieve these goals, we defined the following specific aims:

Aim 1

Prospective phase: Detection of somatic/germline mTOR pathway variants in matched fresh-frozen (FF) brain specimens and blood samples.

- Task 1.1: Design of a single-molecule Molecular Inversion Probes (smMIPs) assay including 9 mTOR pathway genes
- Task 1.2: Matched brain-blood smMIPs analysis to identify germline and brain somatic mTOR pathway variants

Aim 2

Retrospective phase: Detection of somatic *MTOR* variants in unmatched histologically proven type II FCD from formalin-fixed, paraffin-embedded (FFPE) tissues.

- Task 2.1: design of a mMIPs to target 18 *MTOR* somatic mutational hot-spots
- Task 2.2: smMIPs analysis to identify brain somatic *MTOR* variants

Aim 3

Assessment of the predictive value of genotype-histotype findings on the surgical outcome after 2 years of follow-up.

Task 2.1: Correlation between distinctive genetic features, phenotypes and histopathological findings

Task 2.2: Correlation between distinctive genetic features and surgical outcome

Design of the study

This is a multicentre cohort study in which patients were retrospectively and prospectively included.

Patients and methods

Population, setting and inclusion criteria

We included patients of both genders and any age with a diagnosis of FE who have undergone epilepsy surgery.

For the retrospective phase, we enrolled patients who have undergone epilepsy surgery from 2013 to 2016 at ‘‘Claudio Munari’’ Epilepsy Surgery Center (Niguarda Hospital, Milan) with histologically proven type II FCD and FFPE brain tissues available.

For the prospective phase, we consecutively enrolled patients who have undergone epilepsy surgery at Niguarda Hospital from February 2018 to June 2019 and IRCCS Istituto delle Scienze Neurologiche (Bellaria Hospital, Bologna) from December 2018 to May 2021. Patients with an available histological diagnosis of MCDs or without any specific findings (negative) were selected for the genetic analysis.

All patients underwent pre-surgical evaluation to define the epileptogenic zone including clinical history, seizure semiology, interictal EEG, high-resolution MRI, video- EEG monitoring, and neuropsychological assessment. Patients with discordant anatomo-electroclinical findings required invasive evaluation with SEEG recordings. Histologic diagnosis followed the consensus standard operational procedure on Diagnostic Methods by Task Force of Neuropathology from the International League Against Epilepsy (ILAE) Commission [65]. Each patient was assessed at 6 and 12 months after surgery and then yearly by direct visit. Seizure outcome after surgery (surgical outcome) was classified according to Engel outcome scale.

We reviewed data regarding presurgical evaluation, surgery, histological findings and follow-up for all patients. All data were extracted from a prospectively-filled database and collected in ad-hoc database.

This study was approved by our local ethical committee (CE 19125).

Sample collection

Retrospective phase

Available FFPE brain sample tissues of patients with histologically proven FCDII-associated epilepsy were collected and prepared as slides [66]. To compare DNA extraction and NGS performance on FFPE and FF samples, we included matched blood, brain FF MCD and non-MCD tissues of two patients.

Prospective phase

Supposed lesional and non-lesional brain tissues samples were collected during surgical procedures. Lesional brain samples were identified according to the presence of macroscopic abnormalities or the centre of the epileptogenic zone as defined by anatomo-electroclinical data. For non-lesional sample we considered the brain tissues adjacent to the lesional region. Tissues were stock at -80°C within an hour after surgery. Tissues from these regions were also apportioned for histology or banking.

Blood samples were collected and stored in EDTA tubes at 4°C (both retrospective and prospective cases)

Sample preparation and genetic analysis

Sample preparation and genetic analysis were performed at U.O. Genetica Medica, Sant'Orsola Hospital (Bologna), with the technical support of the Center for RNA Technologies – Istituto Italiano di Tecnologia (Genova).

Genomic DNA from peripheral blood was extracted using standard procedures. Genomic DNA, from FFPE tissue slides, was extracted using ReliaPrep FFPE gDNA Miniprep System (A2352; Pro- mega, Madison, WI, USA) according to the manufacturer's protocol. Peripheral blood and FF brain tissues were processed with the QIAamp DNA blood mini kit (51106; Qiagen, Venlo, LI, NL) with modifications to the FF protocol [66].

In order to analysed FFPE tissues, we designed a smMIP sequencing assay using smMIPs to target 926 bp overlapping 18 MTOR (NM_004958) mutational hot-spots associated with

FCDII [39,43,50,55,67]. Eleven probes were synthetized, then pooled together, and libraries were constructed and sequenced as detailed in [66].

For FF samples, we designed a smMIPs assay to cover all exons and splice site regions for all the main genes belonging to mTOR pathway: *AKT3* (NM_05465), *DEPDC5* (NM_00122896), *MTOR* (NM_004958), *NPRL2* (NM_006545), *NPRL3* (NM_001077350), *PIK3CA* (NM_006218) *PTEN* (NM_000314), *TSC1* (NM_000368), *TSC2* (NM_000548).

Data Validation

For each variant identified by smMIPs technique, targeted amplicon primers were designed using Primer3 (https://primer3.ut.ee/). Each primer contained Nextera adapters at 5' followed by 3 degenerate bases before the target complementary sequence, thus allowing to distinguish amplicons generated at each Polymerase Chain Reaction (PCR) cycle. PCRs were performed using Phusion U HotStart High Fidelity DNA Polymersase (F533L, ThermoScientific) in 20 μL of mix reaction final volume following manufacturer's instructions. PCR products were then purified using Agencourt AMPure XP Beads 0.9X (A63880, Beckman Coulter). Barcoding were added with a second PCR step (8 cycles) by using the same enzyme as above with Nextera indexes (i5, i7). Barcoded amplicons were purified again using Agencourt AMPure XP Beads 1.2X, quantified with QuantiFluor® dsDNA System kit (E2670, Promega) and QuantusTM Fluorometer instrument (E6150, Promega) and then pooled in equimolar manner and sequenced on the Illumina MiSeq platform using MiSeq v2 300 cycles, in order to obtain a theoretical coverage of about 10000X. Sequences were analyzed using the same bioinformatic pipeline used for smMIPs data.

In-silico deleteriousness prediction of the novel in-frame insertion

We evaluated the deleteriousness of the validated novel in-frame single-aminoacid insertion using VEST-indel [68], a variant effect scoring tool that uses a Random Forest classifier to predict the effect of in-frame and frameshift indels. We used VESTindel from CRAVAT5 [68] web interface.

Statistical analysis

To assess the predictive value of genotype-histotype findings on the surgical outcome (aim 3) we defined two outcome variables and nineteen explanatory variables.

Outcome variables

1) Patients were dichotomized into 2 classes of surgical outcome: Engel's class I (seizure-free, SF) versus class II-IV (not seizure- free, NSF). The primary outcome variable was 2-year seizure freedom (SF) after surgery at the last follow-up, irrespective of treatment status. Prospective cases who did not achieve 2-year follow-up were excluded from this statistical analysis.

2) Patients were dichotomized into 2 classes: *MTOR* mutation-positive versus *MTOR* mutation-negative. Carrying a somatic *MTOR* variant was the secondary outcome variable.

Explanatory variables

Two variables were continuous: age at seizure onset and epilepsy duration. All the remaining variables were categorical: gender (male/female), familial history of epilepsy (yes/no), perinatal suffering (yes/no), intellectual disability/at least one neuropsychological impairment at Mental Deterioration Battery, seizures during wakefulness/sleep, MRI (positive/negative), SEEG investigation (yes/no), previous surgery (yes/no), resection side (left/right), type of surgery (lesionectomy±corticectomy *vs* hemispherectomy *vs* anteromedial temporal lobectomy), resection site (frontal *vs* temporal *vs* bilobar *vs* multilobar), histological diagnosis of type I/III FCD (yes/no), histological diagnosis of type IIa and IIb FCD (yes/no), histological diagnosis of tuberous sclerosis (yes/no), histological diagnosis of other MCD (yes/no), negative at histological examination (yes/no). Carrying a *MTOR* variant (yes/no) was considered as an explanatory variable for the primary outcome statistical analysis.

Statistical analysis

Continuous variables were presented as mean±SD or median and interquartile range (IQR), categorical variables as absolute (n) and relative frequency (%).

Student's t-test, Mann-Whitney test or Fisher's exact test were used to compare variables between the two surgical outcome classes (2-year SF *vs*. NSF) and between genotypes (*MTOR* mutation-positive *vs*. mutation-negative).

A p-value < 0.05 was considered significant. Statistical analysis was performed with Stata SE 14.2.

Results

Genetic analysis

Retrospective cohort

We collected eighty FFPE brain sample tissues from 50 patients with histologically proven FCDII-associated epilepsy. Sixty were matched lesional and non-lesional tissues of 30 patients, while for 20 patients only lesional tissue was available.

We achieved adequate DNA and sequencing quality in 28 out of 50 cases (56%). The samples of the 28 cases were mostly extracted within 2 years from fixation, showing a statistically significant effect of time from fixation as a major determinant for successful genetic analysis. In fact of the 24 samples removed from sequencing, 15 (62.5%) were extracted from tissues fixed > 2 years earlier with DNAs showing extended damage and degradation.

We identified and validated seven pathogenic *MTOR* somatic variants (found in 25% of sequenced and analysed 28 retrospective patients) (Table 2) [66].

		Lesional brain tissue				Marginal region			
		smMIPs		Amplicon seq		smMIPs		Amplicon seq	
Patient ID	Protein change	VAF	DP	VAF	DP	VAF	DP	VAF	DP
FFPE1	p.Ser2215Phe [10, 11, 13]	5.95%	823X	5.48%	3706X	NA	NA	NA	NA
FFPE3	p.Tyr1450Asp [10]	2.05%	1025X	2.22%	6034X	0%	1425X	0%	2848X
FFPE4	p.Leu1460Pro [11, 13]	4.73%	423X	3.68%	3533X	NA	NA	NA	NA
FFPE6	p.Leu1460Pro [11, 13]	4.57%	350X	2.29%	3630X	0%	579X	0%	3708X
FFPE13	p.Ser2215Tyr [11, 13]	5.08%	767X	4.13%	3653X	NA	NA	NA	NA
FFPE15	p.Ser2215Tyr [11, 13]	3.17%	789X	2.36%	3522X	NA	NA	NA	NA
FFPE17	p.Asp1458_Ala1459InsAsp	5.87%	1381X	3.41%	3698X	NA	NA	NA	NA

DP read depth, *FFPE* formalin-fixed, paraffin-embedded, ID identification, *NA* matched tissue not available, *seq* sequencing, *smMIPs* single-molecule molecular inversion probes, *VAF* variant allele frequency

Table 2: *MTOR* somatic variants (7 patients, retrospective cohort).[66]

The allele fraction had a range of 2–5% and variants were absent in available neighbouring non-focal cortical dysplasia specimens. We computed an alternate allele threshold for calling true variants, based on an experiment-wise mismatch count distribution, well-predicting call reliability.

Prospective cohort

We collected 150 FF brain specimens (lesional and/or non-lesional) and 174 blood samples from 184 patients. At least one tissue sample (blood or brain) was collected for each patient. We selected 63 patients (115 samples) with histological diagnosis of MCDs or negative histology from Niguarda Hospital to be subjected to the genetic analysis (mTOR pathway genes panel). Lesional FF brain specimens were collected in 50 out of 63 cases.

We identified and validated 6 *MTOR* somatic variants in lesional brain tissues and two *DEPDC5*, two *TSC1* and one *TSC2* germline variants without any somatic double hit (11 patients). Detailed genetic findings and related histotypes are shown in Table 3.

Variant	Histology	Somatic/ Germline	VAF (brain lesion)	VAF (blood)
MTOR:NM_004958:c.C6644A:p.S2215 Y	FCDIIb	Somatic	2.61%	0.23%
MTOR:NM_004958:c.C4376A:p.A1459D	FCDIIb	Somatic	2.8%	N.A.
MTOR:NM_004958:c.T4379C:p.L1460P	Tubers/FCDII	Somatic	4.26%	0.31%
DEPDC5:NM_001242896:c.C1474T:p.R492X	FCDIIa	Germline	37.16%	44.33%
DEPCD5:NM_001242896:c.785_789del:p.E265 MfS*8	FCDIIa	Germline	59.63%	42.86%
MTOR:NM_004958:exon47:c.C6644T:p.S2215F	FCDIIb	Somatic	0.39%	0.45%
MTOR:NM_004958:c.T4379C:p.L1460P	FCDIIb	Somatic	1.34%	0.08%
TSC1:NM_000368:c.C2818T:p.Q940X	FCDIIb (TSC*)	Germline	42.96%	33.33%
MTOR:NM_004958:c.C6644T:p.S2215F	FCDIIa	Somatic	3.11%	0.89%
TSC1:NM_000368:c.G737A:p.R246K	Tubers (TSC)	Germline	N.A.	43.33%
TSC2:NM_000548.5:c.138+1G>A	Gliosis** (TSC)	Germline	50.99%	39.29%

Table 3: Genetic results and related histotypes (11 patients, prospective cohort). FCD= focal cortical dysplasia, TSC=tuberous sclerosis complex diagnosis, N.A.=not available. *Based on the genetic result only. **Not performed lesionectomy (disconnection only)

Anatomo-electroclinical data

We prospectively enrolled 170 patients from Niguarda Hospital and 14 cases from Bellaria Hospital who have undergone epilepsy surgery during the recruitment periods.

Sixty-three patients with histological diagnosis of MCDs (FCD I, II, III, TSC, polymicrogyria, multinodular and vacuolating neuronal tumor of the cerebrum) or with negative histology were selected for the genetic analysis.

For the retrospective phase, we collected 50 patients with FFPE brain sample tissues available. Twenty-eight of them had brain samples with histologically proven type II FCD suitable for genetic analysis.

Anatomo-electroclinical data of the analysed prospective (63 cases) and retrospective (28 cases) patients are shown in Table 4. Median age of patients was 25 years. The median follow-up after surgery was 26 months (IQR=24-39 months).

Overall, a genetic aetiology was found in 18 patients (7/28 retrospective cases and 11/63 prospective cases): 13 brain somatic *MTOR* (14%, considering 91 tested cases), 2 *DEPDC5* (3.2%), 2 *TSC1* (3.2%) and 1 *TSC2* (1.6%) germline variants (considering 63 tested cases).

Overall, 74% (67/91) of patients had an excellent surgery outcome.

Considering the mutated cases only, 15 out of 18 patients (83%) reached an Engel class I. Similar proportions were found in each genetic subgroup: 85% of somatic *MTOR* cases, 100% of germline *DEDPC5* cases and 67% of germline *TSC1*/*TSC2* cases are in Engel class I at the last follow-up.

Antiepileptic drugs were stopped in twenty-six patients (28.6%).

Patients	**Total N=91**	**%**	***MTOR* variant**		**P-value**
			NO	**YES**	
			N (%)		
Male	44	48.3	38 (48.7)	6 (46.2)	1.00
Female	47	51.7	40 (51.3)	7 (53.9)	
Median (IQR) age at seizure onset (years)	7 (3-11)		8 (3-11)	6 (2-10)	0.46

Epilepsy family history:					0.51
Positive	26	28.6	21 (26.9)	5 (38.5)	
Negative	65	71.4	57 (73.1)	8 (61.5)	
Perinatal suffering	12	13.2	11 (14.1)	1 (7.7)	1.00
Neuropsychological impairment*	46	51.1	41 (53.3)	5 (38.5)	0.46
Circadian seizure pattern:					0.45
Wakefulness only	31	34.4	28 (36.4)	3 (23.1)	
Sleep only	5	5.6	5 (6.5)	0	
Wakefulness and sleep	54	60.0	44 (57.1)	10 (76.9)	
SEEG investigation:					**0.03**
Yes	33	36.3	32 (41)	1 (7.7)	
No	58	63.7	46 (59)	12 (92.3)	
Brain MRI:					**0.05**
Positive	62	68.1	50 (64.1)	12 (92.3)	
Negative	29	31.9	28 (35.9)	1 (7.7)	
Previous surgery:					0.08
Yes	8	8.8	5 (6.4)	3 (23.1)	
No	83	91.2	73 (93.6)	10 (76.9)	
Lesionectomy ± Corticectomy*	71	78.0	60 (76.9)	11 (84.6)	0.27
Hemispherectomy	3	3.3	2 (2.6)	1 (7.7)	
Anteromedial temporal lobectomy	17	18.7	16 (20.5)	1 (7.7)	
Resection side					0.55
Right	55	60.4	46 (59)	9 (69.2)	
Left	36	39.6	32 (41)	4 (30.8)	
Resection site:					0.12
Frontal	39	42.9	29 (37.2)	10 (76.9)	
Temporal	26	28.6	25 (32.1)	1 (7.7)	
Occipital	2	2.2	2 (2.6)	0	
Insular	1	1.1	1 (1.3)	0	
Bilobar	10	10.1	10 (12.8)	0	
Multilobar	13	14.3	11 (14.1)	2 (15.4)	
Histology:					**<0.001**
Type IIa FCD	26	28.6	25 (32.1)	1 (7.7)	
Type IIb FCD	23	25.3	12 (15.4)	11 (84.6)	
Type I/III FCD	6	6.6	6 (7.7)	0	

Negative histology	27	29.7	27 (34.6)	0	
TSC	5	5.5	4 (5.1)	1 (7.7)	
Other MCDs	4	4.4	4 (5.1)	0	
Engel class:					0.641
I	67	73.6	56 (71.8)	11 (84.6)	
II	6	6.6	5 (6.4)	1 (7.7)	
III	10	11.0	10 (12.8)	0	
IV	8	8.8	7 (9)	1 (7.7)	

Table 4: anatomo-electroclinical data of 91 patients (retrospective and prospective cohort) and results of univariate analysis. Abbreviations: IQR= InterQuartile Range, MRI= Magnetic Resonance Imaging, FCD= focal cortical dysplasia, TSC=tuberous sclerosis, MCDs= malformations of cortical development. * 1 missing

Cases carrying somatic MTOR variants:

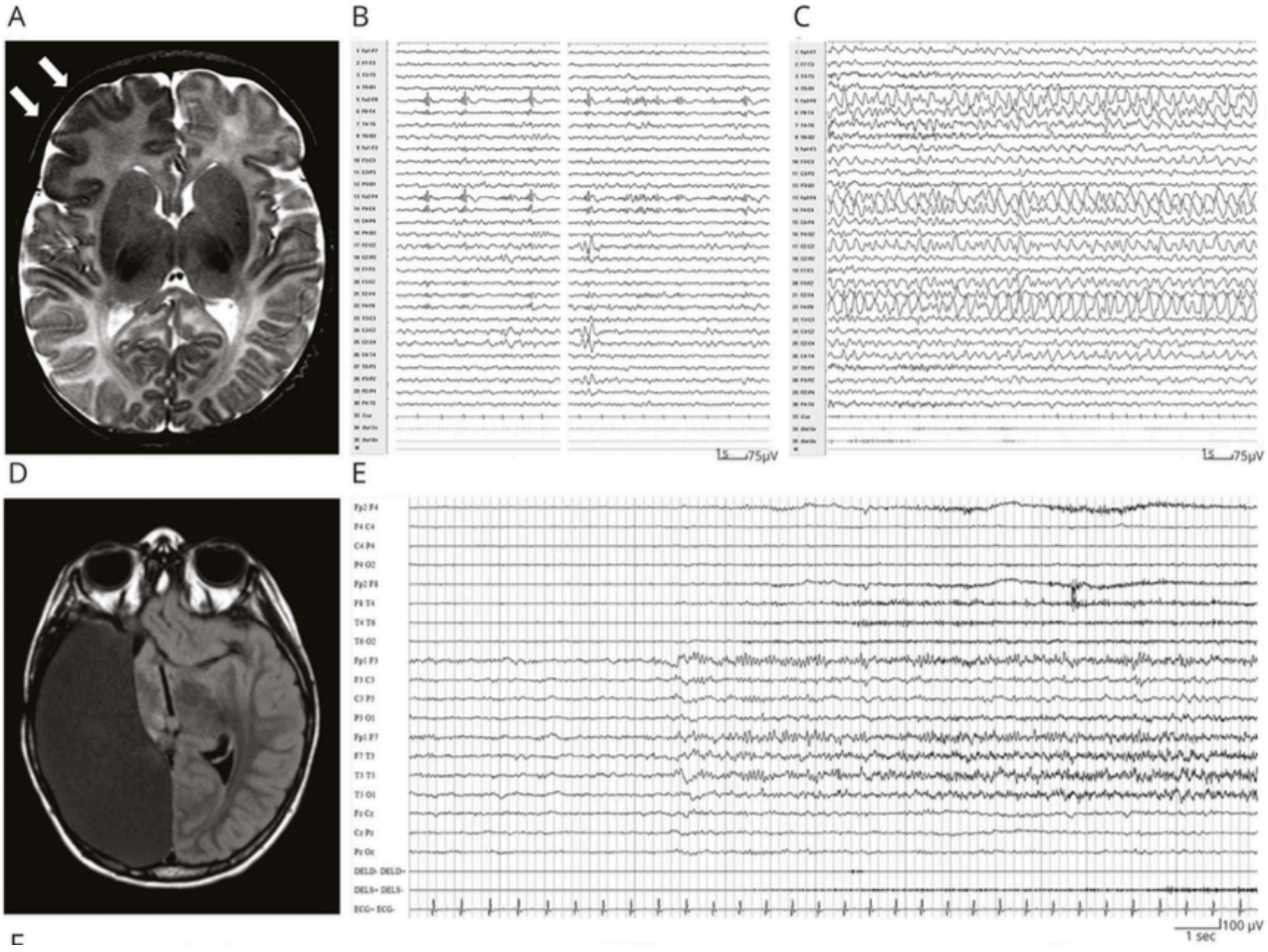
In thirteen patients (6 males) brain somatic *MTOR* variants were found. Five had a positive familial history for epilepsy; intellectual disability (from mild to severe)/neuropsychological impairment was reported in 5 patients. The mean age at seizure onset was 5.1±3.9 years (range 0-11), seizures occurred also during sleep in the majority of cases (77%). All patients except one had a positive brain MR. SEEG investigation was performed in one case to define resection lesional margins.

The majority (12/13 patients) received a histological diagnosis of type II FCD (11 with type IIb); one patient had morphological and immunophenotypic findings suggestive for TSC.

Eleven cases underwent lesionectomy ± corticectomy, a patient with an atypical medial temporal lobe epilepsy (absence of febrile convulsive seizures, early seizure onset, paucysimptomatic seizures) underwent antero-medial temporal lobectomy and one case underwent hemispherectomy (after multiple surgeries), without reaching seizure control. In the remaining patients the Engel class was I (11 cases) and II (1 case) after a median follow-up duration of 36 months.

Focusing on the case undergoing hemispherectomy[69], he was a 11 year- old male born at term after an uncomplicated pregnancy to healthy unrelated parents. During the first month of life, daily focal seizures appeared, with staring and tonic asymmetric posturing, accompanied by a right frontoparietal ictal discharge. At 3 months, asymmetric epileptic spasms also appeared. Brain MR, at 1 month and 3 months, documented extensive blurring of the cortical gray– white matter junction involving the anterior right frontal lobe (figure

2A)[69]. Because of persisting seizures, at 15 months the child underwent a right frontopolar corticectomy. Neuropathology revealed type IIb FCD (figure 2F)[69]. Three months later, seizures reappeared with similar clinical and EEG features (figure 2, B and C)[69]. Subsequent parietal corticectomy with temporo-parieto-occipital disconnection resulted in short seizure-free periods, with persisting right-sided origin, which prompted a complete right hemispherectomy at 5 years. After the operation, the child remained seizure-free for 3 months, after which focal seizures relapsed as sleep episodes, accompanied by left fronto-temporal ictal EEG activity (figure 2E)[69]. Despite multiple AED combinations, daily seizures persisted over the years, manifested at times as status epilepticus. Outcome was classified as Engel Class IV. Follow-up MR showed the consequences of hemispherectomy, with a normal-appearing left hemisphere (figure 2D)[69]. The child has a mild intellectual disability (Full-Scale IQ = 60) with speech impairment and left surgery–induced hemiplegia.



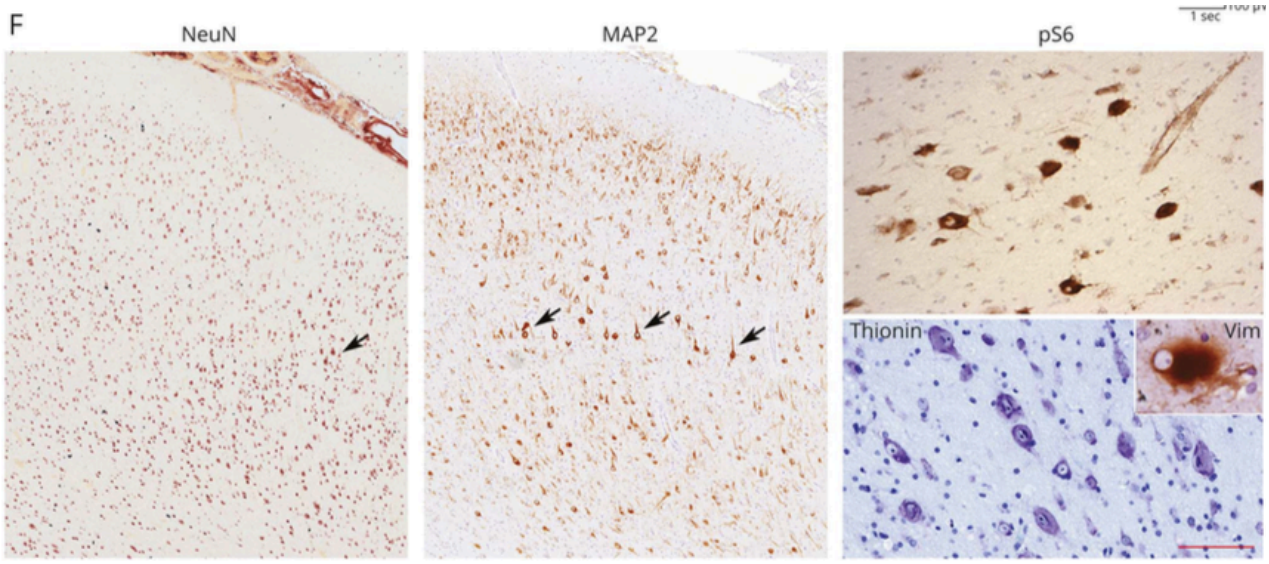


Figure 2: A) Axial T2-weighted MRI section showing an extensive area of cortical dysplasia involving the right frontal lobe (arrows). (B and C) EEG recording at 3 years of life, showing subcontinuous right frontotemporal polyspikes (B), followed by a right frontal ictal discharge. (D) Axial T1-weighted MRI section after complete anatomic hemispherectomy. (E) EEG recording at 7 years shows a left frontotemporal ictal discharge. (F) Representative immunostaining of dysplastic brain tissue from the right frontal lobe showing cortical dyslamination (NeuN), dysmorphic neurons (MAP2, thionin, black arrows) with mTOR hyperactivation (pS6), and balloon cells (vimentin). Scale bar for NeuN and MAP2 = 370 µm, pS6 and thionin = 60 µm, and vimentin (inset) = 35 µm. MAP2 = microtubule-associated protein 2; NeuN = neuronal nuclei; pS6 = phosphorylated RPS6. [69]

Focusing on the case with Engel class II, she was a 16 year- old girl born at 35 weeks due to untimely rupture of fetal membranes after a complicated pregnancy (gestational diabetes, HIV treatment) to unrelated parents. A positive family history of epilepsy both in the maternal (FE) and paternal (generalised epilepsies) branches (second and third-degree relatives) was reported. She had poor academic achievement and needed a support teacher. During the first year of life, multiple per day focal seizures appeared, both during wakefulness and sleep with tonic asymmetric posturing accompanied by a left fronto-centro-temporal discharge. Sometimes convulsive, bilateral tonic-clonic seizures occurred. Brain MR, at 1 year documented T1 hyperintensity lesion involving the left striatum. At 11-year-old the lesion was also visible at the orbito-polar level, anterior lateral dorsum of the frontal lobe and at the insula. At 14 years old she underwent a left frontal lesionectomy (it remains lentiform and caudate nucleus T1 hyperintensities at brain MR). Neuropathology revealed morphological and immunophenotypic findings suggestive of brain lesions referable in the first instance to tuberous sclerosis. She was seizure-free for almost one year. Very recently, she has experienced few seizures during sleep (Engel class IId).

Cases carrying germline DEPDC5 variants

Germline *DEPDC5* variants were found in 2 out of 63 analysed cases (3.2%), without any somatic mutation (double-hit) in the tested mTOR pathway genes.

Both patients had a positive family history of epilepsy in first and second-degree relatives. In particular, one patient had a family history of epilepsy and febrile seizures in the maternal branch (mother, grandmother, two aunts, cousin) with autosomal dominant inheritance pattern.

In addition, they had early seizure onset (0 and 3 years old) with motor onset in one case (clonic seizures) and autonomic onset in the other one. Seizures were not sleep-related. Both cases had drug-resistant FE, positive brain MRI (right frontal FCDs), normal neuropsychological evaluation, coherent epileptiform EEG abnormalities and type IIa FCD at histology. They underwent lesionectomy reaching a complete seizure control (Engel class Ia, follow-up of 27 and 14 months respectively).

In one patient, *DEPDC5* variant was inherited from the affected mother (genetic study performed in other Hospital).

Cases carrying germline TSC1 and 2 variants

Germline *TSC1* variants were found in two patients.

One of them was a 19 year-old male with normal intelligence. Daily focal seizures started at 4 years old. Brain MRI showed a lesion (type II FCD/tuber) in the left F1-F2 sulcus. Interictal EEG was congruous. He underwent lesionectomy with Engel Class Ia after 12 months follow-up and neuropathology revealed type IIb FCD. According to [13], a definite diagnosis of TSC was based on the genetic finding only (no clinical TSC features, unique cortical tuber).

The second patient was a 12 year-old boy with a neurodevelopmental delay, intellectual disability and congenital cardiac rhabdomyomas. At 4 months, epileptic spasms appeared; daily stereotyped focal seizures started from 5 years old. Brain MR documented cortical tubers and subependymal hamartomas. Interictal and ictal video-EEG revealed a left fronto-insular origin. He underwent left insular lesionectomy without seizure control (Engel class

IV) after 6 months follow-up. Neuropathological findings were consistent with the clinical diagnosis of TSC.

De novo germline *TSC2* variant was discovered in a 47-year-old male with a clinical diagnosis of TSC. Brain MR showed bilateral cortical tubers (the largest of which involved the anterior temporal region), subependymal hamartomas and right hippocampal sclerosis. He underwent right temporal disconnection (neuropathology was negative due to the type of surgery) with seizure control (Engel class Ia after 27 months follow-up).

Genotype-phenotype-histotype correlations

We asked whether a particular genotype was correlated with specific histotypes and anatomo-electroclinical features. Thus, we performed statistical correlations between explanatory variables and genotype in the entire cohort of 91 patients.

Carrying a *MTOR* genotype was the outcome variable. All *MTOR* variants were found to be somatic (in the lesional resected brain only).

We considered *MTOR* genotype only because it was analysed in both cohorts and the size of the *MTOR* mutated sample (13 cases, 14%) turned out to be the only one suitable for the statistical analysis.

At univariate analysis, variables significantly associated with *MTOR* genotype were 1) type IIb FCD at histology (P< 0.001); positive brain MR (P=0.05); not performed SEEG investigation (P=0.02). Results of univariate statistical analysis are shown in Table 4.

Genotype-surgical outcome correlations

We then asked whether the detection of brain somatic *MTOR* variants could predict the surgical outcome. The primary outcome was at least 2-year SF after surgery at the last follow-up.

We included 71 out of 91 patients (78%) with a minimum 2-year follow-up after surgery Forty-three cases belonged to the prospective cohort.

Fifty patients (70%) reached the primary endpoint (SF group). The median follow-up duration was 30 months. Median age at seizure onset was 8 years (IQR=2.5-11) and mean age at surgery was 23.8± 14.6 years. Mean epilepsy duration was 15.1 ± 11.8 years.

We performed univariate analysis to study the association between a favourable surgical outcome (2-year SF) and predisposing factors (see explanatory variables).
We did not observe any predictive correlation between explanatory variables and surgical outcome. In particular, carrying brain somatic *MTOR* variants did not influence the surgical prognosis in our cohort.

As regards germline *DEPDC5* (2 cases), *TSC1* (2 cases) and *TSC2* (one case) detected variants, the size of the sample did not allow for statistical correlations. In our cohort all these mutated cases with the exception of one TSC1 patient were seizure-free after surgery.

Discussion

In this study, we aimed to genetically analyse a large surgical population with FEs related to MCDs and negative histology to determine the incidence of mTOR pathway germline/somatic variants and to assess the predictive value of genotype-histotype findings on the surgical outcome.

Our study showed that germline *DEPDC5* and *TSC1/TSC2* variants altogether are found in 8% of our analysed patients (5/63 cases). On the contrary, *MTOR* somatic variants represent the most common genetic finding (14% of the entire cohort), confirming literature data [42]. Particularly, *MTOR* somatic variants account for 17% of our type II FCD individuals. We disclosed a statistically significant association between *MTOR* genotype and type IIb FCD at histology ($P < 0.001$). Nevertheless, one mutated case had type IIa FCD at histology, according to previous reports in which *MTOR* somatic variants can be found in both type II FCDs and HME, depending on VAFs [42,43,50].

Dysmorphic neurones (DNs) and especially, balloon cells (BCs) with astroglial features are the cytological hallmarks of type IIb FCD [7]. Both cells in type II FCD carry pathogenic variants [42] and have been linked to enhanced mTOR signalling [43,70], even if physiological recordings of human type IIb FCD tissues indicates that BCs are almost silent, while DNs are hyperexcitable [71]. The reason why *MTOR* somatic variants are preferentially linked to the presence of BCs remains unclear.

Furthermore, we found no clinical differences between FCD type IIb individuals with or without *MTOR* mutations, except for the reported patient (Figure 2) [69] who underwent sequential, increasingly invasive surgical procedures ultimately resulting in complete hemispherectomy. Although no residual dysplastic tissue was detectable at MRI after hemispherectomy, after 3 months the patient manifested focal seizures unexpectedly originating from the contralateral hemisphere. One hypothesis of this uncommon occurrence is that the *MTOR* mutation originated before hemispheric cleavage in a site that favoured its passing asymmetrically to daughter cells on either side, with only 1 hemisphere receiving a number of mutated cells large enough to develop a macroscopically visible dysplasia. Mutant cells in the contralateral hemisphere, would become epileptogenic later because of their small critical mass, after removal of the leading epileptogenic contralateral dysplasia [69]. Different studies exploring the causes of the hemispheric shift of epileptogenicity after

surgery for large dysplasias, or more in general the causes of surgical failures, had already demonstrated structural abnormalities that escaped early neuroimaging or even macroscopic autoptic neuropathologic studies [72,73]. In one of these studies, conducted before molecular genetic testing of brain tissue was feasible, the authors hypothesized bilateral asymmetric hemispheric abnormalities to be caused by somatic mutations affecting each developing cerebral hemisphere differently with more neurons than expected on the HME side [72].
An alternative hypothesis to explain seizure onset in the seemingly intact hemisphere is that a second somatic mutational hit has occurred in it, involving a different mTOR pathway gene or any of many other epilepsy genes. This second hit, not present in the resected dysplastic tissue, would be sufficient to activate epileptogenesis but not to determine an MRI-visible malformation. Pelorosso *et al*. demonstrated how a somatic *MTOR* mutation in the dysplastic hemisphere and a systemic mosaic RPS6 mutation synergistically concurred in determining a HME/ epilepsy phenotype in which seizure onset had occurred in the seemingly intact hemisphere after hemispherectomy [74].

We could not exclude, however, that the *MTOR* mutation was also present, albeit with lower VAF, in the nondysplastic hemisphere.

The same *MTOR* variant (p.Leu1460Pro) of this patient was also detected in other three patients of our cohort. VAF ranged from 1.34 to 4.73. It belongs to the subset of recurrent mutations found at low-level somatic mosaicism in FCDII/HME [42,43,50,55,63,67] that cause a strong increase of mTORC1 activity and may precipitate epileptogenic activity even at very low VAF levels [55]. Two of them carried type IIb FCD, one had neurophatological findings referable in the first instance to tuberous sclerosis. Nevertheless, to our knowledge, somatic *MTOR* variants were not found to be causative of TSC syndrome. This genetic result and the absence of major/minor clinical TSC features suggest a FCDII-related epilepsy diagnosis, even with an atypical brain MRI localisation. Indeed the lesion involved the frontal lobe, the insula and also the striatum. This patient underwent an incomplete lesionectomy, with striatum residuals. She was seizure-free for one year, then she experienced seizures relapse during sleep (Engel class II).

Anecdotal cases with FCD and a striatum involvement have been reported [75,76]. In these cases, the removal of the EZ including the anterior striatum (during a second surgery) led to

seizure freedom. Histopathological examination of the resected tissue revealed a large number of dysmorphic neurons distributed widely in the cerebral cortex, subcortical white matter and striatum, suggesting that dysmorphic neurons in deep brain structures would play a key role in epileptogenesis [75].

As regards *DEPDC5* patients (2 cases), both had type IIa FCD at histology. DEPDC5, NPRL2 and NPRL3 form the GATOR1 complex, which functions as an inhibitor of mTORC1. Loss-of-function mutations in GATOR1 genes are the most frequent genetic cause of inherited focal epilepsies, including monogenic entities such as familial focal epilepsy with variable foci, autosomal dominant sleep-related hypermotor epilepsy or familial temporal lobe epilepsy [77]. An important clinical feature of the GATOR1-related epilepsies is the prevalence of individuals with FCD II or HME. Type IIa FCD seems to be the most frequent associated histotype [42,48], confirming our findings. Germline *DEPDC5* variants can be also associated with other MCDs such as band heterotopia, polymicrogyria or unilateral pachygyria [48]. Only a portion of epilepsy affected germline *DEPDC5* variant carriers develops FCD, whereas others have nonlesional focal epilepsy [40]. Differences in phenotype, including the development of FCDs seem to be caused by a mutational "double hit" model (germline mutation in *DEPDC5* and somatic variant in the resected brain tissue) [40]. In our two patients, despite the absence of a second somatic hit in the tested genes, type II FCDs are recognizable at brain MR and then, at histological examination. However, somatic mutation in genes out of the panel can not be excluded.

TSC1 and *TSC2* germline variants were found in two and one patients of our cohort, respectively. Two of them had major and/or minor clinical TSC features, thus genetic results confirmed the TSC diagnosis. In contrast, one patient with *TSC1* germline variant had a unique brain cortical lesion (defined as type IIb at histology) without any clinical TSC manifestations. In this case, the genetic result has changed the diagnosis from FCDIIb-related epilepsy to TSC, with clinical, therapeutic and prognostic implications.

Negative histology/non-lesional FE patients account for 30% of our cohort. In these cases, we did not find any variants in the mTOR pathway genes, suggesting that other pathways (e.g. *SLC35A2* related to glycosylation defects) may be involved [42]. In addition, also in type I and III FCDs (6.6%) we did not find any mTOR pathway gene variants.

Finally, we performed statistical correlations between genetic (*MTOR* genotype), anatomo-

electroclinical data and the post-surgery outcome. Seventy percent of patients were seizure-free for at least two years at the last follow-up. We did not find any differences in surgical outcome between *MTOR* mutated and not mutated cases. Nevertheless, in case of seizure recurrence, repeated surgeries up to complete hemispherectomy should be very carefully weighted in children in whom a somatic mutation in *MTOR* is demonstrated after a first operation, as its distribution and cellular consequences can be misleading.

Future larger-scale studies will be needed to improve postsurgical stratifications of patients based on their genetic profile.

Genetic results are beginning to inform therapy by improving the selection of available antiepileptic medications or other treatments [64]. Importantly, mTORC1 can be pharmacologically targeted by derivatives of rapamycin and has been proven to be partially efficient for controlling seizures in TSC syndrome [78]. Rapamycin treatment can prevent brain malformations and epilepsy in animal models, suggesting that inhibition of the mTORC1 pathway could be of interest as a new therapeutic target in patients with FCD or HME related to mTOR pathway genes. To date, type II FCD patients for whom surgery and more than two antiepileptic drugs failed to control seizures are enrolled in an ongoing phase-3 clinical trial using the mTOR-inhibitor everolimus (NCT03198949).

In conclusion, this study suggests that somatic variants of *MTOR* are the most frequent mutations detected in brain specimens of MCDs patients. Germline *DEPDC5, TSC1/TSC2* variants account for a minority of cases.
Somatic *MTOR* variants are found at low-level somatic mosaicism especially in type IIb FCDs and they did not represent a contraindication for epilepsy surgery. Nevertheless, the detection of *MTOR* somatic mutation and the failure in seizure control after the first surgery may represent a red flag for planning further surgical procedures (up to hemispherectomy), due to the possibility of the contralateral hemisphere becoming in turn epileptogenic.

Study limitations

Selection bias is a limitation of our study. In fact, our results do not apply to the entire spectrum of surgical patients because they were obtained in a select population of MCDs and non-lesional cases, in which the probability to find mTOR pathway gene variants is higher.

Furthermore, the genetic screening using the smMIPs technique was different in the retrospective and prospective cohort, in terms of number of tested genes. In fact we did not perform a gene panel in unmatched FFPE brain tissues, but *MTOR* genetic testing only. The possibility to expand the analysis to a larger panel of genes in FFPE tissues was prevented here by the overall poor state of DNA libraries. In addition, our findings regarding the impact of fixation-to-extraction time might vary according to the setting adopted for tissue preparation.

Future perspectives

To date, we have analysed 63 out of 184 (34%) prospectively enrolled cases. More than half of the 121 remaining patients had a histological diagnosis of hippocampal sclerosis and CNS tumours. Recently, other 17 patients received a diagnosis of MCDs. Histotypes of the 121 patients are shown in Figure 3.

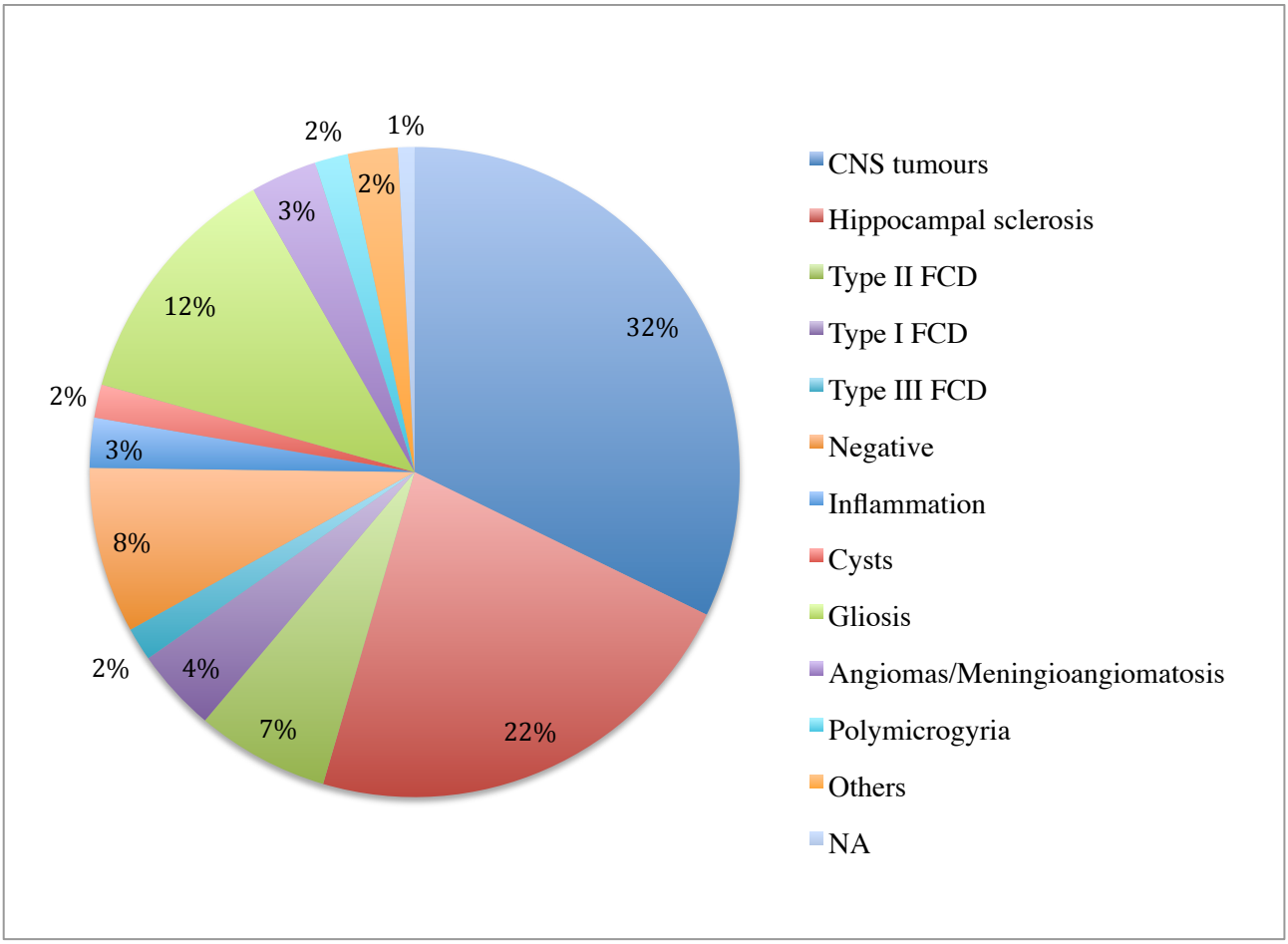


Figure 3: Histotypes of the 121 prospective cases not yet genetically analyzed. FCD= focal cortical dysplasia. NA= not available.

We plan to design a smMIPs assay including a higher number of FE/MCD genes (about 70 genes, including other pathways) to perform genetic analysis on this cohort.

Furthermore, we plan to analyse DNA from cerebrospinal fluid (CSF) of surgically treated FE patients in order to confirm brain somatic variants. In fact, while germline variants can be assessed also during presurgical evaluation through DNA analysis from a blood sample,

brain somatic mutations are until now undetectable during this early stage but only after surgical removal of the EZ. To date, sampling the CSF has proven to be an adequate source for cell-free circulating (cf) brain tumour DNA [79]. Thus the isolation of cfDNA from CSF also for non-tumoral brain diseases as MCDs in which the percentage of cfDNA carrying the somatic mutation is supposed to be higher [80], may allow to detect brain somatic variants before epilepsy surgery, representing a early marker to select cases eligible for surgery *vs* other treatments.

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