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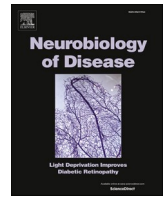
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mTOR pathway: Insights into an established pathway for brain mosaicism in epilepsy

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ABSTRACT

The mechanistic target of rapamycin (mTOR) signaling pathway is an essential regulator of numerous cellular activities such as metabolism, growth, proliferation, and survival. The mTOR cascade recently emerged as a critical player in the pathogenesis of focal epilepsies and cortical malformations. The 'mTORopathies' comprise a spectrum of cortical malformations that range from whole brain (megalencephaly) and hemispheric (hemimegalencephaly) abnormalities to focal abnormalities, such as focal cortical dysplasia type II (FCDII), which manifest with drug-resistant epilepsies. The spectrum of cortical dysplasia results from somatic brain mutations in the mTOR pathway activators *AKT3*, *MTOR*, *PIK3CA*, and *RHEB* and from germline and somatic mutations in mTOR pathway repressors, *DEPDC5*, *NPRL2*, *NPRL3*, *TSC1* and *TSC2*. The mTORopathies are characterized by excessive mTOR pathway activation, leading to a broad range of structural and functional impairments. Here, we provide a comprehensive literature review of somatic mTOR-activating mutations linked to epilepsy and cortical malformations in 292 patients and discuss the perspectives of targeted therapeutics for personalized medicine.

1. The mechanistic target of rapamycin (mTOR) signaling cascade

The mechanistic target of rapamycin (mTOR) is a highly evolutionarily conserved pathway that responds to various environmental signals, such as growth factors, energy signals and nutritional status, and is essential for controlling cell growth, metabolism, protein synthesis, and autophagy (Fig. 1). Aberrant activation of the mTOR pathway is linked to metabolic disorders, neurodegeneration, cancer, and aging, and many research efforts have focused on developing therapies inhibiting the pathway (Liu and Sabatini, 2020).

The serine/threonine protein kinase mTOR (encoded by the *MTOR* gene) is ubiquitously expressed, with high levels in the brain. mTOR is the catalytic subunit of two complexes that differ in structure and function, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), the latter of which was discovered more recently. mTORC1 is rapamycin-sensitive and defined by the regulatory-associated protein of mTOR (Raptor). In contrast, mTORC2 contains the rapamycin-insensitive companion of mTOR (Rictor) and is insensitive to acute rapamycin treatment (Liu and Sabatini, 2020; Laplante and Sabatini, 2012).

At the core of cellular signaling, mTORC1 acts as a crucial hub that receives signals from upstream regulatory proteins, which can be influenced by growth factors (such as insulin), ATP levels, and nutrients (such as amino acids). Upon activation, mTORC1 promotes cell growth and survival by regulating several key cellular processes, including mRNA translation, nucleotide biosynthesis, and autophagy. The modulation of these essential cellular processes is achieved by regulating downstream substrates of mTORC1 signaling, like ribosome S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein. Due to their role in genetic focal epilepsies, the GATOR1 complex and the amino acid-sensing branch of the mTOR cascade have gained recent attention. GATOR1 (*Gap Activity TOWard Rags 1*) is composed of three subunits: (i) Dishevelled, Egl-10, and Pleckstrin domain-containing protein 5 (DEPDC5); (ii) nitrogen permease regulator-like 2 (NPRL2); and (iii) nitrogen permease regulator-like 3 (NPRL3). In response to increasing amino acid levels, the GATOR2 complex inhibits GATOR1, leading to mTORC1 disinhibition and enabling cell growth and proliferation pathways by stimulating protein synthesis, lipid biogenesis, and metabolism while inhibiting autophagy via several intermediate factors (Barpeled et al., 2013; Wolfson et al., 2016; Wolfson et al., 2017). When amino acid levels drop, GATOR1 acts as a GTPase activating protein and

Abbreviations: HME, Hemimegalencephaly; MEG, Megalencephaly; FCD, Focal Cortical Dysplasia; VAF, Variant allele frequency.

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hydrolyzes the GTP bound to the small GTPases RagA/B, thereby preventing mTORC1 from interacting with the small GTPase RAS homolog enriched in brain (RHEB), ultimately leading to the shutdown of the signaling pathway.

The role of mTORC2 signaling has been less explored. mTORC2 contributes to regulating cellular chemotaxis and migration by acting primarily on the reorganization of the actin cytoskeleton as well as promoting cell survival and glucose homeostasis (Saxton and Sabatini, 2017). Recent studies also suggest its implication in the regulation of autophagy in different cellular contexts, possibly via mechanisms complementary to mTORC1 (Sun et al., 2023).

In the brain, mTOR signaling controls neuronal functions, such as synaptic plasticity, learning, and memory (Takei and Nawa, 2014; Lipton and Sahin, 2014). As a result, dysregulation of mTOR signaling has been implicated in several neurodevelopmental and neuropsychiatric disorders (Keppler-Noreuil et al., 2016; Costa-Mattioli and Monteggia, 2013). Single-cell sequencing approaches in the developing human brain and brain organoids have highlighted that mTOR signaling components are highly expressed by outer radial glia (oRG) cells, the human neural stem cells prevalent in the developing human cortex. Dysregulation of the mTOR pathway can result in changes in oRGs cellular morphology, migration, and mitotic behaviors (Lui et al., 2011; Nowakowski et al., 2017; Andrews et al., 2020). mTOR complexes also play an essential role during neurogenesis and gliogenesis, and, later on during development, they participate in the dendritic formation and density, activity-dependent synaptic translation and plasticity, autophagy-mediated pruning of obsolete synapses, oligodendrocyte differentiation and myelination (Takei and Nawa, 2014; Bockeaert and Marin, 2015; LiCausi and Hartman, 2018; LaSarge et al., 2019; Nguyen and Bordey, 2021).

2. mTOR-related cortical malformations

2.1. Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disease affecting multiple body systems with wide variability in presentation (1:6000 live births). TSC is caused by inherited or sporadic loss-of-function mutations in the mTOR inhibitors *TSC1* or *TSC2* (Martin et al., 2017). The *TSC1* and *TSC2* genes encode for the hamartin and

tuberin proteins that, together with TBC1D7, form a GAP complex that inhibits RHEB, thus, inhibiting mTOR signaling. Inactivating mutations in *TSC* genes cause constitutive activation of mTORC1.

The neurological manifestations of TSC include drug-resistant epilepsy and variable degrees of neurodevelopmental abnormalities, such as autistic spectrum disorder and intellectual disability (Northrup et al., 2021). In the brain, TSC can manifest with the development of epileptogenic cortical tubers, subependymal nodules (SENs) and subependymal giant cell astrocytoma (SEGA). Cortical tubers are targeted for surgical resection in drug-resistant epilepsy. Histopathological analysis of resected brain tubers has revealed the presence of morphologically abnormal cells, termed dysmorphic neurons and giant cells, which display mTOR signaling hyperactivation, as shown by phosphorylation of S6K1 and ribosomal S6 protein (Baybis et al., 2004; Miyata et al., 2004; Jungberg et al., 2006). Investigation of a series of fetal TSC brain tissues revealed activation of the mTOR pathway as early as 23 gestational weeks. Cortical tuber formation is a lengthy process that begins with dysmorphic astrocytes appearing at 19–21 weeks gestation, followed by the formation of giant cells at 24 weeks gestation, and lastly, dysmorphic neurons emerging by 36 weeks gestation (Gelot and Represa, 2020). Tubers and SEGAs are thought to arise due to somatic loss-of-function mutations that disrupt the second allele and cause somatic biallelic inactivation (Knudson's 2-hit hypothesis); however, second hits in *TSC1/TSC2* are not always detected (Martin et al., 2017). A study in *TSC2* human brain organoid, instead, postulates that somatic biallelic inactivation occurs during TSC tumor progression and is not needed for tuber formation (Eichmüller et al., 2022).

2.2. Focal cortical dysplasia type II and hemimegalencephaly: a disease continuum

The term “Focal Cortical Dysplasia” (FCD) describes a spectrum of cortical malformations defined by a consensus classification scheme of the International League against Epilepsy (ILAE) in 2011 (Blümcke et al., 2011) that was recently updated to integrate genetics findings (Najm et al., 2022). FCD is classified into three main histopathological subtypes. This review focuses on FCD type II (FCDII), the most common forms of FCD, which belong to the mTORopathies (Wagstyl et al., 2022).

FCDII emerges during prenatal brain development due to abnormal

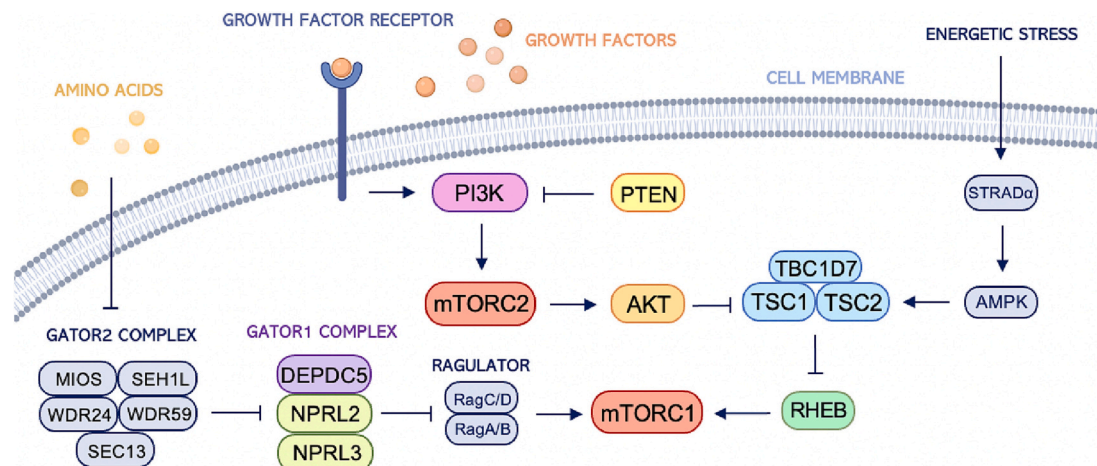


Fig. 1. Upstream regulation of the mTOR-signaling pathway. The mTOR signaling pathway is composed of two complexes with distinct structures and functions: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Activation of mTORC1 is mediated by the upstream PI3K/AKT signaling cascade and the GTPase RHEB, which is inhibited by the tuberous sclerosis complex (TSC). The presence of amino acids at the lysosomal surface inhibits the GATOR1 complex consisting of DEPDC5, NPRL2 and NPRL3. GATOR1 acts as a GTPase-activating protein for RagA/B and RagC/D. This inhibition prevents mTORC1 interaction with the GTP-binding protein RHEB, thus disinhibiting the signaling. Activation of mTORC2 is triggered by growth factors through the IGF1 receptor at the cellular membrane, which activates PI3K. PTEN negatively regulates PI3K activation. When activated, PI3K activates AKT, which inhibits TSC, leading to mTOR1 activation via RHEB.

neuronal migration and differentiation; it is characterized by focally disrupted cortical lamination and the presence of abnormally large pathological cells, dysmorphic neurons in FCDIIA and FCDIIB, and balloon cells in FCDIIB only, similar to the dysmorphic and giant cells observed in cortical tubers in TSC. Both dysmorphic neurons (labeled with Smi311) and balloon cells (labeled with Vimentin) display mTOR hyperactivation as shown by pS6 immunostaining (Fig. 2).

The mTORopathies encompass a range of cortical malformations. FCDII are variable in size: from small lesions restricted to a bottom-of-sulcus dysplasia, confined to one cortical gyrus, or extended to multiple lobes, to large dysplasia affecting an entire hemisphere. In some cases, FCDII are non-visible lesion even using high-resolution MRI. FCDII are preferentially distributed around the superior frontal sulcus, the frontal pole, and the temporal pole (Wagstyl et al., 2022). Megalencephaly (MEG) and hemimegalencephaly (HME) are more extended brain malformations, often associated with altered cortical cytoarchitecture, intractable pediatric epilepsy, intellectual disability, and autistic spectrum disorder. In MEG, brain size and structure are altered bilaterally, whereas, in HME, only one hemisphere is affected. In addition to increased brain size, individuals with HME/MEG may exhibit gyral disorganization and other abnormalities, such as cutaneous vascular malformations and skeletal deformities as seen in megalencephaly–polymicrogyria–polydactyly–hydrocephalus (MPPH) syndrome and megalencephaly–capillary malformation (MCAP) syndrome (Mirzaa et al., 2012). FCDII, HME and MEG represent a disease continuum linked to mTOR dysregulation during brain development: they share clinical and histopathological features, and, in the past decade, clear evidence of the same genetic etiology.

FCDII is a common cause of drug-resistant epilepsy. In most cases, FCDII is amenable to surgical resection of the epileptogenic zone (i.e., the area responsible for seizure generation) to control seizures; in HME cases, the disconnection of the affected hemisphere is usually required (hemispherotomy). FCD represents up to 39% of cases in the pediatric epilepsy surgery population (Blümcke et al., 2017). Analysis of surgical brain tissue has allowed for the discovery of brain-restricted somatic mutations accounting for the hidden genetics in FCDII.

3. Postzygotic mutations and clonal expansion during cortical development

Postzygotic or somatic mutations occurring during embryonic development and accumulating throughout life are present in only a subset of an individual's cells, resulting in genetic mosaicism. The mosaicism distribution and extent depend on the timing of the mutational event (Fig. 3). The proportion of mutated alleles is referred to as variant allele frequency (VAF) or mosaic fraction. While germline heterozygous variants are present in all tissues of the body with a VAF of 50%, somatic mutations can be detected at variable mosaic fractions and may be restricted to a subpopulation of clonal cells in single or multiple tissues (Bizzotto and Walsh, 2022). Somatic mutations are major drivers of cancer development when they result in oncogene activation or tumor suppressor gene inactivation (Tate et al., 2019).

The focal and mosaic nature of FCDII/HME/MEG has been resolved during the past decade with the identification of brain somatic mutations in genes of the mTOR pathway. Somatic mutations in genes of this pathway were first identified in brain tissues from large cortical malformations such as HME/MEG (Lee et al., 2012; Poduri et al., 2012; Rivière et al., 2012). Somatic gain-of-function mutations in mTOR pathway activating genes (*AKT3*, *MTOR*, *PIK3CA*, *RHEB*) and germline loss-of-function mutations of mTOR inhibitors (*DEPDC5*, *NPRL2*, *NPRL3*, *TSC1* and *TSC2*) are now recognized as a significant cause of FCDII/HME/MEG. FCDII/HME/MEG somatic variants in the mTOR pathway are also cancer mutational hotspots and listed in the Catalogue Of Somatic Mutations In Cancer (COSMIC, <https://cancer.sanger.ac.uk>).

Under the assumption that variants do not exert negative effects on cell survival, it is assumed that a mutation occurring earlier during development generates a larger clonal population of mutated cells within an individual. In the context of FCDII/HME/MEG, it is thought that mutations affecting a progenitor cell in the ventricular zone during early development may produce large HME/MEG, whereas mutations occurring later may lead to smaller FCDII lesions typically affecting <5% of brain cells within the malformed area (Blümcke et al., 2021a).

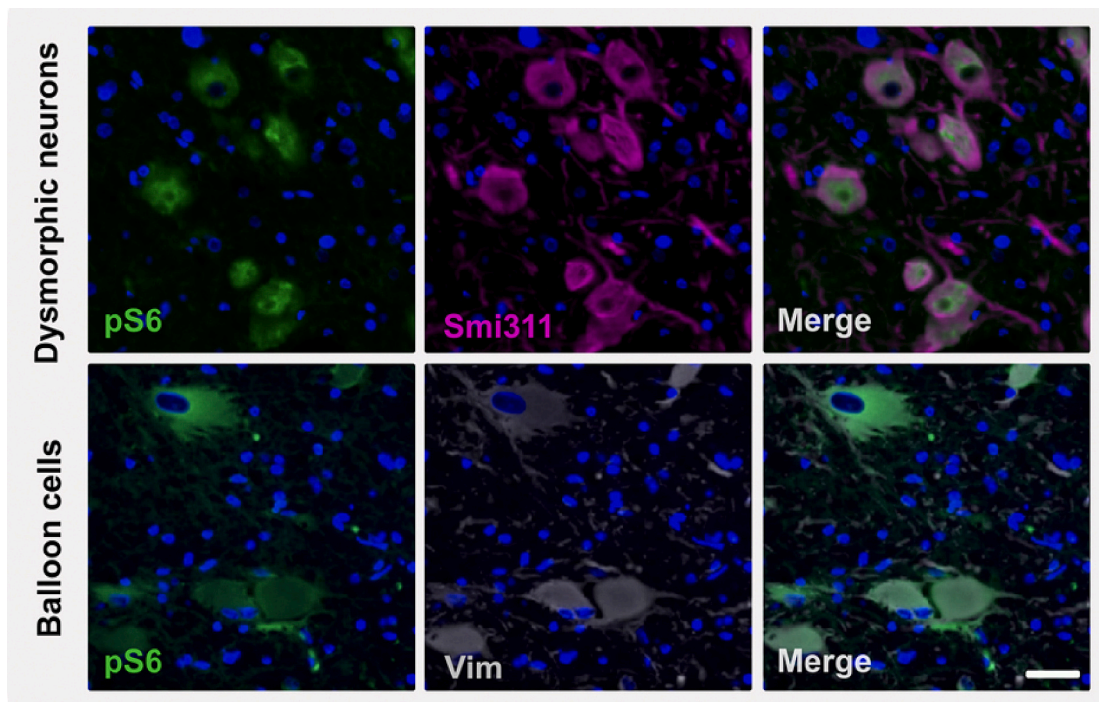


Fig. 2. Characteristic immunoreactivity in dysmorphic neurons and balloon cells in a surgical FCDII case with *MTOR* p.Thr1977Lys mutation. Dysmorphic neurons show strong immunoreactivity for the neurofilament marker Smi311, while balloon cells show strong Vimentin immunoreactivity (Vim). Both dysmorphic neurons and balloon cells exhibit mTOR activation revealed by phosphorylated S6 immunoreactivity (pS6). Scale bar: 50 μ m.

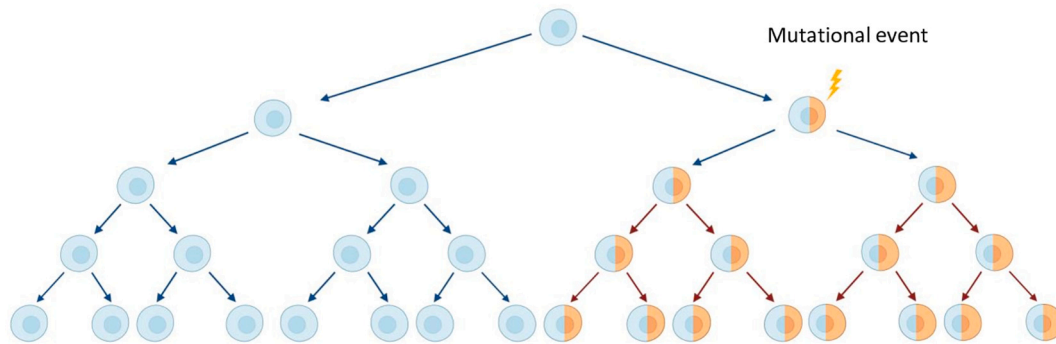


Fig. 3. Somatic mosaicism and clonal expansion of postzygotic mutations during cell division. As cells divide, mutations can become clonally expanded in daughter cells, leading to a mosaic pattern of mutated cells. The frequency of the mutated allele, known as the variant allele frequency (VAF), can depend on the timing of the mutational event or the impact of the mutation on cellular fitness. If the mutation does not negatively impact the fitness of the affected cells, the VAF may increase over time as the mutated cells divide and expand.

4. Landscape of somatic mutations in cortical malformations

We performed a systematic review of PubMed literature from January 2012 to January 2023 to list all somatic and germline mutations reported in surgical epilepsy and FCDII/HME/MEG. We performed a PubMed query using the keywords “somatic” AND “brain” AND “cortical development” AND “epilepsy”, which yielded 80 publications (Fig. 4). Out of those 80 publications, 44 were excluded for various reasons (reviews, animal studies, studies not related to the mTOR pathway...), leaving 36 publications that were included in the study. Additionally, 10 relevant publications were manually curated and added to the analysis by examining the cited references within each article. Overall, we analyzed genetic data from 46 articles and identified 292 patients with pathogenic or likely pathogenic mutations in one of the mTOR pathway genes on cerebral tissue diagnosed with FCDII/HME/MEG, as shown in

Table 1 (Lee et al., 2012; Poduri et al., 2012; Xu et al., 2022; Lai et al., 2022; Bedrosian et al., 2022; Jha et al., 2022; Zimmer et al., 2021; Koh et al., 2021; Blümcke et al., 2021b; Blümcke et al., 2021; Kim et al., 2021; Lee et al., 2021; Lee et al., 2020; Dimartino et al., 2020; Zhang et al., 2020; Baldassari et al., 2019a; Sim et al., 2019; Pelorosso et al., 2019; Niestroj et al., 2019; Salinas et al., 2019; Kim et al., 2019; Zhao et al., 2019; Lee et al., 2019; Ying et al., 2019; Park et al., 2018; Ribierre et al., 2018; Avansini et al., 2018; D’Gama et al., 2017; Hanai et al., 2017; Lim et al., 2017; Lim et al., 2015; Baek et al., 2015; Nakashima et al., 2015; Leventer et al., 2015; Baulac et al., 2015; Jansen et al., 2015; Mirzaa et al., 2016; Griffin et al., 2017; Garcia et al., 2020; Benova et al., 2021; Guerrini et al., 2020; Koboldt et al., 2021; Pirozzi et al., 2022; López-Rivera et al., 2023; Kim et al., 2023; Lim and Lee, 2016). Cases with several mutations in mTOR-activating genes were excluded. This comprehensive literature review highlights a contribution of

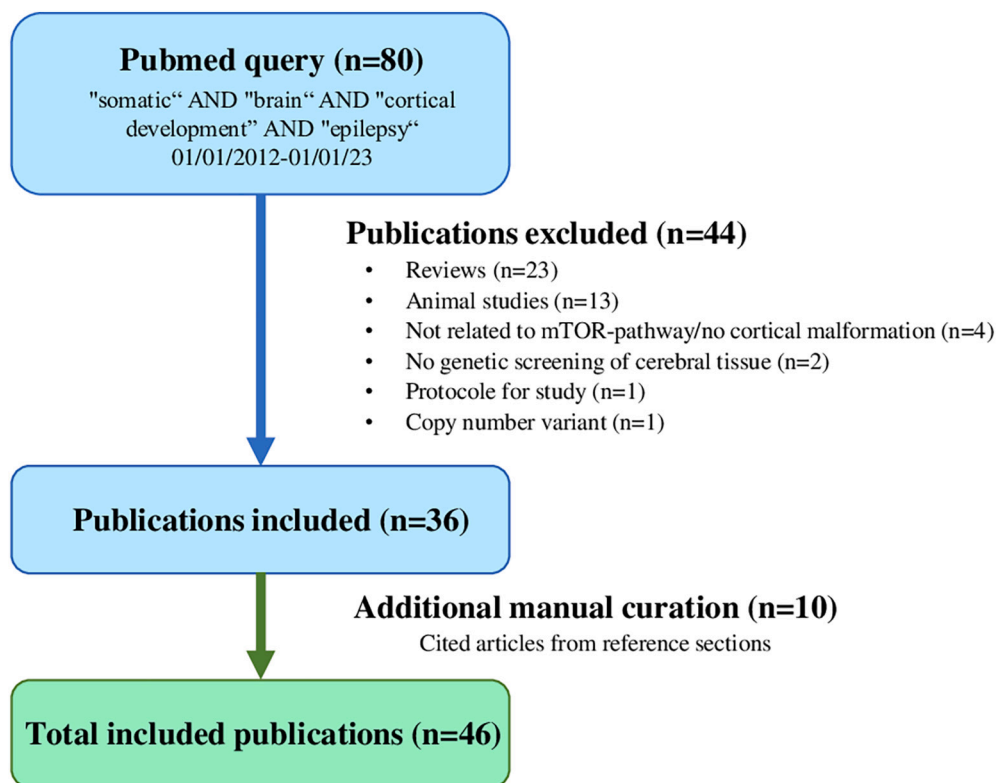


Fig. 4. Flow diagram of article selection process showing the publication search strategy using Pubmed queries. A total of 46 publications that include 292 patients were analyzed.

Table 1

Germline and somatic variants identified in surgical brain tissues of FCDII/HME/MEG cases.

Gene	Nb of patients	FCDII	HME	MEG
Cases with somatic variants				
<i>MTOR</i>	147	130 (68% FCDIIB; 32% FCDIIA)	15	2
<i>PIK3CA</i>	28	2 (100% FCDIIA)	25	1
<i>AKT3</i>	21	12 (100% FCDIIA)	8	1
<i>TSC2</i> ¹	18	16 (87.5% FCDIIB; 12.5% FCDIIA)	2	0
<i>TSC1</i>	15	15 (60% FCDIIB; 40% FCDIIA)	0	0
<i>RHEB</i>	9	6 (50% FCDIIB; 33% FCDIIA; 17% not specified)	3	0
Cases with germline variants				
<i>DEPDC5</i> ^{1,2}	31	30 (10% FCDIIB; 90% FCDIIA)	1	0
<i>NPRL3</i> ³	12	11 (9% FCDIIB; 91% FCDIIA)	1	0
<i>PTEN</i> ²	5	2 (50% FCDIIB; 50% FCDIIA)	3	0
<i>NPRL2</i>	4	4 (100% FCDIIA)	0	0

A total of 292 cases were reviewed (Lee et al., 2012; Poduri et al., 2012; Xu et al., 2022; Lai et al., 2022; Bedrosian et al., 2022; Jha et al., 2022; Zimmer et al., 2021; Koh et al., 2021; Blümcke et al., 2021b; Blümcke et al., 2021; Kim et al., 2021; Lee et al., 2021; Lee et al., 2020; Dimartino et al., 2020; Zhang et al., 2020; Baldassari et al., 2019a; Sim et al., 2019; Pelorosso et al., 2019; Niestroj et al., 2019; Salinas et al., 2019; Kim et al., 2019; Zhao et al., 2019; Lee et al., 2019; Ying et al., 2019; Park et al., 2018; Ribierre et al., 2018; Avansini et al., 2018; D'Gama et al., 2017; Hanai et al., 2017; Lim et al., 2017; Lim et al., 2015; Baek et al., 2015; Nakashima et al., 2015; Leventer et al., 2015; Baulac et al., 2015; Jansen et al., 2015; Mirzaa et al., 2016; Griffin et al., 2017; Garcia et al., 2020; Benova et al., 2021; Guerrini et al., 2020; Koboldt et al., 2021; Pirozzi et al., 2022; López-Rivera et al., 2023; Kim et al., 2023; Lim and Lee, 2016). In addition to patients in the table, 2 cases of HME/MEG were reported with a somatic mutation in *AKT1* (not represented). Abbreviations: FCDII: Type II focal cortical dysplasia; HME: Hemimegalencephaly; Nb: Number; MEG: Megalencephaly. ¹Double-hit germline and somatic mutations were reported for the *DEPDC5* gene (6 cases) and for the *TSC2* gene (1 case). ²One of the reported *DEPDC5* and one of the reported *PTEN* patients presented with double biallelic somatic mutations. ³Three of these patients were reported with a somatic *NPRL3* mutation.

somatic and germline mutations in nine genes (*AKT3*, *DEPDC5*, *MTOR*, *NPRL2*, *NPRL3*, *PIK3CA*, *RHEB*, *TSC1* and *TSC2*) in the spectrum of FCDII/HME/MEG. To note rare mutations were also reported in *AKT1* ($n = 2$) and *PTEN* ($n = 5$). The genetic diagnostic rate was variable across studies, ranging from 16% to 57% in FCDII and 14% to 88% in HME/MEG.

Gain-of-function somatic hotspot mutations in the pathway

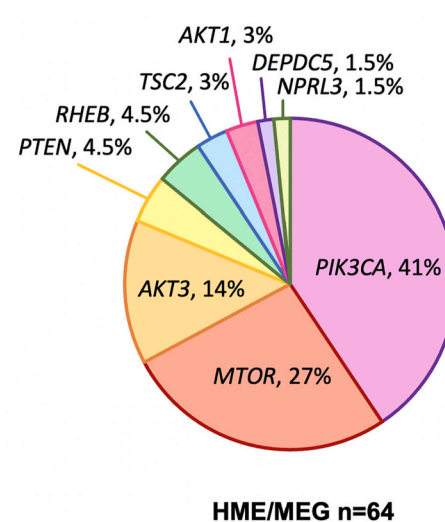
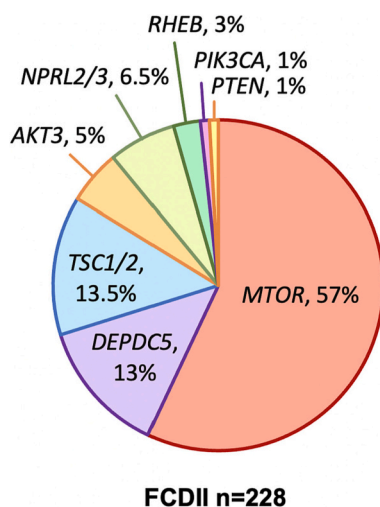


Fig. 5. Gene contribution in FCDII cases ($n = 228$) and HME/MEG cases ($n = 64$). Pie charts showing the percentage of mutations across different genes in FCDII/HME/MEG.

activators account for two-thirds (207/292) of the cases, 71% of which affect the *MTOR* gene itself (147/207) (Table 1). In 29% of the cases (85/292), loss-of-function mutations were reported in mTOR pathway inhibitors: germline and somatic mutations in GATOR1 complex genes (*DEPDC5*, *NPRL2*, and *NPRL3*) and in *PTEN*, and somatic mutations in *TSC1* and *TSC2* genes. Overall, mutations in mTOR-signaling activators (*AKT3*, *MTOR*, *PIK3CA*) are mainly associated to HME/MEG, accounting for 90% of all cases. On the other hand, mutations in genes related to the GATOR1 complex (*DEPDC5*, *NPRL2*, *NPRL3*) and the TSC complex (*TSC1* and *TSC2*) are more commonly associated with smaller malformations (FCDII) (Fig. 5). Double-hit germline and somatic mutations were reported in *DEPDC5* ($n = 6$) and *TSC2* ($n = 1$) (Zimmer et al., 2021; Baldassari et al., 2019a; Sim et al., 2019; Lee et al., 2019; D'Gama et al., 2017; Mirzaa et al., 2016). Double biallelic somatic mutations were also reported in *DEPDC5* ($n = 1$) and *PTEN* ($n = 1$) (Mirzaa et al., 2016; Koboldt et al., 2021).

Among cases of HME/MEG with mutations in *AKT3*, *MTOR*, *PIK3CA* and *RHEB* the VAF ranged from 0.16% to 50% (median 16%) and was above 5% in 91% of cases. In FCDII cases, a lower VAF was reported, ranging from 0.34% to 31% (median 4%), below 5% in 70% of the cases (Fig. 6). Therefore, there is a correlation between the VAF and the size of the malformation, with FCDII lesions having a lower VAF on average compared to HME/MEG. These findings confirm that the time window during which these mutations occur in the developing human brain might have a substantial impact, with HME/MEG-associated mutations likely arising earlier during fetal development and thus affecting a more significant fraction of the developing brain than FCDII-associated mutations.

4.1. Somatic mutations in the *MTOR* gene

mTOR (mechanistic target of rapamycin) is a 289-kDa protein kinase composed of several domains (HEAT repeats, FAT, FRB, kinase, and FATC) (Fig. 7).

Somatic *MTOR* variants in the brain are the predominant cause of FCDII, accounting for 57% of cases (130/228). Nearly 90% of cases with *MTOR* variants were diagnosed with an FCDII (130/147) (Table 1). Somatic *MTOR* variants are more rarely detected in more extensive cortical malformations and only account for 27% of the HME/MEG (17/64) cases (Fig. 5). Median VAF was 4% (ranging from 0.16% to 31%). The FAT and kinase domains are the mutational hotspots, with 74% (17/23) of the variants, accounting for 94% (138/147) of the patients.

We also observed a correlation between the variant type and the

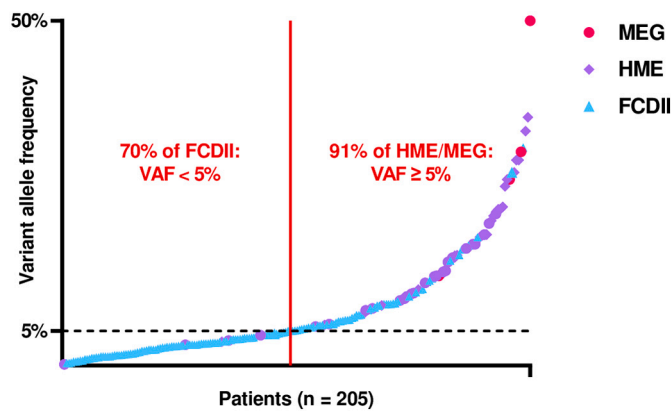


Fig. 6. Relation between the brain VAF and the size of the lesion. Scatter plot showing the distribution of brain variant allele frequency (VAF) in 205 patients with somatic variants in *MTOR* ($n = 147$), *PIK3CA* ($n = 28$), *AKT3* ($n = 21$) and *RHEB* ($n = 9$) genes. FCDII cases are highlighted in green, HME in orange and MEG in pink. The VAF increases with the lesion size, indicating that larger lesions have a higher proportion of mutated cells. Among reported FCDII cases, VAF was below 5% in 70%, and in HME/MEG, VAF was above 5% in 91% of cases. (For accurate interpretation of the color references in the figure legend, the reader is referred to the web version of this article.)

lesion size. The three recurrent mutations p.Leu1460Pro ($n = 15$), p.Ser2215Phe ($n = 35$), p.Ser2215Tyr ($n = 30$) were more frequently found in FCDII lesions (93%, 75/80) compared to the p.Cys1483Arg ($n = 6$), p.Cys1483Tyr ($n = 4$), and p.Ile2500Phe ($n = 4$) variants, which are more often associated with HME/MEG phenotypes (50%, 7/14). The three variants p.Leu1460Pro, p.Ser2215Phe and p.Ser2215Tyr are also found with a lower VAF in FCDII lesions (median 3.8%) than the p.Cys1483Tyr, p.Cys1483Arg and p.Ile2500Phe variants (median 6.6%). This is consistent with a previous study evaluating in vitro the functional effects of *MTOR* mutations and showing variable degrees of mTOR hyperactivation depending on the type of variants and among patient groups (Mirzaa et al., 2016). *MTOR* mutations found in HME/MEG patients, which included p.Cys1483Tyr, showed an intermediate degree of mTORC1 signaling activation, while the variants found in FCDII patients, such as p.Ser2215Phe, p.Ser2215Tyr, and p.Leu1460Pro lead to stronger mTORC1 activity.

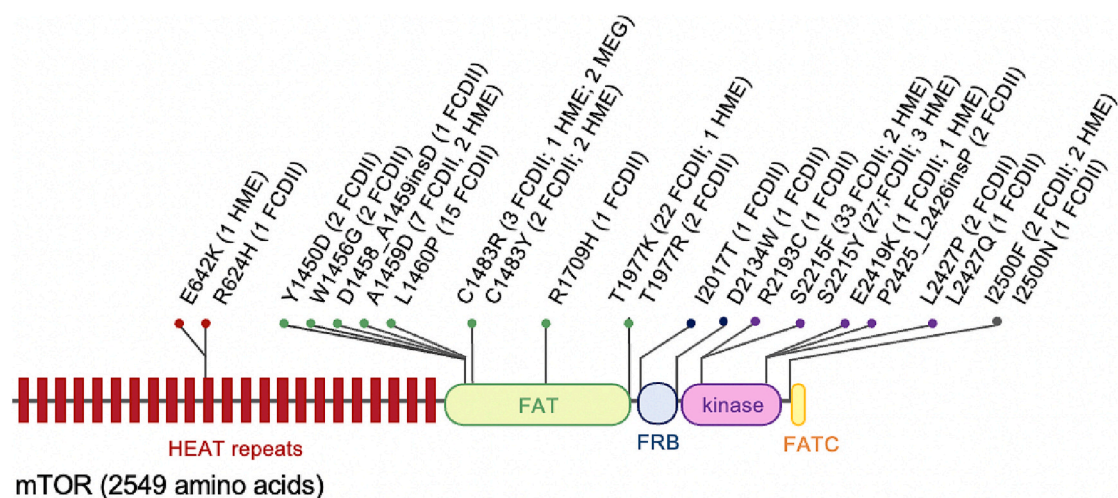


Fig. 7. Schematic representation of the *MTOR* protein showing the location of the brain somatic mutations. In total, 23 distinct somatic mutations among 147 patients with FCDII/HME/MEG were reported. The different domains of the protein include: (i) 20 HEAT repeats domain (amino acids 16–1345), (ii) a FRAP-ATM-TRRAP (FAT) (amino acids 1382–1982), (iii) a FATC domain, (iv) a FKBP12-rapamycin binding (FRB) domain (amino acids 2012–2144), responsible for binding to the rapamycin and its analogs, (v) a kinase domain (amino acids 2156–2469), responsible for the phosphorylation of downstream substrates and (vi) a FATC domain (amino acids 2517–2549) located at the C-terminus.

4.2. Somatic mutations in the *PIK3CA* gene

PIK3CA encodes p110 α , the 110-kDa catalytic subunit of phosphatidylinositol 3-kinase (PI3K), a lipid kinase enzyme that activates AKT through phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-trisphosphate (PIP₃). p110 α comprises several functional domains: ABD, RAS, C2, helical, and kinase (Fig. 8).

To date, 28 cases with brain somatic gain-of-function variants in *PIK3CA* have been reported in the literature, among which 93% (26/28) present with HME/MEG (Table 1), and a median mosaic level of 20% (ranging from 3% to 36%). *PIK3CA* represent the most frequent somatic variants in HME/MEG (41%, 26/64) (Fig. 5).

Three hotspot *PIK3CA* mutations are commonly found in FCDII/HME/MEG, accounting for nearly 90% (25/28) of the cases: p.Glu542-Lys ($n = 9$), p.Glu545Lys ($n = 10$) in the helical domain, and p.His1047Arg ($n = 6$) (Fig. 8). The three mutational hotspots are also known to be the most activating mutations in cancer (Gymnopoulos et al., 2007) and all reported mutations were documented in the COSMIC database.

4.3. Somatic mutations in the *AKT3* gene

AKT3 is a small serine/threonine-protein kinase of 56-kDa. The protein is one of the three closely related serine/threonine-protein kinases (AKT1, AKT2, and AKT3). Upon activation, *AKT3* phosphorylates TSC2, thus causing its destabilization and disrupting its interaction with TSC1, allowing mTOR activation.

Brain somatic mutations in the *AKT3* gene account for 7% of FCDII/HME/MEG patients (21/292) (Table 1). FCDII is the most common lesion diagnosed in patients with *AKT3* brain mosaicism, accounting for 57% (12/21) of the cases, with a median brain VAF of 3% (ranging from 0.6% to 12.3%). Somatic mutations associated with HME/MEG (43%, 9/21) are present with higher levels of mosaicism (median VAF at 13%, ranging from 4.4% to 50%) (Fig. 6). The p.Glu17Lys mutation hotspot is detected in 90% of *AKT3* cases and is also a recurrent variant in cancer. This variant is located in the lipid-binding pocket of the PH domain and increases the binding of *AKT3* to PIP₃. Moreover, one *AKT1* hotspot somatic gain-of-function variant was reported in two patients with HME/MEG (D'Gama et al., 2017; Conti et al., 2015).

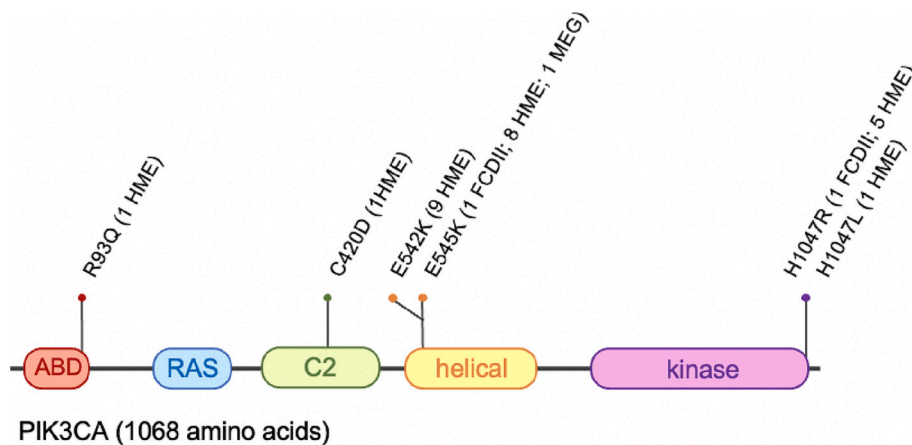


Fig. 8. Schematic representation of the PIK3CA protein showing the location of somatic mutations. In total 6 distinct somatic mutations among 28 patients with FCDII/HME/MEG were reported. The different domains of the protein include: (i) Adaptor-Binding Domain (ABD) (amino acids 15–65) involved in the interaction with the p85 α subunit, (ii) the RAS-binding domain (amino acids 187–289), (iii) the C2 domain (amino acids 330–487), (iv) the helical domain (amino acids 517–694) and (v) the kinase domain (amino acids 765–1051) with the enzymatic activity of the subunit.

4.4. Somatic mutations in the RHEB gene

RHEB is a small GTPase protein allowing mTORC1 activation when present in its GTP-bound state (Fig. 1). Brain somatic mutations in *RHEB* have been described more recently in FCD/HME cases. *RHEB* somatic mutations account for 3% of the cases, with a total of 9/292 patients (Table 1). The two p.Glu40Val and p.Tyr35Leu recurrent mutations are located within or in proximity of the GTP binding region of the protein. Among the *RHEB*-positive cases, 67% (6/9) were diagnosed with FCDII with a median VAF of 9% (ranging from 2.6% to 11%), whereas reported patients with HME (3/9, 33%) presented a median VAF at 18% (ranging from 18% to 22%). In a study describing three patients with a spectrum of FCDII of various sizes associated with pathogenic brain-specific somatic *RHEB* mutations, the somatic variant load directly correlated with the lesion size; laser capture microdissection showed enrichment of *RHEB* variants in dysmorphic neurons and balloon cells (Lee et al., 2021).

4.5. Germline and somatic mutations in the *DEPDC5*, *NPRL2* and *NPRL3* genes (*GATOR1* complex)

GATOR1 complex functions as a negative regulator in the amino acid-sensing branch of the mTOR pathway (Fig. 1). It comprises three subunits: *DEPDC5*, *NPRL2*, and *NPRL3*. Germline mutations in *DEPDC5*, *NPRL2*, and *NPRL3*, are recognized as the most frequent genetic cause of familial focal epilepsies (*GATORopathies*). Cortical malformations, mainly FCDII, are observed in 20% of *GATOR1*-related cases (Baldassari et al., 2019b; Baulac and Baldassari, 2016).

According to Knudson's two-hit model (Knudson, 1971), somatic second-hit mutations have been proposed to explain the focal and mosaic nature of the FCDII lesion within pedigrees with inherited germline mutations in *GATOR1* genes. Somatic second-hit mutations causing a biallelic inactivation have been demonstrated in surgically resected FCDII/HME from 6 patients with germline *DEPDC5* variants (Zimmer et al., 2021; Baldassari et al., 2019a; Sim et al., 2019; Lee et al., 2019; Baulac et al., 2015). The median VAF for the somatic variant was 4% (ranging from 3% to 10%). In two reported cases, the second somatic event was a copy-neutral loss of heterozygosity, a genetic event where there is a loss of one copy of a chromosome region followed by duplication of the remaining copy from the other parent. The variability in phenotype observed within and between families with germline *GATOR1* complex variants may be attributed to second-hit somatic mutations in the brain. In addition, three patients have also been described with a somatic loss-of-function *NPRL3* mutation, presenting with FCDII (Niestroj et al., 2019; López-Rivera et al., 2023; Chung et al., 2023), and one patient was reported with a biallelic somatic hit in *DEPDC5* in an HME patient (Mirzaa et al., 2016). Most *GATOR1* variants

result in loss-of-function (94%; 51/54) due to stop-gain, frameshift indels or splice mutations.

GATOR1 mutations account for 20% (45/228) of FCDII and 3% (2/64) of HME/MEG among the 292 reported cases (Table 1). FCDII is observed in 96% (45/47) of cases with mutations in *GATOR1* encoding genes, and HME is rarely associated (4%, 2/47).

4.6. Germline and somatic mutations in the *PTEN* gene

PTEN is a 403 amino-acid phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that blocks the activation of *AKT* (Fig. 1). Inactivating variants in *PTEN* are a rare cause of FCDII/HME, and only five cases with genetic analysis on cerebral tissue have so far been reported (Table 1, Fig. 5) with high mosaic fractions (>30%). A recent study reported two somatic *PTEN* variants affecting a single HME patient, with the first variant found across multiple cell lineages throughout the entire hemisphere and associated with mild cerebral overgrowth, and the second variant affecting the second allele, confined to posterior brain regions, leading to a segmental area of more pronounced malformation (Koboldt et al., 2021). A double germline and somatic two-hit model has been described in Lhermitte-Duclos disease, a cerebellar tumor (Zhou et al., 2003).

4.7. Somatic mutations in the *TSC1* and *TSC2* genes (*TSC* complex)

The *TSC* complex consists of three proteins, *TSC1*, *TSC2*, and *TBC1D7*, which function together as a negative regulator of mTOR activity. *TSC* acts as a GTPase activating protein for the small GTPase *RHEB*, an activator of mTORC1. By promoting the conversion of *RHEB*-GTP to *RHEB*-GDP, the *TSC* complex inhibits mTORC1 activity and downstream signaling (Fig. 1). Brain somatic variants in *TSC1* ($n = 15$) and *TSC2* ($n = 18$) are reported in 11% of the cases (33/292) (Fig. 5). Most patients were diagnosed with FCDII (94%, 31/33), with a median VAF of 3.7% (Table 1).

4.8. Unsolved FCDII/HME/MEG cases

Over the past decade, advances in next-generation sequencing technology and bioinformatic pipelines for the somatic variant calling have accelerated genetic discoveries in epilepsies. High read-depth sequencing is required to identify brain-restricted somatic mutations, especially low-level mosaic mutations with VAF below 5%. While most studies are based on targeted sequencing using a capture panel at deep coverage (>1000 \times sequencing depth), allowing the high sensitivity required for the detection of low mosaic level mutations, an alternative approach that is well suited for the molecular profiling of hotspot mTOR pathway variants is the droplet digital polymerase chain reaction

(ddPCR). ddPCR allows ultra-sensitive and highly targeted screening of mutations of interest, as recently shown in a study achieving a 29% diagnostic rate in FCDII by testing six mutational hotspots in the genes *PIK3CA* (p.Glu542Lys, p.Glu545Lys, p.His1047Arg), *AKT3* (p.Glu17Lys) and *MTOR* (p.Ser2215Phe, p.Ser2215Tyr) (Pirozzi et al., 2022).

In surgically resected tissues containing very few mutated cells (e.g., rare and sparse dysmorphic neurons or balloon cells), ultra-low-level somatic mutations may be undetectable by deep sequencing in bulk brain tissues. A recent study used fluorescence-activated cell sorting (FACS)-mediated enrichment of mTOR hyperactivated neurons prior to sequencing to detect ultra-low mosaic fractions below 0.2% in bulk tissue by whole genome sequencing (Kim et al., 2023). Despite these recent advances, about 20–40% of FCDII cases remain unsolved (Baldassari et al., 2019a; Kim et al., 2023). In panel-negative cases, several recent studies based on deep whole-exome and whole-genome sequencing have been performed to attempt to identify new FCD/HME causal genes (Bedrosian et al., 2022; López-Rivera et al., 2023; Chung et al., 2023) and detect small structural variants (Kim et al., 2023), which may also be detected with array-based comparative genomic hybridization (Kim et al., 2023; Conti et al., 2015).

5. Detecting brain mosaicism: implications for genetic diagnosis

In the context of drug-resistant epilepsy, genetic diagnosis of mTOR pathway activating mutations could reveal significant prognostic insights and have an impact in regular clinical practice. For instance, FCDII due to somatic brain mutations may have a tendency toward a favorable epilepsy surgery outcome (Moloney et al., 2021). On the other hand, GATOR1-related epilepsies may be associated with an increased risk of sudden unexpected death in epilepsy (SUDEP) (Bacq et al., 2022; Bagnall et al., 2016). The important role of a genetic diagnosis has also been acknowledged in the neuropathological FCD classification systems, which now include molecular classification (Najm et al., 2022).

Mosaic variants arising in brain tissue are increasingly recognized as a hidden cause of focal epilepsy. However, detecting somatic mutations in patients with FCDII/HME requires brain tissue analysis, which is invasive and challenging. In larger malformations such as HME/MEG, somatic mutations in *PIK3CA* and *AKT3* may be detected in saliva-derived DNA samples, thus allowing a genetic diagnosis also in patients that would not be eligible for surgery (Pirozzi et al., 2022). Other methods are being developed to detect brain somatic mutations when brain tissue is unavailable. Liquid biopsy of cell-free DNA (short fragments of non-encapsulated DNA) released into the cerebrospinal fluid (CSF) or the bloodstream, has shown clinical applications in cancer diagnostics and disease monitoring, including detecting somatic mutations in malignant brain tumors (Wang et al., 2015). In one study, somatic mutations in *PIK3CA*, *BRAF*, and *SLC35A2* genes were detected by ddPCR in CSF-derived cell-free DNA from 25% of epilepsy surgery patients with confirmed somatic mutations in the brain tissue (Kim et al., 2021). Another study used the same approach to detect mosaic somatic mutations in the mTOR pathway genes *TSC1* and *PIK3CA* in CSF-derived cell-free DNA obtained via dural puncture before surgery in two patients with FCDIIB and HME (Ye et al., 2022). Establishing genetic diagnosis of epilepsy-associated cortical malformations before surgery might inform on prognosis and facilitate early adoption of potential precision therapies.

Another alternative approach for detecting brain somatic mutations is to analyze the trace of brain-derived DNA on the surface of explanted stereoelectroencephalography (SEEG) electrodes used in presurgical epilepsy workup to localize the epileptogenic zone. A proof-of-concept study detected a somatic high-*VAF* *MEN1* mutation from cells adhered to the surface of SEEG depth electrodes in a patient with drug-resistant focal epilepsy and bilateral periventricular nodular heterotopia (Montier et al., 2019). Another recent publication described a somatic pathogenic *KCNT1* loss-of-function mutation at high-*VAF* detected from SEEG electrodes, with a mosaic gradient showing a correlation with the

SEEG electrophysiological findings: the region with the highest mutant allele fraction was located in the most epileptogenic area (Jiang et al., 2022). Similar results were also recently obtained in FCDII patients with low-*VAF* somatic mutations in *DEPDC5* and *AKT3* by Checri et al. (Checri et al., 2023). These proof-of-concept studies emphasize future opportunities for integrating genetic testing from SEEG electrodes into the presurgical evaluation of refractory epilepsy patients to guide precision medicine.

6. mTOR pathway hyperactivation and epileptogenesis

Recent studies indicate that cytomegalic dysmorphic neurons in FCDII harbor somatic mutations with enriched mutation load, establishing a clear link between the presence of the mutation and cytomegaly. A study has shown that FCDII somatic variants are enriched in neuronal cells compared to non-neuronal cells (D'Gama et al., 2017). Another study used laser capture microdissected pools of dysmorphic neurons and balloon cells to show that these abnormal cells carry the mutations (Lee et al., 2021; Baldassari et al., 2019a). Therefore, the density of dysmorphic neurons and balloon cells in bulk tissues plays an important role in the detection of somatic variants. FCDII brain specimens with somatic mutations exhibit a mutational gradient of somatic mosaicism: tissues with a higher density of dysplastic cells have higher mosaicism rates (Baldassari et al., 2019a). Conversely, the mutational load is lower in the tissue surrounding the epileptogenic zone, and adjacent normal tissue lacks somatic mutations (Lee et al., 2020; Baldassari et al., 2019a; Lee et al., 2019; Ribierre et al., 2018; Dobyns and Mirzaa, 2019).

Hyperactivity of the mTORC1 pathway has been demonstrated in multiple animal models of mTORopathies, by functional assessments of epilepsy-causing genetic variants in the mTOR pathway in vitro and in surgical brain tissue from patients with FCDII/HME. Immunostaining for downstream substrates of mTORC1 activation, such as phosphorylated ribosomal protein S6, is commonly used to assess mTORC1 activity. Resected FCDII/HME specimens consistently display enhanced mTORC1 activation due to somatic mutations in mTOR pathway activating genes or to germline GATOR1 mutations. Importantly, there is evidence of mTORC1 hyperactivation regardless of the presence of somatic or germline variants (Baldassari et al., 2019a). This observation supports the idea that all FCDII cases are mosaic mTORopathies.

The exact mechanisms through which mTORC1 hyperactivity leads to neuronal hyperexcitability and seizures are not yet fully understood. However, excessive activation of mTORC1 appears to contribute to epileptogenesis by disrupting the formation of neural circuits and altering established neural networks. It is thought that neuronal hyperexcitability may directly result from mTORC1 hyperactivation, but network disruption caused by structural cortical malformation could also contribute. The presence of dysmorphic neurons and balloon cells is considered a neuropathological hallmark of aberrant mTOR signaling, as they consistently exhibit increased mTORC1 activation and are enriched in somatic mutations in FCDII. In vitro electrophysiological studies on FCDII tissue have shown that dysmorphic and immature neurons play a crucial role in generating and propagating epileptic discharges, while balloon cells lack epileptogenicity (Abdijadid et al., 2015).

mTOR hyperactivation has been linked to abnormalities in the morphology of dendritic spines and glutamatergic synaptic transmission. These changes in neuronal structure and function likely contribute to the development of epilepsy. In a mouse model of *DEPDC5*-related FCDII generated using in utero electroporation and CRISPR/Cas9 technology to replicate a second-hit somatic mutation in cortical pyramidal cells, *DEPDC5* inactivation resulted in abnormal dendritic and spine shaping, increased excitatory transmission, and epileptogenesis (Ribierre et al., 2018).

Numerous preclinical mouse models of mTORopathies have been developed to model somatic mutations in vivo and study the

consequences of knockdown or conditional knockout of mTOR pathway inhibitors or overexpression of mTOR pathway activators. Mouse FCD models have been developed using in utero electroporation to produce focal and mosaic cortical expression of mutant *MTOR* and *RHEB* (Lim et al., 2015; Hsieh et al., 2016). Such mutant mice displayed dysmorphic neurons and spontaneous seizures that can be rescued by rapamycin treatment. *DEPDC5* knockout rats displayed mTOR-hyperactive cytomalgalic dysmorphic neurons and lowered seizure thresholds (Marsan and Baulac, 2018). Neuron-specific conditional *DEPDC5* knockout mice display the main human mTORopathy phenotypes, including increased phosphorylated S6 immunostaining, thickened cortex, cytomalgalic neurons, electro-clinical seizures, and SUDEP-like events (Bacq et al., 2022; Yuskaitis et al., 2018). These FCD animal models are reviewed elsewhere (Nguyen and Bordey, 2021).

7. mTOR pathway in the era of personalized medicine

Personalized medicine aims to use an individual's genetic and molecular information to improve clinical decisions and optimize treatments. Most currently available epilepsy treatments are not targeted and only relieve symptoms without addressing the underlying disease. In focal epilepsies and FCDII, the first-line therapy for seizures involves antiseizure medications. However, surgical intervention becomes the preferred course of action if the patient fails to respond. Although resective neurosurgery efficiently controls seizures in most patients, a fraction of cases is not eligible and thus has limited possibilities of a genetic diagnosis.

Enhanced mTORC1 activation is consistently observed in FCDII surgical resections as the result of either loss-of-function mutations in pathway inhibitors such as *DEPDC5*, *NPRL3*, *NPRL2*, *PTEN*, *TSC1* and *TSC2* or gain-of-function mutations in pathway activators such as *AKT3*, *MTOR*, *PIK3CA* and *RHEB*, and even in specimens without detectable somatic mutations (Baldassari et al., 2019a). Rapamycin has also been shown to reduce seizures in rodent models of FCDII (Nguyen and Bordey, 2021).

Therefore, most studies on brain mTORopathies have focused on potential therapeutic options for reducing mTORC1 activity. Several drugs and therapeutic approaches based on antisense oligonucleotides targeting the mTOR pathway hyperactivation are available (or being developed), especially in the context of cancer. The effectiveness of mTOR inhibitors as a treatment for drug-resistant epilepsy caused by FCDII is not yet determined. In cases where immediate surgery is not possible, mTOR inhibitors may be used as a bridging therapy to reduce seizures. They may also be considered as an alternative treatment for surgically inaccessible FCD or persistent seizures after epilepsy surgery. Importantly, because mTOR signaling has ubiquitous roles in the body, global mTOR inhibition may have deleterious side effects that need to be considered, especially in children.

Everolimus, an mTOR inhibitor, was reported to reduce seizure frequency in a large-scale trial of TSC patients (French et al., 2016). Alternatively, everolimus can be used to complement or as a substitute for the surgical procedure in TSC (Bobeff et al., 2021). Since mTOR inhibitors have shown safety and effectiveness in treating drug-resistant epilepsy in TSC, they offer a potentially useful treatment option for epilepsies related to mTOR-activating mutations in the context of FCDII/HME (Auvin and Baulac, 2023). In a study, sirolimus was administered to 16 patients diagnosed with epilepsy-related FCDII, but the reduction of focal seizures did not meet statistical significance (Kato et al., 2022). Moreover, since the mTOR pathway is also involved in the regulation of synaptic plasticity for learning and memory, the development of therapies targeting the mTOR pathway in patients with mTORopathies has the potential to be beneficial not only for the control of seizures but also for the amelioration of cognitive functions.

Mosaic multisystem disorders known as *PIK3CA*-related overgrowth syndromes can be characterized by congenital lipomatous overgrowth, vascular malformations, and skeletal abnormalities with somatic

PIK3CA mutations. Treatment with alpelisib, a selective PIK3CA inhibitor, improved clinical outcomes in individuals with *PIK3CA*-related overgrowth syndromes (Venot et al., 2018). Miransertib, an AKT inhibitor, has also shown promising results in reducing seizures and improving cognitive engagement in a child with HME in the context of *PIK3CA*-related overgrowth syndrome (Forde et al., 2021). PIK3CA and AKT inhibitors represent, therefore, potential targeted therapies for cortical malformations caused by somatic mutations in *PIK3CA* or *AKT3* genes.

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Anna Gerasimenko: Data curation, Methodology, Writing – original draft. **Sara Baldassari:** Writing – original draft, Supervision. **Stéphanie Baulac:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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