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Cutting Edge Approaches to Detecting Brain Mosaicism Associated with Common Focal Epilepsies: Implications for Diagnosis and Potential therapies

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Abstract

Introduction: Mosaic variants arising in brain tissue are increasingly being recognized as a hidden cause of focal epilepsy. This knowledge gain has been driven by new, highly sensitive genetic technologies and genome-wide analysis of brain tissue from surgical resection or autopsy in a small proportion of patients with focal epilepsy. Recently reported novel strategies to detect mosaic variants limited to brain have exploited trace brain DNA obtained from cerebrospinal fluid liquid biopsies or stereo-electroencephalography electrodes.

Areas covered: The authors review the data on these innovative approaches published in PubMed before June 12, 2021, discuss the challenges associated with their application, and describe how they are likely to improve detection of mosaic variants to provide new molecular diagnoses and therapeutic targets for focal epilepsy, with potential utility in other non-malignant neurological disorders.

Expert opinion: These cutting-edge approaches may reveal the hidden genetic aetiology of focal epilepsies and provide guidance for precision medicine. **(150/150 words)**

Key Words: brain lesion; CSF liquid biopsy; high-depth exome sequencing; focal epilepsy; stereo-electroencephalography; somatic mosaicism

Article highlights

- Mosaicism is increasingly being recognized as an important cause of focal epilepsy
- Current routes to detect mosaicism are via privileged access to brain tissue from surgical resection or autopsy
- Alternative approaches are needed to identify mosaic pathogenic variants in brain when tissue is not clinically accessible
- Cerebrospinal fluid or stereo-electroencephalography electrodes provide novel approaches to detect somatic mosaicism in brain
- These techniques may reveal the hidden genetic aetiology of the largest group of epilepsies, the focal epilepsies, with the hope of identifying new therapeutic targets

1. Genetics of focal epilepsies (FEs) and contribution of mosaic variants

Focal epilepsies (FEs) are characterised by recurrent unprovoked seizures arising from networks limited to one hemisphere [1] and account for 60% of all forms of epilepsy [2]. Despite the introduction of many new anti-seizure medications (ASMs) in the past two decades, one-third of patients continue to have uncontrolled seizures [3]. While ~20-40% of patients with FE have structural epileptogenic abnormalities on neuroimaging ('lesional' FEs), the remainder have no detectable abnormality ('non-lesional' FEs) [4, 5].

FEs were historically considered non-genetic disorders owing to the observation that FE often followed an 'acquired' brain injury, such as stroke, severe head trauma or brain infection. In reality, different lines of evidence, including epidemiological data, twin studies, clinical descriptions of familial FE syndromes, and molecular investigations, point to strong genetic contributions to FEs [6]. Furthermore, a growing body of work has implicated genetic factors in lesional FEs [2, 7], including those associated with different types of brain malformations [8-11]. Uncovering the molecular genetic causes of FEs opens the door to precision therapies, which hold the promise of transforming outcomes for people living with epilepsy [12].

Somatic mosaicism has long been recognized as a genetic mechanism in cancer, but it was generally regarded as a rarity of limited importance in non-malignant disorders [13]. More recently, the role of somatic mosaicism in a wide variety of disorders has been highlighted [14]. Indeed, based on early observations in epilepsy genetics, it has been posited that, in addition to obvious inherited epilepsies and those shown to be due to *de novo* mutagenesis in parental gametes, there may be a sizeable 'hidden genetics' component of the FEs due to somatic mutations giving rise to mosaic variants [8, 15]. As opposed to germline *de novo* mutations, where there is a mutation present in a parental gamete, a mosaic mutation occurs post-zygotically, may be confined to one or more tissues, such as the brain, and may be difficult or impossible to detect by sequence analysis of conventionally acquired DNA samples from venous blood [8]. The discovery of mosaic somatic variants restricted to brain tissue from brain malformations associated with FEs, including unilateral malformations such as hemimegalencephaly (HME) [16], Sturge-Weber syndrome [17, 18], and small focal cortical dysplasias (FCDs) [19-22] established the key role of this mechanism in the

FEs. Notably, within the brain lesion, the mosaic pathogenic variant may be present in only a fraction of the cells; yet, this is sufficient to disrupt neuronal development and lead to a brain malformation and FE.

Many of the initial reports of somatic variants were in genes belonging to the mechanistic (formally mammalian) target of rapamycin (mTOR) pathway, building on the initial discovery of germline mutations in these genes being associated with FCDs [16, 19-21, 23-26]. Subsequent larger cohort studies suggest that pathogenic variants in these genes account for ~20-30% of FCDs, HMEs and other lesions [22, 27, 28]. Elegant studies have revealed a mutation gradient in the resected tissue with higher mosaicism levels in the most epileptogenic region [29]. Furthermore, recent reports have identified a second pathogenic variant limited to the lesion in the second allele of the same gene or in another gene from the mTOR or a different molecular pathway [25, 28, 30, 31].

Interestingly, recent studies have implicated a novel pathway in the pathophysiology of FCDs. They detected post-zygotic variants in the glycosylation gene *SLC35A2* in resected brain tissue from a surprisingly large proportion of lesional (~4-16%) and non-lesional (~17-30%) FE cases, demonstrating that diverse pathways are involved [22, 27, 32, 33]. The extent of the contribution of somatic variation in these and other pathways to non-lesional FEs is a critical knowledge gap for which molecular data are still limited but are beginning to accrue [8, 11, 22, 27, 33]. The goal is to optimize methods to detect mosaic variants in known and novel genes to understand how one or more variants contribute independently or synergistically to causation or risk of FE.

The major limitation to reaching this goal is access to brain tissue which is only available from a small proportion of FE patients who are suitable for resective

epilepsy surgery [8, 11] or at post-mortem. This leaves unanswered the question of how mosaic variants in the brain contribute to the common forms of FE. Most patients with FE do not require epilepsy surgery as their seizures are readily controlled with ASMs. If their epilepsy is drug-resistant, they may not be a surgical candidate as seizure origin may not be localizable or seizures may be multifocal. Novel, minimally invasive approaches are required to detect mosaic variants in patients with FE using alternate sources of DNA, particularly for patients with no brain tissue available for analysis. In this review, we discuss two recent strategies (**Figure 1**) developed to facilitate the detection of brain-only mosaic variants: (i) analysis of cell-free DNA (cfDNA) in cerebrospinal fluid (CSF) liquid biopsies; and (ii) screening of trace DNA genome obtained and amplified from stereo-electroencephalography (SEEG) depth electrodes. Improved detection of mosaic variants has great potential to improve personalized precision medicine, not only by providing guidance for the use of currently available ASMs, but also molecular insights to identify new therapeutic targets [34].

2. Using cerebrospinal fluid (CSF) liquid biopsy to detect brain mosaicism

In FE patients with brain malformations, the genetic diagnostic yield is low when screening peripheral blood leukocytes, even when high depth sequencing is used, because most of the pathogenic mosaic variants are brain-only and thus cannot be detected in blood [10]. Brain-only mosaicism has also been suggested to contribute to non-lesional FEs [33]. In the absence of resected or autopsied brain specimens, one potential surrogate for brain tissue to provide a molecular diagnosis for FEs is via cell-free DNA (cfDNA) derived from CSF liquid biopsy.

DNA circulates in plasma and other bodily fluids in a cell-free state, considered to be the product of programmed cell death [35]. The size of cfDNA fragments is predominantly around 167 base pairs, equivalent to the length of DNA wrapped around each nucleosome [35]. Analysis of cfDNA obtained from plasma has been established in obstetric practice [36], for diagnosis, monitoring and precision therapy guidance in oncology [37], and as a biomarker for allograft rejection monitoring following organ transplantation [38]. As for central nervous system (CNS) tumors, somatic mutations have been identified in the plasma cfDNA of a subset of patients with primary CNS lymphoma or glioma [39]. Another promising source of cfDNA from CNS tumors is CSF and interrogation of CSF-derived cfDNA was first reported in 2015 [40, 41]. Patients with various CNS tumors were subsequently found to have significantly enriched tumor-derived cfDNA in CSF [42] in which somatic mutations were detected [40, 41, 43-47]. Studies comparing the diagnostic performance of CSF and plasma cfDNA demonstrate, not surprisingly, although cfDNA concentration is higher in plasma [40], cfDNA from CSF provides better representation of and detection sensitivity for the somatic alterations in brain tumors [39, 40, 48].

The first proof-of-principle study demonstrating that CSF liquid biopsy can be used for the molecular diagnosis of lesional FE was reported this year [49]. cfDNA was extracted from CSF obtained from 28 patients with FE and 28 controls without epilepsy. Droplet digital PCR (ddPCR) was used for absolute quantitation of cfDNA concentration. The median concentration of cfDNA was approximately 1.5ng/mL CSF in patients with FE and 0.18 ng/mL CSF in controls, which was sufficient for downstream analysis [49]. Although this difference in concentration was significant, this finding is based on a relatively small cohort and needs to be replicated.

The study then directly interrogated CSF cfDNA from three patients using ddPCR for molecular diagnosis. Patient 1 with bilateral posterior subcortical band heterotopia and Patient 2 with left inferior temporal gyrus focal cortical dysplasia IIb each had a known mosaic pathogenic variant (*LIS1* c.190A>T and *TSC1* c.1741_1742delTT, respectively) in resected brain tissue [10, 50]. These pathogenic variants were reliably detected in their CSF cfDNA at 9.4% and 7.8% variant allele frequency (VAF), in contrast to their VAF in tissue of 5.8% and 2.3%, respectively [49]. More significantly, the third patient with left mesial temporal ganglioglioma did not have a molecular diagnosis prior to CSF liquid biopsy. A *BRAF* c.1799T>A (p.Val600Glu) pathogenic variant was initially found in CSF cfDNA at 3.2% VAF and subsequently validated in brain tissue at 20.4% VAF [49]. Cell-of-origin analysis showed that brain-specific methylation markers were significantly enriched in CSF cfDNA, confirming that the cfDNA was mainly brain-derived [49].

Kim et al. [51] subsequently reported independent validation oSF liquid biopsy in 3 out of 12 patients with drug-resistant FE using a different protocol: unlike the first study where cfDNA was extracted from the whole CSF samples, in this study CSF samples were collected and divided into 1mL aliquots, and cfDNA was extracted from each aliquot. cfDNA concentration was measured at 0.38ng/μl (range 0.05-2.7ng/μl) using high-sensitivity double stranded DNA Qubit assays [51]. ddPCR was performed to target mosaic variants in each patient's CSF cfDNA aliquots. However, given the limited cfDNA obtained from the aliquots, pre-amplification was performed prior to standard ddPCR, which was also distinct from the first study where ddPCR was directly performed without pre-amplification. The authors reported genetic diagnosis in CSF cfDNA from 3 patients, including a patient with hemimegalencephaly (HME) and a somatic *PIK3CA* p.Glu545Lys variant, a patient

with a mild malformation of cortical development with oligodendroglial hyperplasia and a somatic *SLC35A2* p.Gln168* variant, and a patient with ganglioglioma and the recurrent somatic *BRAF* p.Val600Glu variant. In this study, false positive signal was detected in almost all variant ddPCR assays run on controls, a direct consequence of pre-amplification [51, 52]. Indeed, for the *BRAF* Val600Glu assay, the negative controls had significantly higher VAF (0.073%) than their true-positive patient (0.0014%) [51].

It is important to highlight other limitations. Firstly, in both studies [49, 51], targeted genetic testing was performed by ddPCR, which does require prior knowledge of the exact pathogenic variant. Secondly, almost all patient CSF samples were collected via dural puncture during neurosurgery which is not a feasible route of collection for patients who are not surgical candidates. Importantly, for Patient 1 in the first study [49], the CSF sample was collected via lumbar puncture with successful downstream detection of the pathogenic variant, demonstrating this minimally invasive route is feasible for patients with FE who are not surgical candidates.

Despite these limitations, both studies validated CSF cfDNA as a viable surrogate for brain tissue to detect brain mosaicism (**Figure 2**). A lumbar puncture can now be considered in a patient who is not a candidate for epilepsy surgery to investigate potential molecular genetic causes. There are highly recurrent mosaic brain mutations (e.g. *BRAF* Val600Glu) which account for a significant proportion of all pathogenic variants for certain aetiologies (e.g. FEs associated with ganglioglioma) [11, 16, 21, 26, 29, 53, 54]. Screening for many recurrent variants one by one using the standard single-plex ddPCR protocol is not practical considering the extremely limited amount of cfDNA obtained from CSF. However, in

studies of CNS tumors, multiplex ddPCR has been applied using several sets of primers and probes targeting different genomic regions to achieve simultaneous detection of multiple somatic variants [55, 56]. In light of these studies, multiplex ddPCR might also be established to test recurrent somatic mutations causing FE, in order to achieve rapid screening using limited cfDNA amounts, noting that at present recurrent somatic variants only account for a small number of lesional FEs. Next-generation sequencing, such as targeted gene panel and unbiased exome or genome sequencing, could also be applied to CSF samples from both lesional and non-lesional FEs to yield a molecular diagnosis. However, most brain somatic mutations causing FEs with brain malformations have very low VAF in brain tissue (as low as 1-2%) [11, 57-59] and may be present at even lower frequencies in the CSF. In the future, unbiased, high-depth screens with optimized low-input genomic capture will be required to establish broader utility of this minimally invasive diagnostic method.

3. Exploiting trace brain tissue from stereo-electroencephalography (SEEG) depth electrodes

Pioneered by Drs. Jean Talairach and Jean Bancaud in the 1950s and increasingly adopted worldwide in the last decade, SEEG relies on the stereotactic placement of multiple depth electrodes in the brain to record interictal and ictal EEG activity with the ultimate goal of identifying an epileptogenic focus amenable to epilepsy surgery [60]. For patients with drug-resistant FE undergoing SEEG investigations, the trace brain-derived DNA on the surface of SEEG depth electrodes provides another alternative source for molecular diagnosis. In late 2019, the first method of sampling lesional brain tissue from the cells adhered to the surface of

SEEG depth electrodes for downstream genetic analysis was reported by Montier et al [61].

The authors described a patient with drug-resistant FE and bilateral periventricular nodular heterotopia (PNH) who underwent a SEEG investigation targeting the PNH areas and both hippocampi [61]. Trace cellular material was collected from the SEEG electrodes and subjected to whole-genome amplification. Amplified DNA from the left and right PNH areas, and paired blood-derived genomic DNA, were then subjected to high-depth exome sequencing (mean depth: 155-fold) and standard clinical variant analysis, as outlined in **Figure 3**. Sanger sequencing on left and right PNH DNA samples was performed for variant validation.

Genetic analysis of the paired PNH- and blood-derived DNA samples failed to detect any variants in known familial or sporadic PNH genes, but did reveal a mosaic *MEN1* duplication (c.1546dupC) variant predicted to lead to frameshift (p.R615PfsX15) in 16.7% of sequenced reads in PNH DNA. The presence of this variant in PNH DNA, and its absence from blood, was confirmed independently by Sanger sequencing. This variant had been reported at high frequency as a germline heterozygous change in patients with multiple endocrine neoplasia type 1 (MEN1) syndrome with *in vitro* functional studies confirming it impairs nuclear localization [62]. Germline mutations in *MEN1* are well-known to cause MEN1 syndrome, and double-hit mutations (a constitutional germline mutation with a second somatic mutation) in *MEN1* can cause MEN1 syndrome related tumors such as ependymoma, lipoma and parathyroid tumor [63, 64]. However, this gene has not been related to PNH. It is possible that constitutional mutation or double-hit mutations in *MEN1* can cause MEN1 syndrome, while mosaicism may lead to other phenotypes (e.g. PNH) through

mechanisms like cellular interference between cell populations with and without mutations, already established for some X-linked disorders [65-67].

This study had a number of limitations. First, this was an 'N-of-1' study, and requires validation in additional cases. Second, there is no known association between the *MEN1* gene and neuronal migration defects or epilepsy in MEN1 syndrome, apart from the possibility of seizures arising due to endocrinological dysfunction [68]; nor has PNH been reported in patients with MEN1 syndrome. As mutations of *MEN1* are not a known cause of PNH or FE, at this stage it can only be considered a candidate gene. Third, whole-genome pre-amplification of DNA induces PCR artefacts that are detectable on sequencing, meaning stringent analysis and sensitive independent validation is required to distinguish true mosaic variants. Fourth, loss of heterozygosity of the *MEN1* gene, commonly detected in MEN1 syndrome tumor cells [68], could not be reliably assessed using the short-read exome data.

Despite these limitations, this proof-of-concept study demonstrated the utility of interrogating trace brain-derived DNA on the surface of SEEG electrodes as a novel, minimally invasive method for mosaic variant detection in FE. Future studies will likely overcome the limitations outlined above. Given the precedent set by this study and the increasing use of SEEG around the world, additional FE patients undergoing SEEG investigations will have their depth electrode tissue studied, enabling detection of somatic mosaic variants in known brain malformation and epilepsy genes. Furthermore, evidence for the extent of mosaicism can be obtained from patients undergoing clinical SEEG investigations targeting multiple, including distant, brain regions, confirming previous observations of a mosaic gradient in somatic pathogenic variants [29]. Rigorous, independent validation of low frequency mosaic

variants, using highly sensitive technologies like amplicon sequencing or droplet digital PCR at the single molecule level, is already possible, even via alternate tissue sources such as CSF as outlined earlier [49, 51].

4. Expert opinion

Epilepsy affects 3-5% of the population accounting for 0.5% of the global burden of disease [69]. Of the up to 70 million affected people worldwide, one-third have drug-resistant epilepsy, most commonly FE [3, 70]. Epilepsy is associated with increased risks of disability, morbidity and mortality as well as heightened costs to society [71]. Despite recent advances in diagnostic approaches, the underlying cause in most patients with FEs remains elusive [6].

Identification of mosaic pathogenic variants is critical to take clinical management into the exciting arena of precision medicine. It leads to diagnostic certainty, alleviating a patient's, or their family's, concern about why they have epilepsy. Secondly, it informs precise genetic counselling about recurrence risk, or lack thereof, in the case of a post-zygotic mutation. Thirdly, molecular diagnoses can guide selection of existing therapies, such as sodium channel blockers (i.e. carbamazepine) to treat *KCNQ2*-related early-onset epileptic encephalopathy, N-methyl-D-aspartate receptor antagonists (i.e. memantine) to treat *GRIN2A*-related early-onset epileptic encephalopathy, or potassium channel openers (i.e. quinidine) to treat *KCNT1*-related epilepsy of infancy with migrating focal seizures [34]. Determining a molecular diagnosis also provides opportunities for precise therapeutic trials of repurposed or novel drugs [34, 72-75], such as mTOR inhibitors (i.e. everolimus) [72], as well as for use of emerging therapies, such as fenfluramine and cannabidiol [73-75].

Testing patients with FEs without brain tissue available by assaying CSF liquid biopsies or trace SEEG depth electrode tissues for mosaic variants in brain provides a promising path to increase moelcular diagnostic rates. The discovery of new genes for non-lesional FEs will improve targeted and unbiased screening approaches for this large group of patients. Increasing recognition of the contribution of brain mosaicism to FEs will lead to development of the first clinical testing guidelines and recommendations for assessment of the significance of mosaic variants. New therapeutic targets will emerge as novel pathways are implicated, and existing FDA-approved drugs may be repurposed. Even more exciting is the promise of targeted gene therapies, yet the challenges of getting these therapies to a brain lesion at the right time during development to prevent FE cannot be underestimated.

Abbreviations

ASM, anti-seizure medication; cfDNA, cell-free DNA; CNS: central nervous system; CSF, cerebrospinal fluid; ddPCR, droplet digital PCR; FCD, focal cortical dysplasia; FE, focal epilepsy; HME, hemimegalencephaly; MEN1, multiple endocrine neoplasia type 1; MTOR pathway: mechanistic target of rapamycin pathway; PNH, periventricular nodular heterotopia; SEEG, stereo-electroencephalography; VAF: variant allele frequency

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Declaration of interests

IE Scheffer has served on scientific advisory boards for UCB, Eisai, GlaxoSmithKline, BioMarin, Nutricia, Rogcon, Chiesi, Encoded Therapeutics, Knopp Biosciences and Xenon Pharmaceuticals; has received speaker honoraria from GlaxoSmithKline, UCB, BioMarin, Biocodex, Chiesi, Liva Nova and Eisai; has received funding for travel from UCB, Biocodex, GlaxoSmithKline, Biomarin and Eisai; has served as an investigator for Zogenix, Zynerba, Ultragenyx, GW Pharma, UCB, Eisai, Xenon Pharmaceuticals, Anavex Life Sciences, Ovid Therapeutics, Epigenyx, Encoded Therapeutics and Marinus; and has consulted for Zynerba Pharmaceuticals, Atheneum Partners, Ovid Therapeutics, Care Beyond Diagnosis, Epilepsy Consortium and UCB. She may accrue future revenue on pending patent WO61/010176 (filed: 2008): Therapeutic Compound; has a patent for SCN1A testing held by Bionomics Inc and licensed to various diagnostic companies; has a patent molecular diagnostic/theranostic target for benign familial infantile epilepsy (BFIE) [PRRT2] 2011904493 & 2012900190 and PCT/AU2012/001321 (TECH ID:2012-009). PP has received speaker honoraria or consultancy fees to his institution from Chiesi, Eisai, LivaNova, Novartis, Sun Pharma, Supernus, and UCB Pharma, outside the submitted work. He is an Associate Editor for *Epilepsia Open*. The authors have no other relevant affiliations or financial involvement with any organization or entity

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Figure Legends

Figure 1. Innovative Strategies for Detection of Somatic Mosaic Variants in Brain

Potential strategies include genetic analysis of cell-free DNA (cfDNA) in cerebrospinal fluid (CSF) or genomic DNA amplified from trace stereo-EEG (SEEG) electrode tissue.

Adapted from "Brain" and "Lumbar puncture", by BioRender.com (2021). Retrieved from https://biorender.com/icon/human-anatomy/head-and-neuroanatomy/brain-lateral-damaged/ and https://biorender.com/icon/human-anatomy/skeletal-system/lumbar-puncture/

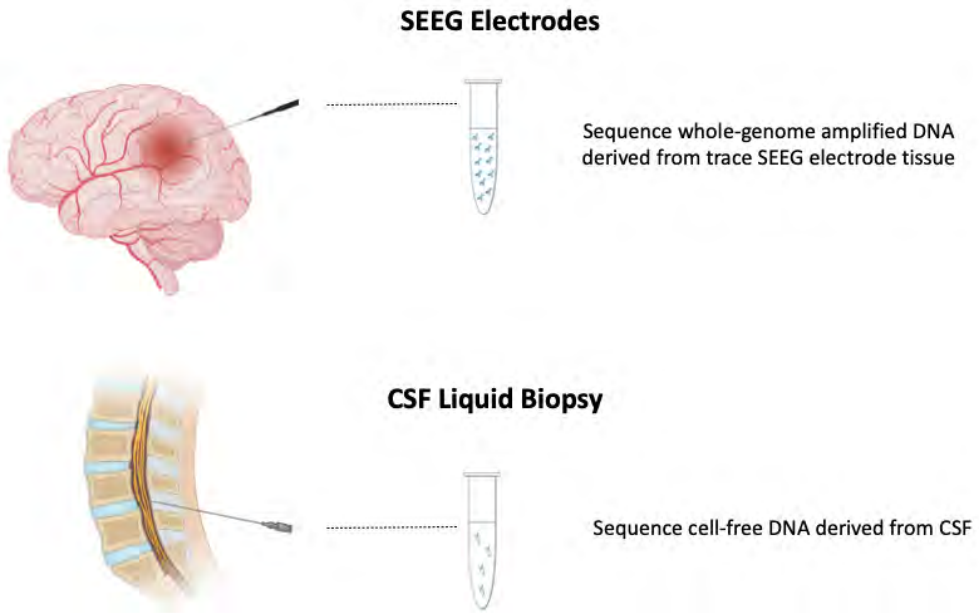

Figure 2. Potential Strategies for Cerebrospinal Fluid (CSF) Liquid Biopsy to Detect Brain Mosaicism

ddPCR has utility for targeted screening of recurrent somatic variants while unbiased, high depth massively parallel sequencing can be used for genome-wide interrogation.

ddPCR: droplet digital PCR**; WES:** whole exome sequencing**; WGS:** whole genome sequencing

Adapted from "Droplet digital PCR system" and "Sequencer (Illumina HiSeq)", by BioRender.com (2021). Retrieved from https://biorender.com/icon/lab-and-

objects/machinery-and-tech/sequencer-illumina-hiseq/ and https://biorender.com/icon/lab-and-objects/machinery-and-tech/droplet-digital-pcr-system/

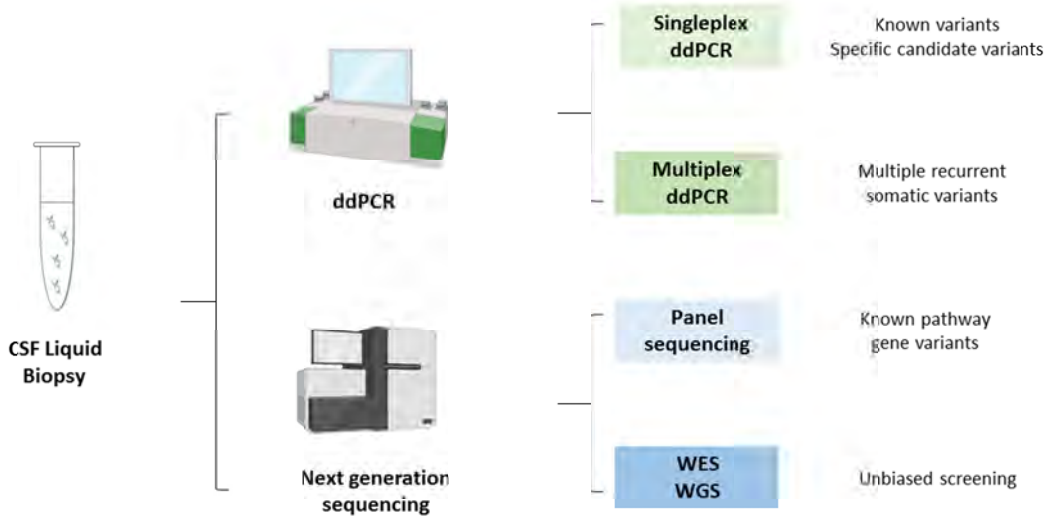

Figure 3. Protocol for Detection of Brain Somatic Mosaicism using Trace DNA from Stereo-EEG (SEEG) Electrodes

Trace DNA obtained from SEEG electrodes is whole-genome amplified, followed by deep exome sequencing. Bioinformatic analysis reveals multiple somatic candidate variants.

VAF: variant allele frequency; **VUS**: variant of unknown significance

Adapted from "Sequencer (Illumina HiSeq)", by BioRender.com (2021). Retrieved from https://biorender.com/icon/lab-and-objects/machinery-and-tech/sequencer-illumina-hiseq/