


# BRAIN COMMUNICATIONS

## Cerebrospinal fluid liquid biopsy for detecting somatic mosaicism in brain

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Brain somatic mutations are an increasingly recognized cause of epilepsy, brain malformations and autism spectrum disorders and may be a hidden cause of other neurodevelopmental and neurodegenerative disorders. At present, brain mosaicism can be detected only in the rare situations of autopsy or brain biopsy. Liquid biopsy using cell-free DNA derived from cerebrospinal fluid has detected somatic mutations in malignant brain tumours. Here, we asked if cerebrospinal fluid liquid biopsy can be used to detect somatic mosaicism in non-malignant brain diseases. First, we reliably quantified cerebrospinal fluid cell-free DNA in 28 patients with focal epilepsy and 28 controls using droplet digital PCR. Then, in three patients we identified somatic mutations in cerebrospinal fluid: in one patient with subcortical band heterotopia the *LIS1* p. Lys64\* variant at 9.4% frequency; in a second patient with focal cortical dysplasia the *TSC1* p. Phe581His\*6 variant at 7.8% frequency; and in a third patient with ganglioglioma the *BRAF* p. Val600Glu variant at 3.2% frequency. To determine if cerebrospinal fluid cell-free DNA was brain-derived, whole-genome bisulphite sequencing was performed and brain-specific DNA methylation patterns were found to be significantly enriched ( $P=0.03$ ). Our proof of principle study shows that cerebrospinal fluid liquid biopsy is valuable in investigating mosaic neurological disorders where brain tissue is unavailable.

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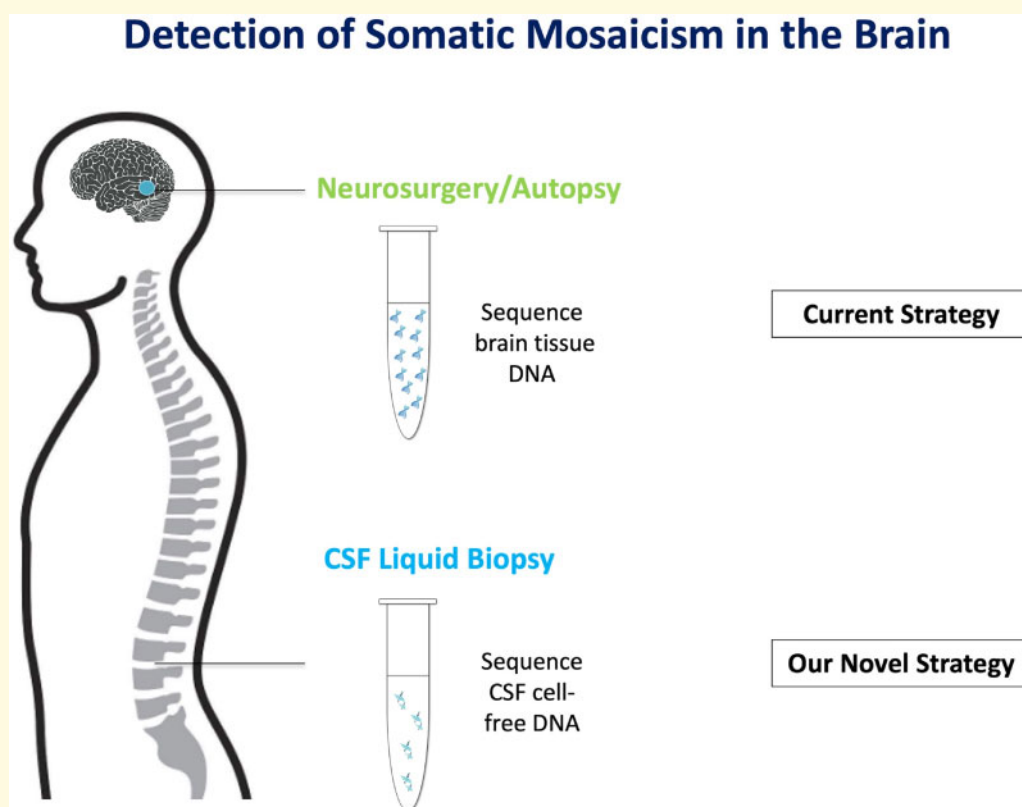
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**Abbreviations:** CNS = central nervous system; CSF = cerebrospinal fluid; ddPCR = droplet digital PCR; VAF = variant allele frequency; WGBS = whole-genome bisulphite sequencing

### Graphical Abstract



## Introduction

Cell-free DNA is externalized, fragmented DNA found in bodily fluids and is the product of programmed cell death. Fragments are predominantly short with a predominant peak at ~167 base pairs (bp) (Chandrananda *et al.*, 2015). Analysis of cell-free DNA, typically from peripheral blood, is now clinically used as a liquid biopsy for diagnosis and monitoring of cancer (Crowley *et al.*, 2013), obstetric

practice (Bianchi and Chiu, 2018) and as a biomarker for allograft rejection after organ transplantation (Goh *et al.*, 2017). Interrogation of CSF-derived cell-free DNA allows detection of mosaic somatic mutations in malignant brain tumours (Pan *et al.*, 2015; Wang *et al.*, 2015; Garcia-Romero *et al.*, 2019), but its application to diagnosis of non-malignant brain diseases has not been explored.

While a significant proportion of patients with neurological and neurodevelopmental disorders have disease-

causing germline mutations (Allen *et al.*, 2013; Iossifov *et al.*, 2014), many remain unsolved after routine genetic testing. Recent studies indicate that some of these unsolved cases may be explained by somatic mutation (Thomas and Berkovic, 2014). Mosaic somatic mutations have been identified in patients with neurological disorders including brain malformations and epilepsy (Poduri *et al.*, 2012; D’Gama *et al.*, 2017; Winawer *et al.*, 2018; Sim *et al.*, 2019; Ye *et al.*, 2019) and autism spectrum disorder (D’Gama *et al.*, 2015). Somatic mutations occur post-zygotically and, if confined to brain, are difficult or impossible to detect by conventional sequence analysis of lymphocyte-derived DNA (Poduri *et al.*, 2013). At present, the only established route to discovery of somatic mutations in brain is via the privileged situation of having brain tissue from surgical or autopsy specimens; this is not generally applicable to common epilepsies and other neurological and neurodevelopmental disorders. An alternative route is needed to detect the brain-only somatic mutations when brain tissue is not available. Here, we provide proof of principle that CSF liquid biopsies can be used as a surrogate to detect somatic mosaicism, without the need for brain tissue.

## Materials and methods

### Study approval

The Human Research Ethics Committees of The Royal Children’s Hospital (Project No. 29077F) and Austin Health, Melbourne, Australia (Project No. H2007/02961) approved this study. Written informed consent was obtained from all participants or their parents or legal guardians according to the Declaration of Helsinki.

### Clinical cohorts

For initial CSF cell-free DNA quantitation, we studied 28 patients with epilepsy (mean age,  $7.8 \pm 5$  years old) and 28 controls without epilepsy (mean age,  $46.9 \pm 15.6$  years old). They were recruited through Austin Health, Royal Children’s Hospital, and Queensland Children’s Hospital, Australia. All patients had drug-resistant, lesional, focal epilepsy requiring surgical treatment (Supplementary Table 1). Controls had been referred for non-epilepsy presentations (e.g. persistent headache). CSF was collected in Cell-Free DNA BCT<sup>®</sup> (Strek, La Vista, NE) tubes via dural puncture immediately after craniotomy (epilepsy patients) or lumbar puncture (controls). Mean CSF volume collected was 2 ml (range, 0.25–10.5 ml).

For molecular diagnosis, CSF samples were obtained by lumbar puncture (patient 1, separate from the 28 patients used for the quantitation) and peri-operative dural puncture (patients 2 and 3, from the cohort of 28 patients used for quantitation) in patients with known or suspected brain mosaic mutations.

## DNA extraction

Cell-free DNA was extracted from CSF using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany): CSF was centrifuged at  $1000 \times g$  at  $4^{\circ}\text{C}$  for 10 min then extracted according to the manufacturer’s protocol. For blood and brain, genomic DNA was extracted using standard methods (Qiagen QIAamp DNA Maxi Kit or AllPrep DNA/RNA Kit, Hilden, Germany).

## Droplet digital PCR

For quantitation of total cell-free DNA in CSF, we used a PrimerPCR<sup>™</sup> Mutation Assay (Catalog: 10049047; Bio-Rad, Hercules, CA) that we previously optimized in-house (Hildebrand *et al.*, 2018) to detect the GNAQ c.548G wild-type allele (NM\_002072.5:c.548G) as a reference for cell-free DNA copies (Supplementary Table 2). Total cell-free DNA copies and cell-free DNA concentration (copies/ml CSF and ng/ml CSF) were calculated using reported formulas (Supplementary Methods).

For molecular diagnosis, we used the in-house LIS1 NM\_000430.4:c.190A>T (p.Lys64\*) ddPCR assay (Damiano *et al.*, 2017), a customized TSC1 NM\_000368.5:c.1741\_1742delTT (p.Phe581His\*6) ddPCR assay (Thermo Fisher Scientific, Waltham, MA), or a PrimerPCR<sup>™</sup> BRAF NM\_004333.6:c.1799T>A (p.Val600Glu) assay (ID: dHsaCP2000027 and dHsaCP2000028; Bio-Rad, Hercules, CA) (Supplementary Table 2). Droplet generation, PCR cycling and droplet reading were performed on the QX200 ddPCR system according to the manufacturer’s recommendations (Bio-Rad). Limit of detection tests for each assay were conducted using Gblock synthetic DNA fragments (Supplementary Methods). The limit of detection for all three assays was 0.25% variant allele frequency (VAF) (Supplementary Figs 1–3).

## Whole genome bisulphite sequencing and cell-of-origin analysis of cell-free DNA

To determine origin of CSF cell-free DNA, we examined cell-free DNA for brain-specific DNA methylation patterns. We analysed four control CSF samples and patient 1’s CSF sample by whole-genome bisulphite sequencing (WGBS) followed by methylK analysis (Supplementary Methods). Sequencing libraries were prepared using Accel-NGS Methyl-Seq DNA Library Kit (Swift Biosciences, USA) according to the manufacturer’s instructions for low-input methylomes. Pronex beads were used to deplete adapter dimers and enrich library fragments prior to sequencing. We sequenced 2–5 million paired-end reads for each CSF sample. All samples had 0.024–0.051x coverage for cytosines in the CG dinucleotide sequence context and 0.025–0.055x coverage for cytosines in the CH dinucleotide sequence context. In addition, we acquired five WGBS data sets from Swift

Biosciences that were produced from peripheral blood plasma cell-free DNA in which the libraries were made with the same library preparation kit and sequenced to a comparable depth to CSF samples.

To identify the cell-of-origin of CSF cell-free DNA, we established an analytical method for assigning single WGBS reads. Briefly, publicly available WGBS from 11 cell types-of-interest [e.g. blood Cells (ENCODE) and central nervous system (CNS) cell types (Lister *et al.*, 2013)] provided reference cell-type data sets from which DNA methylation fractional measurements were converted into cell-type specific reference sequences (FASTA). Sub-sequences (k-mers) were indexed from all cell-type references ( $n=11$ ) using Kallisto software (Bray *et al.*, 2016). Trimmed and truncated WGBS reads from CSF ( $n=5$ ) and plasma ( $n=5$ ) cell-free DNA were assigned (pseudo-aligned) to their cell-of-origin by hash table lookup using Kallisto software (Bray *et al.*, 2016) and only high-quality reads assigned to DNA methylation reference sequences of a unique cell-type were counted and used for statistical analysis (Supplementary Methods, Supplementary Fig. 4).

## Statistical analysis

Student's *t*-tests were used to compare counts of WGBS reads assigned to CNS cells and blood cells between CSF cell-free DNA and plasma cell-free DNA. All statistical tests were performed using SPSS Statistics (SPSS 25, SPSS Inc., Chicago, IL, USA). A *P*-value of  $<0.05$  was considered statistically significant.

## Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary Material. Raw data of this study are available from the corresponding authors, upon reasonable request.

## Results

First, to establish that CSF cell-free DNA can be reliably quantified, we studied 28 patients with focal epilepsy and

28 controls. We quantified cell-free DNA in CSF samples by interrogating the GNAQ c.548G wild-type allele as a reference for cell-free DNA copies using ddPCR. Cell-free DNA copies were successfully detected and reliably quantified in all patient CSF samples (examples of ddPCR output are shown in Supplementary Fig. 5). The median value of cell-free DNA concentration was 502 copies/ml CSF ( $\sim 1.5$  ng/ml CSF) in paediatric patients with epilepsy and 61 copies/ml CSF ( $\sim 0.18$  ng/ml CSF) in adult controls, respectively (Supplementary Table 3 and Fig. 6). This demonstrated that cell-free DNA could be reliably detected in CSF from subjects with epilepsy and controls.

Next, to establish molecular diagnosis, we investigated CSF cell-free DNA from three patients. Two patients had a known mosaic pathogenic variant in brain tissue which was also found in CSF cell-free DNA. In the third patient, the mutation was first identified by CSF cell-free DNA analysis and later confirmed in brain tissue.

Patient 1 was a 41-year-old woman with drug-resistant focal epilepsy, mild intellectual disability and bilateral posterior subcortical band heterotopia. She had the somatic mosaic *LIS1* mutation c.190A>T (p.Lys64\*) in blood (13% VAF) (Jamar *et al.*, 2014), saliva ( $\sim 13\%$  VAF) and a 25-year-old formalin-fixed paraffin-embedded brain tissue ( $\sim 5\%$  VAF) (Damiano *et al.*, 2017). We obtained 6 ml of CSF via lumbar puncture and extracted cell-free DNA (444 copies/ml,  $\sim 1.3$  ng/ml). The *LIS1* p. Lys64\* mutation was detected in CSF cell-free DNA on two independent tests with a mean VAF of 9.4% (Table 1, Fig. 1A, Supplementary Table 4 and Fig. 7A).

Patient 2 was a 3-year-old girl with a solitary, left inferior temporal gyrus focal cortical dysplasia type IIB, but no other clinical or imaging features of tuberous sclerosis. She had the brain-only somatic *TSC1* mutation c.1741\_1742delTT (p.Phe581His\*6) detected at 2.8% VAF by deep sequencing in the resected lesion. We obtained 1.5 ml of CSF via dural puncture prior to resective surgery and isolated cell-free DNA (789 copies/ml). The *TSC1* p. Phe581His\*6 mutation was detected at 7.8% VAF in CSF; using the same ddPCR assay, we re-confirmed this mutation in brain tissue (2.3% VAF), and it was undetectable in blood (Table 1, Fig. 1B and Supplementary Table 4 and Fig. 7B).

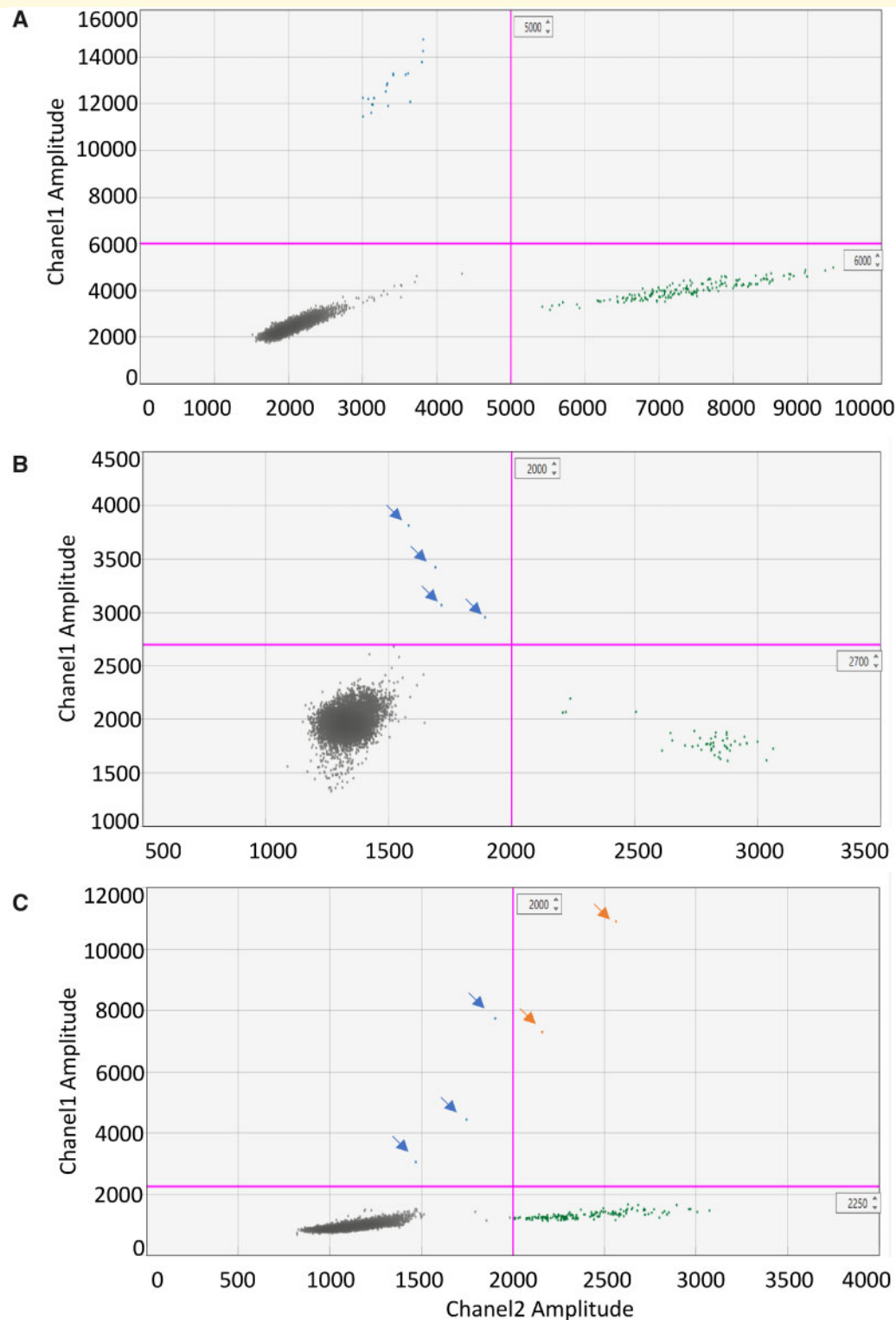
**Table 1** Genetic diagnosis of patients with epilepsy and brain malformations

	Clinical diagnosis	CSF volume (ml)	cfDNA concentration (copies/ml CSF)	Gene mutation	Brain VAF (%) <sup>a</sup>	CSF VAF (%) <sup>a</sup>	Blood VAF <sup>a</sup>
<b>Patient 1</b>	Bilateral posterior subcortical band heterotopia	6	444	<i>LIS1</i> c.190A>T (p.Lys64*)	5.8 <sup>b</sup>	9.4	14.5%
<b>Patient 2</b>	Left inferior temporal gyrus focal cortical dysplasia IIb	1.5	789	<i>TSC1</i> c.1741_1742delTT (p.Phe581His*6)	2.3	7.8	Undetectable
<b>Patient 3</b>	Left medial temporal ganglioglioma	3	1058	<i>BRAF</i> c.1799T>A (p.Val600Glu)	20.4	3.2	Undetectable

Abbreviation: VAF, variant allele frequency.

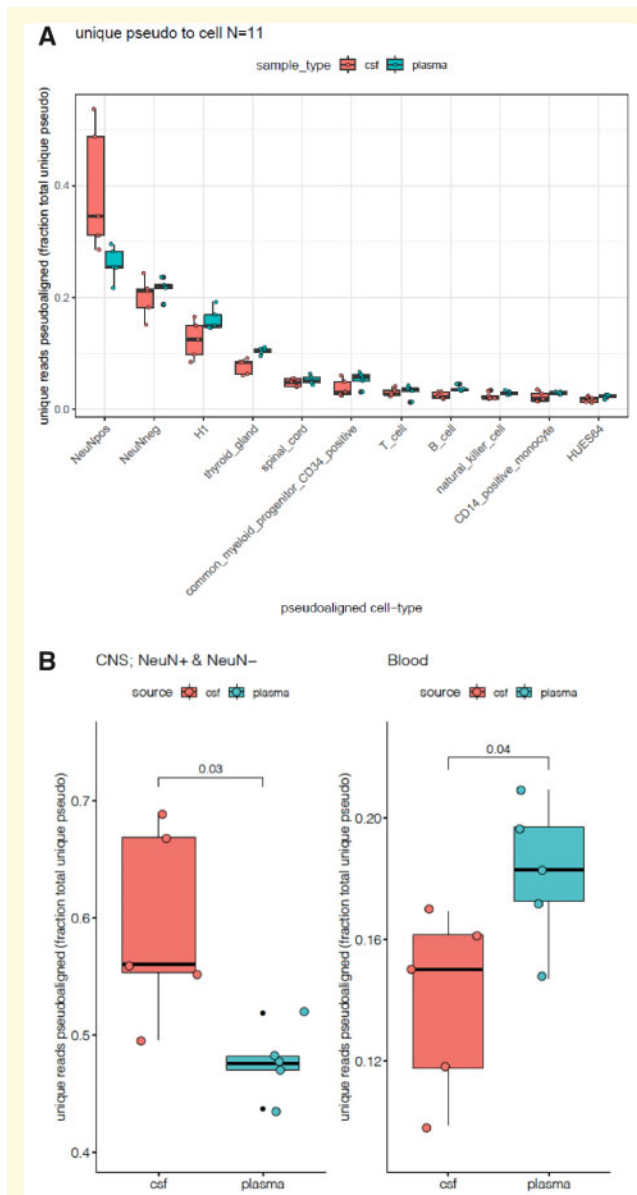
<sup>a</sup>Measured by ddPCR.

<sup>b</sup>Damiano *et al.* (2017).



**Figure 1** ddPCR 2D-plot for molecular diagnosis in CSF from three patients with lesional focal epilepsy. **(A)**. 2D-Plot showing droplets positive (blue, upper left quadrant) for *LIS1* Lys64\* in patient 1. CSF cell-free DNA was tested twice with similar VAF, then data were combined. Mutant concentration 3.61 copies/ $\mu$ l; wild-type concentration 35 copies/ $\mu$ l; VAF: 9.4%. **(B)** 2D-plot showing droplets positive for *TSC1* p. Phe581His\*6 in patient 2. CSF cell-free DNA: mutant concentration 0.47 copies/ $\mu$ l; wild-type concentration 5.58 copies/ $\mu$ l; VAF: 7.8%. **(C)** 2D-plot showing droplets positive for *BRAF* Val600Glu in patient 3. CSF cell-free DNA: mutant concentration 0.42 copies/ $\mu$ l; wild-type concentration 12.7 copies/ $\mu$ l; VAF: 3.2%. Green droplets are wild-type copies, orange droplets are double-positive copies and grey droplets are empty. VAF: variant allele frequency.





**Figure 2 Enrichment of CNS-derived cell-free DNA in CSF samples. (A)** Unique pseudo-alignment of cell-free DNA from CSF and plasma to reference sequences/k-mers from 11 tissues. **(B)** Unique sequence pseudo-alignments of cell-free DNA from CSF and plasma to brain and blood cells. CSF-derived cell-free DNA has significantly higher number of unique reads pseudo-aligned to brain compared to plasma-derived cell-free DNA ( $P=0.03$ ), whereas plasma-derived cell-free DNA was significantly enriched for unique reads pseudo-aligned to blood cells ( $P=0.04$ ).

Patient 3 was a 2-year-old boy with a left medial temporal ganglioglioma who had received no molecular testing. We obtained 3 ml of CSF via dural puncture prior to resective surgery and isolated cell-free DNA (1058 copies/ml). Since 50–60% of patients with ganglioglioma have a brain-only recurrent somatic *BRAF* mutation c.1799T>A (p.Val600Glu) (Koelsche et al., 2013; Berghoff and Preusser, 2014), we screened for this mutation using a

targeted ddPCR assay. We detected this mutation at 3.2% VAF in CSF cell-free DNA; subsequent testing confirmed that the mutation was present at 20.4% VAF in his ganglioglioma, and undetectable in his blood (Table 1, Fig. 1C and Supplementary Table 4 and Fig. 7C).

Finally, to confirm that cell-free DNA in CSF is brain-derived, we examined cell-free DNA for brain-specific DNA methylation patterns. We showed for CSF cell-free DNA that a greater proportion of WGBS reads pseudo-aligned to brain cells than blood cells (Fig. 2A and Supplementary Fig. 4). We assessed brain cell-derived (NeuN+ neurons, NeuN-glia) and blood cell-derived (CD34+ myeloid progenitors, T-cells, B-cells, Natural Killer cells and CD14+ monocytes) contributions to cell-free DNA within CSF and plasma. We observed that CSF-derived cell-free DNA was significantly ( $P=0.03$ ) enriched for unique reads pseudo-aligned to brain cells, whereas plasma-derived cell-free DNA had a significantly ( $P=0.04$ ) higher number of unique reads pseudo-aligned to blood cells (Fig. 2B). The small fraction of NeuN k-mer within plasma likely reflects a lack of specificity in established methods for deconvolution of CpH methylation in the pseudo-aligned reads from blood genomes. Our findings indicate that the cell-free DNA in CSF is largely brain derived.

## Discussion

Somatic mutations have been well studied in malignant tumours, but their role in neurological and neurodevelopmental disorders remains poorly understood. With the improving throughput and depth of genetic screening technologies, the contribution of somatic mutations is increasingly being recognized.

There are many unsolved brain diseases for which a genetic aetiology has been invoked. In epilepsy, however, only a minority of patients have a known molecular cause. By study of privileged brain tissue samples, usually from epilepsy surgery, some cases, including but not limited to brain malformations, are now known to be due to somatic mutation in the brain (Poduri et al., 2012; D’Gama et al., 2015, 2017; D’Gama and Walsh, 2018; Winawer et al., 2018). The discovery of these mutations in post-mortem tissue in individuals with autism spectrum disorder (D’Gama et al., 2015) and Alzheimer’s disease (Park et al., 2019) implicates this mechanism more broadly in neurodevelopmental (D’Gama and Walsh, 2018) and neurodegenerative disorders (Lodato and Walsh, 2019). It has even been repeatedly hypothesized that somatic mutations contribute to the pathogenesis of Parkinson disease (Engholm and Gasser, 2013; Proukakis et al., 2013). Analysing brain tissue in living patients with autism spectrum disorder, Alzheimer’s disease or Parkinson disease is not feasible as these patients do not have surgery (except deep-brain stimulation in

rare cases where tissue is not resected) as part of their clinical treatment.

Our results show that there is enough cell-free DNA in CSF to make a targeted liquid biopsy possible. We found cell-free DNA concentration is higher in the patients with epilepsy compared to the controls. This might be because seizures can trigger an increase in apoptosis of brain cells, leading to shedding of more cell-free DNA into CSF. But there are multiple confounding factors that may contribute to the difference: (i) age: the patients were children while the controls were adults and apoptosis is elevated in the brain during development; (ii) CSF collection: patient CSF was collected via dural puncture whereas control CSF was collected via lumbar puncture. It would have been ideal to obtain CSF via dural puncture from some controls, but we did not have institutional ethics approval for this.

Our findings suggest that CSF liquid biopsy is a viable surrogate for brain tissue to establish molecular diagnosis of brain somatic mosaicism, with implications for clinical care. These findings may provide new insights into the molecular genetic factors behind epilepsy and brain malformations, as well as other neurodevelopmental and neurodegenerative diseases potentially linked to brain somatic mutations where neurosurgery is not standard of care.

There are three main challenges to clinical implementation of CSF liquid biopsy for diagnostic purposes. First, the technical issue of obtaining high-quality DNA from CSF. We have shown that the level of CNS-derived cell-free DNA in the CSF of epilepsy patients is sufficient to enable targeted molecular interrogation of somatic mutations. Second, the biological issue of detection of low concentrations of mutant DNA in CSF, especially if the mutation is presumed localized to a small lesion; detection of mutations in patients 2 and 3 shows that this challenge may be overcome. Third, we performed only targeted mutational analysis in three patients to establish the proof of principle of this approach. The remaining patients were not tested due to lack of a candidate variant from tissue analysis for targeted assays. In the future, unbiased, high depth screening, with optimized low-input genomic capture for CSF cfDNA, will be required to identify a novel somatic mutation genome-wide in establishing broader clinical utility.

## Conclusion

As precision treatments for neurogenetic disorders are being investigated, such as mTOR inhibitors for cortical dysplasia, implementing CSF liquid biopsy for aetiological diagnosis will become increasingly important as a surrogate for brain-derived DNA to diagnose brain-specific somatic disorders.

## Supplementary material

Supplementary material is available at *Brain Communications* online.

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## Competing interests

I.E.S. has served on scientific advisory boards for UCB, Eisai, GlaxoSmithKline, BioMarin, Nutricia and Xenon Pharmaceuticals; editorial boards of the *Annals of Neurology*, *Neurology* and *Epileptic Disorders* may accrue future revenue on pending patent WO61/010176 (filed: 2008): Therapeutic Compound; a patent for *SCN1A* testing held by Bionomics Inc., and licensed to various diagnostic companies, has received speaker honoraria from GlaxoSmithKline, Athena Diagnostics, UCB, BioMarin, Biocodex, Eisai and Transgenomics; has received funding for travel from Athena Diagnostics, UCB, Biocodex, GlaxoSmithKline, Biomarin and Eisai. S.F.B. discloses payments from UCB Pharma, Novartis Pharmaceuticals, Sanofi-Aventis and Jansen Cilag for lectures and educational presentations, and a patent for *SCN1A* testing held by

Bionomics Inc., and licensed to various diagnostic companies. The remaining authors declare no competing interests.

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