



Identification of clinically relevant biomarkers of epileptogenesis — a strategic roadmap

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Abstract | Onset of many forms of epilepsy occurs after an initial epileptogenic insult or as a result of an identified genetic defect. Given that the precipitating insult is known, these epilepsies are, in principle, amenable to secondary prevention. However, development of preventive treatments is difficult because only a subset of individuals will develop epilepsy and we cannot currently predict which individuals are at the highest risk. Biomarkers that enable identification of these individuals would facilitate clinical trials of potential anti-epileptogenic treatments, but no such prognostic biomarkers currently exist. Several putative molecular, imaging, electroencephalographic and behavioural biomarkers of epileptogenesis have been identified, but clinical translation has been hampered by fragmented and poorly coordinated efforts, issues with inter-model reproducibility, study design and statistical approaches, and difficulties with validation in patients. These challenges demand a strategic roadmap to facilitate the identification, characterization and clinical validation of biomarkers for epileptogenesis. In this Review, we summarize the state of the art with respect to biomarker research in epileptogenesis and propose a five-phase roadmap, adapted from those developed for cancer and Alzheimer disease, that provides a conceptual structure for biomarker research.

Approximately 2.4 million new cases of epilepsy are diagnosed annually, and more than an estimated 50 million people have epilepsy worldwide¹. Some forms of epilepsy manifest months or even years after an initial epileptogenic insult (for example, a stroke, trauma or status epilepticus) or identification of a genetic defect (for example, tuberous sclerosis complex (TSC)) (TABLE 1). During this latency period, an epileptogenic process that involves (at least for epilepsies caused by epileptogenic insults) neurodegeneration, neurogenesis, gliosis, blood–brain barrier (BBB) damage and neuroinflammation is in motion and leads to circuitry reorganization and/or abnormal excitability².

In principle, these epilepsies could be prevented if effective treatments were administered during the pre-symptomatic latency phase. Almost 27 million traumatic brain injuries (TBIs) occur each year worldwide³ — if ~15% of these injuries lead to epilepsy (TABLE 1), over 4 million cases of epilepsy could be prevented each year. However, such treatments are not yet available⁴; existing anti-epileptic drugs are symptomatic agents

that can prevent seizures but not the development of epilepsy⁵.

One difficulty with the development of anti-epileptogenic therapies is that running clinical trials of such treatments is challenging. Only a subset of individuals who experience a potentially epileptogenic brain insult actually develop clinical epilepsy, so clinical trials of anti-epileptogenic therapies can become extremely costly and unethical because of the need to recruit large numbers of individuals, many of whom will never develop epilepsy and are therefore unnecessarily exposed to therapy-related safety risks. In addition, resolving a treatment effect would be difficult owing to patient variability, and the follow-up period needed would be unreasonably long for an inhomogeneous study population⁶. For example, one estimate suggests that ~750 patients with a head injury would need to be randomly assigned to receive treatment or placebo for an 80% chance of detecting a 50% reduction in the development of epilepsy (defined as two unprovoked seizures) in a follow-up period of 3 years, and that such a trial would

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Key points

- Many forms of epilepsy manifest and/or are diagnosed months or even years after an epileptogenic insult or identification of a genetic defect.
- Epilepsies that result from an insult or genetic defect could be amenable to secondary prevention but preventive treatments are not available.
- Development of preventive therapies is complicated by the fact that only a subset of at-risk individuals develop clinical epilepsy.
- Biomarkers are needed to clearly identify individuals who have the highest risk of developing epilepsy after an epileptogenic insult.
- We propose a strategic roadmap designed to facilitate the identification, characterization and clinical validation of biomarkers for epileptogenesis.

cost up to USD \$40 million⁶. These difficulties with trials of anti-epileptogenic treatments could be addressed if we had biomarkers that could clearly identify individuals who are at the highest risk of developing epilepsy after a brain insult. Unfortunately, no such biomarker is currently available^{7,8}.

The development of biomarkers of epileptogenesis is addressed in the goals of the US National Institute of Neurological Disease and Stroke (NINDS) working group to accelerate therapies for anti-epileptogenesis and disease modification. The overall goals of this working group are: to identify populations of individuals with epilepsy or at risk of developing epilepsy that are optimal for the investigation of new anti-epileptogenesis and disease-modification therapies; to align relevant animal models to these populations and identify common molecular and cellular pathways linked to epileptogenesis; to recommend the steps needed to develop and validate translational biomarkers; and to develop strategies to address barriers and challenges and thereby accelerate development of new therapies. An additional goal is to inform the NINDS Epilepsy Therapy Screening Program as it develops new preclinical

workflows to identify potential anti-epileptogenic and disease-modifying therapies. The work is organized into subgroups: Preclinical Science; Regulatory and Industry; Clinical; and Biomarkers and Translational Science. Each subgroup works to identify gaps and determine the goals, strategies, outcomes and next steps to advance development of therapies.

In this Review, we summarize the current state-of-the-art for the main biomarkers under investigation, assess the quality of preclinical and clinical evidence, and present the recommendations of the Biomarkers and Translational Science subgroup. We also propose a roadmap — based on existing structured roadmaps in the fields of cancer and Alzheimer disease (AD) — to focus research on the most important knowledge gaps and facilitate the development of biomarkers that predict with high sensitivity and specificity whether an individual will develop epilepsy.

Terminology

For the purposes of this review, we use the FDA–NIH definition and classification of biomarkers, the BEST (Biomarkers, Endpoints, and other Tools) recommendations⁹. Accordingly, a biomarker is “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions” and can be classified according to type (for example, molecular, histological, radiographic or physiological) and category (BOX 1). Three categories of biomarkers refer to the disease (susceptibility or risk, diagnostic and prognostic), three to the therapeutic intervention (predictive, pharmacodynamic or response, and safety), and one (monitoring) can refer to the disease or to the treatment.

Given these definitions and classifications, and given that the aim is to identify biomarkers that predict who will develop epilepsy among individuals who have experienced an epileptogenic insult or who carry an epileptogenic gene variant, we focus on two specific types of biomarker: biomarkers that are prognostic of epilepsy (or, in other words, diagnostic of an ongoing epileptogenic process), and monitoring biomarkers that enable, through serial analyses, identification of when an epileptogenic process is set in motion.

State of the art

We first explored the available evidence for biomarkers of epilepsy at large and epileptogenesis in particular. We defined a search strategy to interrogate the literature (see Review criteria) with the aim of identifying not only potential biomarkers that have been assessed with an appropriate statistical analysis, but also promising candidate biomarkers even if they were not yet validated. Publications from leaders in the field of epilepsy and epileptogenesis were also included on the basis of the expertise of these individuals. This approach can provide a general overview of the degree to which the field has matured. In this way, we identified a variety of biomarkers and potential biomarkers of epileptogenesis (TABLE 2) that fall into various categories; the most promising findings are discussed in the following sections.

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Table 1 | Epileptogenic insults, the associated risk of epilepsy, and rodent models

Insult or trigger	Estimated risk of epilepsy ^a	Rodent models	Refs
Genetic variation and mutation	~32% for generalized epilepsy and ~9% for focal epilepsy, but varies with gene and risk loci	Knockout and transgenic mouse and rat models for multiple genes identified as risk factors	82–84
Status epilepticus	20–40%, depending on age and aetiology; higher in refractory status epilepticus	Induction with focal or systemic chemoconvulsants (for example, kainate or pilocarpine) or electrical stimulation	84–87
Traumatic brain injury (open, closed or penetrating)	1–50%, depending on type and severity	Fluid percussion injury, controlled cortical impact, weight drop, blast injury	88–91
Stroke (ischaemic or haemorrhagic)	3–29%, but higher in some populations (younger adults)	Focal cerebral ischaemia, global cerebral ischaemia, intracerebral haemorrhage	90,92–95
Neonatal encephalopathy	7–10%, but varies with follow-up and treatment, and can be higher with neonatal seizures	Middle cerebral artery occlusion, continuous or intermittent hypoxia	96–98
Infection	7–8%, but higher in resource-poor countries and varies according to pathogen	Theiler murine encephalomyelitis virus	90,99,100
Brain tumour	Up to 90% but varies according to tumour	Glioma-associated epilepsy	101,102
Autoimmune encephalitis	Generally ~5%, but can be higher for some autoantibodies	Infusion of (human) autoantibodies	103–106

^aRisks vary according to aetiology, age and other factors for each insult; see references for more details.

Molecular biomarkers

Molecular biomarkers can be any molecule (metabolites, proteins or nucleic acids) in biofluids, where they can be free, bound to proteins or enclosed in microvesicles, such as exosomes. Molecular biomarkers are, in many ways, the most attractive type of biomarkers, as they can often be measured in an easily accessible compartment (for example, the blood or cerebrospinal fluid (CSF)), meaning minimal expertise is required and the cost is low. However, molecular biomarkers of epileptogenesis are the furthest from clinical validation and use. Given that molecular biomarkers in the blood are physically remote from the site of pathology in epileptogenesis, mechanistic links will need to be established; for example, the molecule may be enriched within neural tissue or its increase in peripheral levels might be triggered by a known epileptogenic process. Some candidate molecular biomarkers of epileptogenesis have been identified and are discussed below.

Plasma and serum proteins. Specific molecular alterations in plasma or serum could reflect alterations in brain function that are associated with epileptogenesis, including dendritic remodelling, neuronal hyperexcitability, axonal injury, BBB disruption and neuroinflammation. Several studies have shown that altered levels of brain-enriched proteins in the plasma or serum could be biomarkers of epilepsy and possibly of epileptogenesis.

These proteins include ubiquitin C-terminal hydrolase 1 (UCH-L1), neuronal specific enolase (NSE), glial fibrillary acidic protein (GFAP), calcium-binding protein S100 β , matrix metalloproteinase 9 (MMP-9) and high mobility group box 1 (HMGB1)^{10–15}, all of which are abundant in neural tissue. Unfortunately, the evidence for the use of these proteins as biomarkers of epilepsy is currently weak. Few of the relevant studies included a receiver operating characteristic (ROC) analysis of the results, sample sizes were generally limited, and patient cohorts were mostly heterogeneous. In addition, the specificity of these proteins for epilepsy compared with other diseases has not been established. Whether these candidate markers are of any value for identifying epileptogenesis has not been tested.

Despite the lack of progress in this area, plasma and serum proteins have great potential as biomarkers owing to their accessibility and relatively straightforward quantification. Therefore, substantial efforts should be invested in advanced proteomics–bioinformatics approaches for plasma and serum proteomic profiling.

Non-coding RNAs. MicroRNAs (miRNAs) are non-coding RNA molecules that have emerged as potential molecular biomarkers of epileptogenesis. Mature miRNAs are ~20 nucleotides in length and post-transcriptionally regulate protein levels in cells. The human genome contains an estimated 2,300 miRNAs (~500–700 are present in laboratory rodents)¹⁶. Numerous characteristics of miRNAs make them good candidates as biomarkers: they are present in biofluids, including the blood and CSF, are more stable than mRNAs and proteins, and are quick and inexpensive to assay. Some miRNAs are enriched or exclusively expressed in the brain, and animal studies have shown that the composition of miRNAs in the circulation reflects the type and severity of brain injury^{17–19}. Dysregulation of miRNAs has been observed in brain tissue from patients with epilepsy, and functional studies have demonstrated that manipulations of miRNA by use of antisense oligonucleotides can have anti-seizure effects in rats and mice^{20,21}.

Circulating miRNAs after status epilepticus in animals have been analysed in several studies (TABLE 2). Studies that involved continuous video or electroencephalography (EEG) monitoring have revealed that plasma levels of brain-enriched and neuroinflammation-linked miRNAs change at early and late phases of epileptogenesis^{22–24}. However, the miRNAs identified differed between studies and the ability of these miRNAs to distinguish between animals that developed epilepsy and those that did not was not investigated. However, two studies in mice have shown that an experimental anti-epileptogenic miRNA therapy administered after status epilepticus partly normalizes plasma levels of some miRNAs that have been identified as biomarkers of human temporal lobe epilepsy and potential biomarkers of epileptogenesis^{24,25}. In ongoing studies, blood miRNA profiles are being analysed in animals that have undergone TBI and are being monitored for development of epilepsy²⁶. These studies could determine whether specific miRNA biomarkers are associated with the epileptogenic trigger.

Receiver operating characteristic (ROC) analysis

A standard method to determine the sensitivity and specificity of a proposed biomarker.

Box 1 | **Categories of biomarkers**

The FDA–NIH Biomarker Working Group categorize biomarkers as follows. Descriptions are from the BEST (Biomarkers, Endpoints, and other Tools) Resource⁹.

Susceptibility/risk

Biomarkers that indicate the potential for developing a disease or medical condition in an individual who does not currently have clinically apparent disease or the medical condition.

Monitoring

Measured serially for assessing the status of a disease or medical condition or for evidence of exposure to (or effect of) a medical product or an environmental agent.

Diagnostic

Used to detect or confirm the presence of a disease or condition of interest or to identify individuals with a subtype of the disease.

Prognostic

Used to identify the likelihood of a clinical event, disease recurrence or progression in patients who have the disease or medical condition of interest.

Predictive

Used to identify individuals who are more likely than similar individuals without the biomarker to experience a favourable or unfavourable effect from exposure to a medical product or an environmental agent.

Pharmacodynamic/response

Used to show that a biological response has occurred in an individual who has been exposed to a medical product or an environmental agent.

Safety

Measured before or after an exposure to a medical product or an environmental agent to indicate the likelihood, presence or extent of toxicity as an adverse effect.

To date, no study has been done to identify biofluid miRNA biomarkers of epileptogenesis in humans. One study published in 2020 has revealed changes in blood levels of miRNAs among patients who developed late seizures after intracerebral haemorrhage, a finding that might be relevant to human epileptogenesis²⁷. However, most clinical studies in this area have investigated miRNA levels in patients with pre-existing epilepsy. These studies have identified several miRNAs that seem to distinguish patients with epilepsy from controls with good sensitivity and specificity, and some of these miRNAs are the same as those identified in animal models²⁸. Some clinical studies have also shown that levels of circulating miRNAs change in response to epilepsy therapy^{29,30} and some evidence suggests that different seizure types are associated with different plasma miRNA profiles²⁸.

In future studies, animal models of epilepsy with different aetiologies should be used to profile the circulating miRNAs present during epileptogenesis. Similar studies are needed in humans. Levels of several miRNAs in biofluids are known to be altered after TBI in humans, but epilepsy has not been assessed as an outcome in these patients^{31,32}. Blood profiling studies in patients with TSC who had not yet developed epilepsy was a focus of the European **EPISTOP** project. Results from other large preclinical and clinical projects might also close some of the gaps in our knowledge of miRNAs and epileptogenesis; such studies include **EpimiRNA**, **EPITARGET** and **EpiBioS4Rx**.

Finally, other non-coding RNAs have been identified as possible biomarkers of seizures or epilepsy. These include fragments of transfer RNAs (5'-GlyGCC,

5'-AlaTGC and 5'-GluCTC), which are thought to be released from brain cells as part of an injury or stress response and increase in the plasma before a seizure³³. As technology progresses towards point-of-care devices for non-coding RNA testing^{34,35}, these remarkable RNAs could be incorporated into future clinical trials of anti-epileptogenic therapies.

CSF biomarkers. Given its closeness to the brain parenchyma and its central role in intracranial fluid movement and clearance, the CSF might be the biofluid that is most likely to yield protein or other biochemical markers of epileptogenesis. Stroke and TBI are causes of acquired epilepsies in which CSF is often obtained, but analysis of CSF to identify potential biochemical markers of epileptogenesis has been done in only a few studies after TBI and in none after stroke.

In one clinical study, the association of CSF and serum levels of the pro-inflammatory cytokine IL-1 β with the development of post-traumatic epilepsy was investigated in 256 adults of European descent³⁶. Multivariate analysis showed that a higher CSF to serum ratio of IL-1 β levels during the acute phase of injury was associated with an increased risk of developing post-traumatic epilepsy after moderate to severe TBI. However, this study did not include an ROC analysis, and the sample size was relatively small. No additional studies have assessed IL-1 β as a prognostic, CSF-based biomarker of epileptogenesis. Animal studies have identified several candidate CSF biomarkers of epilepsy including UCH-L1, fibronectin, tetranectin, SOD1 and TGF β 1 (REFS^{37–39}), but not of post-traumatic epilepsy or epileptogenesis. CSF levels of these markers are also high in drug-resistant epilepsy, which may or may not be caused by TBI.

The value of CSF for identification of protein biomarkers of post-traumatic epilepsy cannot be overstated. Future clinical studies need to include serial sampling of the CSF and blood during the acute phase of injury and during clinical follow-up. The availability of high-sensitivity protein assay platforms combined with rigorous statistical analyses and data interpretation via systems biology will help to make CSF proteomics a centrepiece of future clinical studies⁴⁰.

Imaging biomarkers

Imaging modalities, either alone or in combination, enable monitoring of a myriad of biological processes and therefore have the potential to provide mechanistic biomarkers of epileptogenesis and epilepsy. Several modalities, including several MRI approaches, PET and single-photon emission computed tomography (SPECT) have already been applied to the study of epilepsy-related biomarkers, mostly in animals, and have the potential to identify biomarkers of epileptogenesis.

MRI is ideal for biomarker studies, as it is non-invasive, safe and translatable to clinical studies. One of the most widely available and least complex imaging approaches in the study of epilepsy is MRI measurement of hippocampal volume and signal intensity. In an animal model of post-traumatic epilepsy, hippocampal T1 ρ was found to be a prognostic biomarker for the development of increased seizure susceptibility³⁸. In humans,

Table 2 | Proposed biomarkers of epileptogenesis

Biomarker	Pattern of changes	Animal model studied	Human epilepsy studied	Performance metrics	Ref
Molecular biomarkers: plasma miRNAs					
miR-21, miR-142, miR-146	Upregulation during latency (miR-21 and miR-142) and in chronic epilepsy (miR-146a)	Electrically evoked status epilepticus in rats	None	No ROC analysis	22
miR-9a, miR-300, miR-598	Upregulation (miR-9) or downregulation (miR-300, miR-598) during latency	Pilocarpine-induced status epilepticus in rats	None	No ROC analysis	23
miR-93, miR-182 and miR-574	Upregulation (miR-93, miR-182) and downregulation (miR-574)	Status epilepticus induced by intra-amygdala kainic acid in mice, pilocarpine in mice, perforant pathway stimulation in rats	Temporal lobe epilepsy	Profiling, qPCR validation and ROC in humans (AUC using combination of three, 0.88)	24
Molecular biomarkers: CSF					
IL-1 β	Higher CSF to serum ratio predicts PTE	None	PTE	Multivariate analysis, $P=0.008$	36
Imaging biomarkers					
T2-weighted MRI signal	Reduced T2 signal in the amygdala	Hyperthermic seizures in rats	None	AUC 0.91	107
Hippocampal myo-inositol levels	Elevated myo-inositol levels	Pilocarpine-induced status epilepticus in rats	None	AUC 0.83	78
T1-GAD, T2	Increased BBB permeability in the piriform network	Paraoxon-induced status epilepticus in rats	None	AUC 0.96	57
EEG biomarkers					
Shortening of sleep spindle duration at transition N3 to REM	A spindle duration of <2.1 s differentiated animals with TBI that developed epilepsy from those that did not	LFP in rats	None	AUC 0.91, sensitivity 86%, specificity 80%	64
Perilesional pHFOs in the first 2 weeks after TBI	pHFOs are present only in animals that will develop epilepsy	LFP in rats	None	No ROC analysis	65
Absolute value of the slope of EEG theta power over time	Decreasing power of theta activity across recording sessions	Multiple rat and mouse models	None	AUC 0.91–0.99, specificity >90%, sensitivity >90%	66
Epileptiform activity after TBI	Early seizures, epileptiform discharges, lateralized periodic discharges, generalized periodic discharges, lateralized rhythmic delta	None	PTE	No ROC analysis	72
Epileptiform activity in asymptomatic TSC	Epileptiform discharges	None	TSC	100% of patients with epileptiform discharges developed epilepsy; negative predictive value 64%	74
EEG flattening (decreased EEG signal amplitude across frequency bands) following neonatal hypoxia–ischaemia	EEG power >10 μV^2	None	Neonatal hypoxia–ischaemia	Positive predictive value 86% for seizures, but high false negative rate	75
Disruption of the relationship between brain and cardiac electrical activity	Disrupted relationship between long R–R interval in ECG and EEG activity	Post-malaria infection (mouse)	Post-infection epilepsy	Specificity and sensitivity 100%	67
Behavioural biomarkers					
Seizure threshold and phenotypic features	Reduced seizure threshold and severity, and behavioural alterations	Lithium–pilocarpine-induced status epilepticus in rats	None	AUC 0.96 for combined tests	77
Cognitive deficit	Accelerated forgetting and reduced learning rate	Pilocarpine-induced status epilepticus in rats	None	Learning AUC 0.79, forgetting AUC 0.84	78

AUC, area under the curve; BBB, blood–brain barrier; CSF, cerebrospinal fluid; ECG, electrocardiography; EEG, electroencephalography; GAD, gadolinium; LFP, lateral fluid percussion; N3, stage III non-REM sleep; miR, microRNA; pHFO, pathological high-frequency oscillation; PTE, post-traumatic epilepsy; qPCR, quantitative polymerase chain reaction; REM, rapid eye movement; ROC, receiver operating characteristic; TBI, traumatic brain injury; TSC, tuberous sclerosis complex.

an ongoing study — known as the FEBSTAT study — involves serial scans of children with febrile status epilepticus to track the evolution of hippocampal injury and relate it to viral infection and the development of epilepsy⁴¹. The final results will enable comparison of data from animal models and humans.

Other possible MRI approaches to the discovery of candidate biomarkers of epileptogenesis include cortical thickness measurements, diffusion tensor imaging tractography, and resting state functional MRI to measure fluctuations in the blood oxygenation level-dependent signal after a potentially epileptogenic event⁴². These approaches could provide data on the evolution of structural changes and network alterations during epileptogenesis. Magnetic resonance spectroscopy can also be used to measure changes in neuronal and glial dysfunction that might contribute to epileptogenesis⁴³. New high field strength MRI approaches to the measurement of metabolites could provide information about the contribution of these molecules to epileptogenesis; one example is glutamate, although MRI cannot distinguish between the metabolic pool and neurotransmitter pool⁴⁴, which could be a hindrance.

PET allows imaging of more physiological processes — for example, neurotransmitter function and metabolism — than MRI. However, PET studies are sensitive to the specific characteristics of the radioligands used and their binding to metabolites and proteins. Nevertheless, PET has been used in several studies to assess potential mechanisms of epileptogenesis. For example, a combination of hippocampal surface and ¹⁸F-DG-PET data predicted epilepsy development after fluid percussion injury in rats⁴⁵. In another study in rhesus monkeys with epilepsy, binding of the type 1 cannabinoid receptor ligand ¹⁸F-MK-9470 was increased in the seizure onset zone, and glucose metabolism was low during the progressive decrease in seizure threshold that occurs during the kindling process, a plausible model of epileptogenesis⁴⁶. In humans, PET has shown that the 5-HT_{1A} receptor is reduced in temporal lobe epileptic foci⁴⁷. The role of 5-HT_{1A} receptors in epilepsy is still debated, but their blockade has had beneficial effects in experimental models⁴⁸. Data on 5-HT_{2A} receptors are still inconclusive. For example, in a rat electrical kindling model, PET indicated greater binding in kindled than in non-kindled rats, but autoradiography did not produce the same finding⁴⁹. This discrepancy might have been a result of a reduction in serotonergic central activity, which can be detected by *in vivo* PET neuroimaging but not by *in vitro* 5-HT_{1A} receptor autoradiography. Other potential targets for PET radioligands in studies of epileptogenesis include metabotropic glutamate receptors⁵⁰, as excitatory amino acids appear to play an important role in epileptogenesis, as well as in associated structural and functional reorganization in regions such as the hippocampus⁵¹.

PET also enables assessment of inflammation through the use of radioligands that bind to translocator protein (TSPO), which is highly expressed in activated microglia and reactive astrocytes. TSPO expression is increased in patients with mesial temporal sclerosis and focal cortical dysplasia^{52,53}. TSPO PET studies in kainate-induced and

electrically induced status epilepticus in rodents have provided evidence that inflammation is associated with spontaneous recurrent seizures^{54–56}.

Various imaging modalities can be used to visualize BBB breakdown, a process that is thought to be an important contributor to epileptogenesis⁵⁷. In a study in rats, multimodal imaging with ⁶⁸Ga-DTPA-PET, ^{99m}Tc-DTPA-SPECT, T2-weighted MRI and T1-weighted MRI after infusion of gadolinium contrast agents showed that BBB permeability is increased after status epilepticus⁵⁸. Similarly, imaging can be used to detect inflammation that is known to be associated with BBB opening. For example, high expression of vascular cell adhesion molecule 1 (VCAM1), which is a marker of leukocyte extravasation across vascular endothelium, can be imaged with MRI and antibody-coated iron oxide particles, or with SPECT with ¹²⁵I-labelled particles⁵⁹. Clinical application of BBB imaging with either dynamic, contrast-enhanced or dynamic, susceptibility contrast MRI is technically challenging, but this approach has been used in one study to predict seizure onset in post-traumatic epilepsy and recurrence of seizures in patients with neurocysticercosis⁶⁰. These data may provide a link between animal models and human epilepsy.

Unfortunately, none of the studies discussed has had sufficient power to calculate the sensitivity or specificity of the candidate imaging biomarkers identified. Moreover, neither the animal studies nor the clinical studies that have been done have involved formal blinding and randomization that are usual for clinical trials. Furthermore, imaging data on human epileptogenesis are sparse. PET studies have rarely included patients with new-onset epilepsy, as opposed to established epilepsy. Consequently, the available data do not support the use of imaging as a validated biomarker for human epileptogenesis studies. Emerging modalities, such as BBB integrity and diffusion tensor imaging, could have roles in establishing study stratification criteria or defining outcome biomarkers, but additional data need to be collected with these modalities, ideally as part of large multicentre studies with standardized acquisition parameters and analysis procedures. An attempt to standardize MRI techniques for rat studies of post-traumatic seizures revealed inter-site differences of 10% for fractional anisotropy and 3% for magnetization transfer values, and such variation would need to be accounted for in multicentre studies⁶¹.

In planning studies that involve imaging, several aspects of the imaging modalities need to be considered; for example, availability, cost, technical complexity, reliability, suitability for use in children, and the ability to scale up for multicentre and longitudinal studies. Safety is also an important consideration. Emerging evidence suggests that gadolinium contrast agents can accumulate in the brain with repeated use, although the clinical implications are uncertain⁶². PET studies in children, and in adults to a lesser extent, are limited by radiation exposure guidelines. Imaging studies to investigate epileptogenesis involve further specific challenges. For example, one pitfall in preclinical and clinical studies is that imaging markers of established epilepsy, such as hippocampal atrophy or

Autoradiography

A technique in which an image is produced on an X-ray film or nuclear emulsion by the pattern of decay emissions from the distribution of a radioactive substance in a cellular or histological preparation.

hypometabolism, might have different implications for epileptogenesis. In addition, uncertainties remain with respect to processes that have been implicated in epileptogenesis. For example, BBB breakdown might be strongly influenced by seizure frequency and timing, and inflammation can be a contributor to and a consequence of seizure development, and imaging markers might not be able to distinguish the two⁶³.

EEG biomarkers

EEG enables non-invasive, real-time measurement of brain activity. Many variations of EEG technology exist, but in its most basic form, EEG measures local field potentials created by neuronal activity. EEG can reveal changes in the frequencies of background brain activity (that is alpha, beta, delta, theta and gamma activity), and enables identification of electrographic seizure activity and of epilepsy-associated activity, such as interictal spikes and pathological high-frequency oscillations (HFOs).

EEG has multiple benefits with respect to biomarkers. Brain activity can be monitored with extremely high temporal resolution and relatively high spatial resolution (which can be improved by the use of high-density EEG). The technique is non-invasive, and is commonly used in all clinical settings, from comprehensive academic epilepsy centres to community hospitals. Expertise in the approach is already widespread among epileptologists, who are well-versed in reading EEG recordings to identify abnormal brain activity. Finally, EEG analysis can be highly quantitative, a key factor for the development of useful biomarkers. However, two main drawbacks must be considered. First, the activity measured is largely cortical; subcortical activity cannot be measured accurately. Second, EEG can generate large datasets that are challenging to analyse and store.

Preclinical and clinical studies have produced promising results that support investment in the development of EEG-based biomarkers of epileptogenesis. In rodent models, shortening of sleep spindles⁶⁴, detection of pathological HFOs⁶⁵, loss of stability in theta power over time⁶⁶, and abnormal EEG activity in combination with disturbances in heart rate⁶⁷ all predicted development of epilepsy after a brain injury or insult. The results of these preclinical studies suggest that EEG biomarkers have potential but highlight key challenges in the use of such EEG-based biomarkers in humans. First, many standard scalp EEGs are limited to the measurement of brain activity with frequencies up to 100 Hz, but strong evidence from animal models shows that meaningful, activity-based biomarkers of post-traumatic epileptogenesis can be detected at frequencies above 100 Hz^{65,68}, meaning that predictive information is currently being missed⁶⁹. Second, HFOs can generally be detected only with invasive techniques that require depth electrodes rather than the commonly used scalp electrodes^{70,71}. Third, repeated EEG monitoring of the same patient might be needed to reveal dynamics in brain activity that can help predict the development of epilepsy⁶⁶, but standard of care often does not involve repeated EEG monitoring, especially when no acute seizures occur during initial monitoring. One study has shown that a

loss of theta power over time, even without seizure activity, is highly predictive of epileptogenesis, even with as few as two independent monitoring sessions⁶⁶.

Despite these challenges, some clinical studies have indicated that EEG-based biomarkers of epileptogenesis have promise. These studies have shown that EEG detection of early epileptiform activity (defined as epileptiform discharges, lateralized periodic discharges, generalized periodic discharges or lateralized rhythmic delta)⁷² and early seizures⁷³ are associated with an increased risk of developing post-traumatic epilepsy. Similarly, early epileptiform abnormalities detected with EEG were extremely valuable for predicting development of epilepsy in a study of infants with TSC⁷⁴. In another study, EEG flattening (decreased EEG signal amplitude across frequency bands) after neonatal hypoxia and/or ischaemia strongly predicted later development of epilepsy, although this approach was also associated with a high rate of false-negatives⁷⁵.

ROC analysis in preclinical studies has identified strong candidate biomarkers of epileptogenesis. Shortening of sleep spindles, the absolute value of the theta slope and disruption of the relationship between brain and cardiac electrical activity are all measures for which the area under the curve (AUC) value is >0.9, which indicates robust prediction of subsequent epilepsy^{64–67}. Equivalent studies in humans that included rigorous ROC analysis to identify diagnostic biomarkers are sparse, but epileptiform activity in asymptomatic individuals with TSC⁷⁵ and epileptiform discharges after TBI are promising candidates^{72,73}.

Methodological improvements and changes in the standard of care could accelerate development of EEG-based biomarkers of epileptogenesis. Clinical EEG monitoring equipment can be updated relatively easily to enable measurement of high-frequency activity. Most clinical EEG systems can record frequencies up to 1–2 kHz, although storage of the large data files produced can be problematic. In addition, use of high-density EEG arrays and tripolar EEG electrodes will improve spatial resolution and enable a wider range of frequencies to be monitored⁷⁶. Although costly, repeated EEG monitoring of at-risk patients could enable identification of EEG dynamics that are highly predictive of epileptogenesis. In addition, standardized methods are needed for EEG interpretation to reduce variation between experts. Finally, steps to improve early identification of patients who are at risk of epilepsy, increase the frequency of EEG monitoring and facilitate long-term monitoring of patients will support the development of robust EEG biomarkers of epileptogenesis.

Behavioural biomarkers

Behavioural biomarkers are changes in one or more behaviours that predict a disease state. A validated behavioural biomarker of epileptogenesis could provide a non-invasive, continuous, functional marker of disease.

Studies in animal models have provided evidence that some behavioural changes can be considered as biomarkers of epileptogenesis. In a study in rats⁷⁷, seizure threshold and several phenotypic features (assessed

Sleep spindles

Trains of distinct waves with frequency at 11–16 Hz detectable on the EEG during sleep.

Box 2 | Five-phase framework for developing epileptogenesis biomarkers**Phase 1: Preclinical exploratory studies****Primary aims**

1. Identify leads for potentially useful epileptogenesis biomarkers.
2. Prioritize identified leads.

Phase 2: Initial clinical assessment**Primary aims**

1. Estimate the frequency of true-positive and false-positive results or perform receiver operating characteristic analysis.
2. Assess ability to distinguish between individuals who will and will not develop epilepsy.

Secondary aims

1. Optimize procedures for assays and ensure reproducibility within and between laboratories.
2. Determine the relationship between biomarker measurements in animal models in phase 1 and those in clinical specimens.
3. Assess whether biomarker status varies with sex, age and other variables in healthy controls, and/or with disease variables, such as epileptogenic insult or genetic defect.

Phase 3: Retrospective studies using longitudinal data available in repositories**Primary aims**

1. Assess the capacity of the biomarker to detect the development of epilepsy.
2. Define criteria for a positive screening test in preparation for phase 4.

Secondary aims

1. Explore the effects of covariates on the value of the biomarker; if the biomarker discriminates well only in certain subpopulations, appropriate populations could be selected for prospective screening with the biomarker.
2. Compare biomarkers to select the most promising.
3. Develop algorithms to determine the likelihood of positive results with the use of biomarker combinations.
4. Determine the required interval between biomarker testing if repeated testing is of interest in phase 4.

Phase 4: Prospective diagnostic accuracy studies**Primary aims**

Determine the diagnostic accuracy of biomarkers in the clinical setting by calculating frequencies of positive and false-positive detection.

Secondary aims

1. Describe the characteristics of disease detected by the biomarker test, particularly with regard to the potential benefits of early detection.
2. Assess the feasibility of case-finding programmes and the likelihood that individuals with positive test results will adhere to work-up schedules and treatment recommendations.
3. Make preliminary assessments of the effects of biomarker testing on disease-associated costs.
4. Monitor disease diagnosed clinically but not detected by biomarker testing to identify subpopulations of patients in which the biomarker does not identify epileptogenesis.

Phase 5: Disease burden reduction studies**Primary aims**

Estimate reductions in mortality, morbidity and disability associated with biomarker testing.

Secondary aims

1. Obtain information about the costs of biomarker testing and treatment per life saved or quality-adjusted life-years gained.
2. Assess adherence to testing and work-up in various settings.
3. Compare different biomarker testing protocols and approaches to treating individuals with positive tests in terms of effects on mortality, costs or both.

with a test battery including approach–response, touch–response, finger–snap and pick–up tests) were assessed during the 3 weeks after lithium–pilocarpine-induced status epilepticus to determine whether any of these

behavioural features, either alone or in combination, were sufficiently sensitive and specific as biomarkers of epileptogenesis. The ROC analysis showed that the combination of all behaviours assessed enabled almost perfect prediction of the development of spontaneous recurrent seizures (AUC 0.96, $P < 0.01$). In another study of rats that underwent pilocarpine-evoked status epilepticus, rates of learning were reduced and rates of forgetting were increased in rats that developed epilepsy compared with rats that did not⁷⁸. A ROC analysis in this study showed that the AUC for slower learning was 0.79 and the AUC for accelerated forgetting was 0.84, suggesting that both behaviours are predictive of epilepsy development in this model.

Whether these exploratory findings translate from animal models to humans remains to be seen. However, behavioural studies to investigate the potential of multiple behaviours, either alone or in combination with other biomarker types, seem warranted. Such behavioural biomarkers, if validated, could be developed for non-clinic settings and/or mobile devices, might be more acceptable than physiological markers for participants, and could be less costly than other biomarker types. Given that age and culture can influence behavioural patterns, collection of normative data will be critical.

Roadmap

A rapidly increasing amount of research is being done to identify molecular, imaging, EEG and behavioural biomarkers of epileptogenesis. However, substantial limitations remain, including incomplete statistical validation, a lack of human studies, limited knowledge of how markers apply to epileptogenesis rather than ongoing epilepsy, and high costs. Therefore, there is an urgent need to define a strategic roadmap to facilitate the identification, characterization and clinical validation of biomarkers for epileptogenesis.

We have developed a five-phase roadmap for biomarker research, adapted from those established for cancer and AD^{79,80}. We acknowledge that this roadmap is an initial proposal that might be amended in future and that the process is not necessarily linear, so that deviations might be necessary for specific applications. Nevertheless, this proposed roadmap provides a conceptual structure for biomarker research and a checklist of issues that should be addressed at each phase of development.

We base our roadmap on those developed for cancer and AD, which are structured into five phases:

1. Phase 1: preclinical exploratory studies with the aim of identifying promising directions.
2. Phase 2: initial clinical assessment to estimate the frequency of true-positive and false-positive results.
3. Phase 3: retrospective studies of data in repositories to assess the ability of the candidate biomarker to detect the disease before its clinical manifestation.
4. Phase 4: prospective studies to determine the diagnostic accuracy of core biomarkers in the clinical setting.
5. Phase 5: disease burden reduction studies with the aim of measuring the extent to which use of the biomarker reduces the burden of disease.

Negative predictive value
The probability that a patient does not have the disease when the biomarker is negative.

Positive predictive value
The probability that a patient has the disease when the biomarker is positive.

We set out our roadmap for biomarkers of epileptogenesis, which is a modification of this scheme, in BOX 2. A synoptic comparison of this roadmap with those for cancer and AD is provided in Supplementary Tables 1–5. A SWOT analysis has identified some of the key issues involved in implementation of this roadmap (FIG. 1).

Hurdles and challenges

Major hurdles in the development of biomarkers for epileptogenesis are inevitable given the nature of the disease. The process of epileptogenesis is irregular over time, and a given biomarker might be detectable at a given time point in the process but not at others. In this respect, identification of biomarkers that can be used for monitoring, together with serial sampling to assess these biomarkers, are critical for identifying the trend of pathobiological changes. However, this monitoring approach makes for high costs, and the higher the cost of a biomarker, the less likely it is to be used clinically, especially in screening. For this reason, staged use of different biomarkers could be an advantage. For example, a non-invasive, low-cost blood-based monitoring biomarker could provide a tool for serial assessment of patients and to verify the development of epileptogenesis. Such a tool might not be intended as diagnostic but could serve as a screening tool to determine

when higher-cost or more invasive diagnostic procedures are warranted. The primary goal in the development of such a screening tool would be to attain a high negative predictive value but not necessarily a high positive predictive value. For example, a working group on AD biomarkers have proposed that a candidate biomarker for primary care screening in AD should have a negative predictive value of >90% but a positive predictive value of ~50%⁸¹.

Most studies that have been published to date are in the biomarker discovery phase and indicate differences between patients (or animals) with epilepsy and controls. Few studies have involved more rigorous comparisons based on classification of patients, which could be important because many, if not most, biomarkers will be specific for a given epilepsy aetiology (for example, syndrome-specific or insult-specific). In addition, biomarker specificity for epileptogenesis relative to other phases of the disease and to other diseases must be determined. Similarly important is to determine the biomarker sensitivity and specificity for assessing the effects of interventions because biomarker-based detection of therapeutic effects would be instrumental in the development and validation of preventive anti-epileptic therapies.

In principle, identification of a biomarker should be easiest when a precipitating insult or an identified genetic risk are followed by development of epilepsy in a particularly high proportion of patients. In this respect, mild to moderate TBI, neonatal hypoxic–ischaemic encephalopathy, CNS viral infections and TSC (where the disease can often be identified in utero on the basis of cardiac abnormalities) provide promising clinical frameworks for the development of meaningful biomarkers of epileptogenesis.

Other hurdles relate to technical aspects, including alignment of study design and outcome measures between experimental and clinical studies, standardization and harmonization of analysis platforms between study sites, validation across laboratories (possibly through multi-centre preclinical and clinical trials) and consideration of all pre-analytical patient-related and procedure-related factors. Dichotomous biomarkers, which are either present or not, can be better than continuous biomarkers, which are always present but vary in their level. For example, the degree of change is an issue with continuous biomarkers, meaning that statistical significance alone might not be sufficient for practical use and ROC analysis should be paired with other statistical approaches, such as volcano plots. In cases in which the baseline levels of the biomarker are very low (for example, many circulating miRNAs) upregulation is likely to generate broader changes, and therefore be more informative, than downregulation.

Priorities and recommendations

On the basis of the above considerations, we have identified priorities and recommendations to accelerate identification of biomarkers of epileptogenesis. First, we need adequate infrastructure and technical support. For example, we need to develop a high-quality, epilepsy-specific database and repository. This project will need to be a global enterprise because multiple

	Helpful	Harmful
Internal	<p>Strengths S</p> <ul style="list-style-type: none"> • Biomarkers are used in and work for other diseases (for example, cancer) • Acute insults, such as injury, infection or genetic abnormalities, enable identification of at-risk individuals • Combinations of biomarkers could improve prediction 	<p>Weaknesses W</p> <ul style="list-style-type: none"> • Only initial studies are currently available, with no rigorous characterization of biomarkers • Biomarkers in categories other than diagnostic are also needed; markers of risk, susceptibility and prognosis are a priority • The diversity of epilepsy means different biomarkers might be needed for different aetiologies • Preparation of samples is not harmonized between centres • Phenotypes and the quality of samples collected are not characterized well • Competence in informatics is needed
External	<p>Opportunities O</p> <ul style="list-style-type: none"> • Some promising results have already been obtained • The aims are technically feasible • Technical improvements are on the horizon; for example, the Human Brain Project and the BRAIN Initiative • Repositories provide an opportunity for data sharing • Many modalities for biomarker analysis are standard of care; for example, blood tests, EEG and ECG • Some biomarkers are ready to move to phase III studies; for example, EEG for TSC • CDEs and quality control examples are available 	<p>Threats T</p> <ul style="list-style-type: none"> • Future investments might be limited if barriers to the development of anti-epileptogenic therapies are not lowered • Existing data repositories are of low quality and heterogeneous • The field might not be prepared • Acquisition of some biomarkers, such as imaging markers, could have adverse effects

Fig. 1 | **SWOT analysis for the biomarker development roadmap.** The analysis includes strengths, weaknesses, opportunities and threats identified for the development of biomarkers of epileptogenesis according to the roadmap we propose (BOX 2). This analysis was developed by face-to-face and web-based discussions between the authors. CDEs, common data elements; ECG, electrocardiography; EEG, electroencephalography; TSC, tuberous sclerosis complex.

databases on different informatics platforms are difficult to merge and interrogate together, so common (or at least inter-communicating) foundations are essential. In addition, we need the development of high-level informatics and statistical approaches, not only to handle big data but also to integrate use of multimodal biomarkers.

Second, we should identify the clinical situations that provide the best opportunities for identification of the first biomarkers of epileptogenesis. As stated above, examples are TBI, hypoxic-ischaemic encephalopathy, infections and TSC. In studying these conditions, identification of molecular markers, and specifically mechanistic markers, should be the aim, as these types of markers can identify therapeutic targets in addition to teaching us about the pathobiology. Ideally, biomarker identification studies should be paired with the testing of anti-epileptogenic therapies, as these therapies will be needed to benefit from predictive biomarkers. This approach is increasingly used^{15,24}.

Third, we believe that consensus needs to be established with respect to the roadmap we have proposed. This consensus would be instrumental for moving the

field forward, which will require partnership with industry, and in particular the assay development industry. Finally, we believe that encouraging ongoing biomarker identification research is mandatory. We are still in the initial phase of a process that promises to dramatically improve the diagnosis and therapy of epilepsy. Any contributions of ideas and data will be essential to reaching these goals.

Conclusions

Identification of biomarkers for epileptogenesis and, more widely, for different types of epilepsy would greatly facilitate diagnosis, clinical trials, therapy and, ultimately, improve quality of life of patients and reduce the social costs of epilepsy. We are at the beginning of a process, and time and effort will undoubtedly be required to adequately validate the first biomarkers for epileptogenesis and subsequently introduce them into clinical use. We trust that the proposed roadmap will facilitate this process.

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Author contributions

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Competing interest

K.S.W. is on the scientific advisory board for and is shareholder of Blackfynn, and is a consultant for Xenon Pharmaceuticals. All other authors declare no competing interests.

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Review criteria

We searched PubMed for articles published up to August 2020, including experimental and clinical studies published in English. Generic searches using free terms (such as "epilepsy" AND "biomarker") had low specificity and retrieved a large number of articles (2,812 articles), the majority of which provided no clear evidence to support the notion that a particular molecular, imaging, electroencephalography (EEG) or behavioural alteration could be considered a biomarker of epilepsy or epileptogenesis. Use of medical subject heading (MeSH) terms (such as "Epilepsy"[Majr] AND "Biomarkers"[Majr]) had low sensitivity and retrieved few

articles (149 articles). Similarly, unsatisfactory results were obtained when using the generic search "epileptogenesis" AND "biomarker" or "Biomarkers"[Majr] (339 and 45 articles, respectively). Therefore, we refined the generic search with specific biomarker types (molecular, imaging, EEG, behavioural) by adding one or more subtopic-specific additional query term. Eligible papers were then read and filtered on the basis of experimental methodology — studies that included a receiver operating characteristic (ROC) analysis or an equivalent methodology for ascertaining discriminative value were prioritized. In addition, articles from leaders in the field of epilepsy and epileptogenesis were included on the basis of the investigators' expertise. Consequently, the final selection of biomarkers discussed in this Review comes from a narrative rather than a systematic search. For Table 1, rates and other information were compiled from reviews or, in some

cases, original references identified by use of tailored search terms for each risk factor.

Supplementary information

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