Advances in the development of biomarkers for epilepsy


Over 50 million people worldwide have epilepsy. In nearly 30% of these cases, epilepsy remains unsatisfactorily controlled despite the availability of over 20 antiepileptic drugs. Moreover, no treatments exist to prevent the development of epilepsy in those at risk, despite an increasing understanding of the underlying molecular and cellular pathways. One of the major factors that have impeded rapid progress in these areas is the complex and multifactorial nature of epilepsy, and its heterogeneity. Therefore, the vision of developing targeted treatments for epilepsy relies upon the development of biomarkers that allow individually tailored treatment. Biomarkers for epilepsy typically fall into two broad categories: diagnostic biomarkers, which provide information on the clinical status of, and potentially the sensitivity to, specific treatments, and prognostic biomarkers, which allow prediction of future clinical features, such as the speed of progression, severity of epilepsy, development of comorbidities, or prediction of remission or cure. Prognostic biomarkers are of particular importance because they could be used to identify which patients will develop epilepsy and which might benefit from preventive treatments. Biomarker research faces several challenges; however, biomarkers could substantially improve the management of people with epilepsy and could lead to prevention in the right person at the right time, rather than just symptomatic treatment.

Introduction

More than 50 million people worldwide have epilepsy and an estimated 2–4 million people are newly diagnosed with epilepsy each year.1 However, despite over 20 antiepileptic drugs being available, seizures remain uncontrolled in about 30% of patients. The research community has focused substantial attention on developing antiepileptic drugs with increased efficacy and fewer adverse effects.2 Additionally, awareness of the need to identify individuals at risk of developing epilepsy and to develop preventive or disease-modifying treatments is increasing.

One of the major impediments to improving epilepsy treatment options is the heterogeneity of epilepsies. Many different genetic and pathophysiological factors, alone or in combination, can underlie an increased risk of developing a seizure disorder. Among these are different forms of brain injuries such as stroke, traumatic brain injury (TBI), or perinatal and prenatal injury, and CNS malformations or tumours. This broad variety of disease entities implies that a large range of mechanisms can lead to establishment of an epileptogenic focus, and that potentially diverse mechanisms of functional impairment and seizure generation exist. Moreover, even in the comparatively homogeneous groups of patients in whom an initial injury can be identified, such as those with stroke or TBI, on average, less than 20% of patients will develop epilepsy within 1–2 years,3 implying further complexity and potentially intra-syndrome heterogeneity. Accordingly, awareness is increasing that both antiepileptic drugs that would ideally be tailored to the cause for developing epilepsy (eg, a brain injury or genetic mutation). Consequently, identification of biomarkers that can help to guide diagnosis and treatment has been at the centre of research efforts in the past decade. A result of these efforts is that the medical assessment of patients with first-time seizures or initial injuries is often comprehensive, and thus detailed clinicopathological data are available.

Pitkänen and Engel4 defined a biomarker for epileptogenesis as “an objectively measurable characteristic of a biological process that reliably identifies the development, presence, severity, progression, or localization of an epileptogenic abnormality”. An epileptogenic abnormality is the pathophysiological substrate responsible for the start or continuation of epilepsy, or both. This definition captures the important features of the epilepsies that would benefit from the availability of diagnostic and prognostic biomarkers. An ideal diagnostic biomarker should provide information about clinical status, such as the extent and localisation of the epileptogenic area or the severity of the epilepsy. They might also provide information about sensitivity of clinical symptoms to specific treatments. Prognostic biomarkers, similarly, are of potentially high clinical relevance, but are more difficult to identify than diagnostic markers. They are defined as biomarkers that allow prediction of future clinical features, such as the speed of progression or severity of epilepsy, or the prediction of remission or cure, and could be used to identify features of the development of epilepsy, a process termed epileptogenesis.4–6 This term refers to a process whereby CNS tissue acquires the capability to generate the abnormal and spontaneous electrical activity that underlies seizures. In addition to unprovoked seizures, epilepsy is often associated with somatic, cognitive, psychiatric, and behavioural comorbidities, such as memory impairments.4–6 The development of these comorbidities is an important feature of epileptogenesis. Thus, a further relevant category of biomarkers could be those that can be used to predict the occurrence of comorbidities or sudden...
unexpected death. In the discovery of prognostic biomarkers, a crucial additional challenge is that these biomarkers are dependent on disease stage. Thus, even in a cross-section of clinically well-defined people with epilepsy, the stage of epileptogenesis (ie, time after the initial precipitating event) is likely to differ. Hence, the expected proportion of the population who are positive for a given biomarker might depend on the timing of sampling relative to disease progression. Perhaps more importantly, as a consequence, the use of combinatorial biomarkers is also likely to vary depending on the stage of the epileptogenic process.

Epilepsy specialists might argue that the surrogate endpoints (eg, number of epileptic seizures) often used in clinical trials can be regarded as biomarkers and can be used to predict the effect of the treatment. However, in our view, surrogate endpoints do not strictly fall within the definition of biomarker and will not be discussed in this Review.

Why is it so important to identify diagnostic and prognostic biomarkers for epilepsy? One of the major objectives in the discovery of diagnostic biomarkers is to individualise and optimise treatment. Over 30 preclinical proof-of-concept studies using animal models of genetic epilepsies, cortical malformations, status epilepticus, or TBI have either shown favourable antiepileptogenic or comorbidity-modifying effects, or both. However, none of these experimental studies have led to a clinical trial of an antiepileptogenic drug. Designing appropriately powered clinical trials is not possible even in well-defined groups of patients with epilepsy, because of the heterogeneity of epileptogenesis and the endogenous recovery mechanisms of the brain after injury. As a consequence, adequately powered antiepileptogenic trials will only be possible after the identification of biomarkers that allow stratification of participants on the basis of the predicted risk of epileptogenesis.

In this Review, we describe the proof-of-concept preclinical studies and the first clinical studies of biomarker discovery in epilepsy (table). So far, most studies examining the validity of biomarkers, in particular prognostic biomarkers, have relied on animal models with clearly defined initial injury, such as experimental temporal lobe epilepsy (TLE) models caused by an initial status epilepticus, or stroke or TBI models; in human beings, a large portion of the data have been obtained from patients with TLE. Thus, most of the biomarker data available are from a subset of epilepsies, and might not be generalisable across the whole spectrum of the epilepsies.

**Genetic biomarkers**

Genetic mutations can cause epilepsy, make the brain more vulnerable to develop epilepsy after an acquired brain injury, such as ischaemic stroke or TBI, and influence a patient’s response to a given treatment. Genetic markers have great potential because DNA is readily available from patients at risk admitted to hospital after potentially epileptogenic injuries such as TBI, new-onset status epilepticus, or stroke. Several genetic variants have been linked to the modulation of seizure threshold after injury or to acquired epilepsy (appendix). Some of these genes are involved in GABAergic neurotransmission, such as the GABA receptor subunit genes GABRB1, GABRB2, GABRG3, and the GAD1 gene; and the ADH5A1 gene. Susceptibility to TLE has also been associated with polymorphisms in genes involved in serotonergic transmission, for example HTR1B and SLC6A4. Association of genetic variants with epilepsy has also been shown for genes involved in the regulation of neuronal excitability (eg, SCN1A, PDYN, MTHFR, or ASIC1a), genes that encode pro-inflammatory mediators (eg, CD40), interleukin 1β, interleukin 1α, interleukin 1 receptor antagonist protein), and genes involved in protection against oxidative stress (eg, ALDH2, NFE2L2, or PRNP). A possible involvement

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<td>Wang et al (2015)11</td>
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EEG=electroencephalogram. sICAM5=soluble intercellular adhesion molecule 5. TARC=thymus and activation regulated chemokine.
of glial cells is shown by the association between a single nucleotide polymorphism (SNP) in the ATP-sensitive KCNJ10 gene, whose protein product is expressed by macroglia,\(^4\) and seizure susceptibility,\(^1\) as well as the association between risk of TLE and a SNP in KCNJ10 in combination with SNPs in AQP4, a gene encoding a protein crucial for the conductance of water through the cell membrane.\(^4\) A SNP in CALHM1 has been suggested to affect TLE susceptibility because it can alter the protein’s ability to control cytosolic calcium concentrations or modulate amyloid β concentrations, or both.\(^4\) Finally, polymorphisms in genes related to cell–cell interactions (eg, PCDH7,\(^3\) NRG1,\(^4\) and BDNF\(^5\)) and genes related to key components of the innate immune system and inflammatory response that have a role in neuronal plasticity and excitability (eg, C3)\(^6\) have been described.

However, most of the association studies of common variants that reportedly increase susceptibility to epilepsy have been small, none have been replicated, and they have been limited to pre-selected candidate polymorphisms or genes. No common variants are in clinical use as predictors of the development of epilepsy. In recent years, a few better-powered genome-wide association studies have been done and their findings have revealed possible associations between genetic variants and epilepsy syndromes or populations of patients.\(^4\) In a meta-analysis of 8696 cases and 26 157 controls,\(^4\) an association was identified between all types of epilepsy and variation in the SCN1A gene, which had previously been associated with epilepsy syndromes that are typically characterised by febrile seizures. However, this finding cannot be used to predict the individual risk of epilepsy. Association data alone are unlikely to be sufficient for prediction of individual risk without consideration of other predictors.

**microRNAs**

MicroRNAs (miRNAs) are small non-coding RNAs that regulate post-transcriptional gene expression.\(^6\) They are differentially expressed in the brain under pathological conditions and might therefore represent both therapeutic targets and diagnostic or prognostic biomarkers for neurological diseases, including epilepsy.\(^5\)\(^,\)\(^10\) Several studies have assessed the potential of miRNAs as biomarkers for different aspects of the epileptogenic process.\(^5\)\(^,\)\(^11\) Zucchini and colleagues\(^5\) examined the expression of human miRNAs in hippocampal granule cells of surgical specimens from patients with intractable TLE and identified 12 miRNAs that were differentially expressed in patients with granule cell dispersion. One of these, hsa-miR-487a, has been verified in an extended cohort of seven patients with intractable TLE and seven controls.\(^8\) Whether the miRNA signature of the resected tissue could be used as a biomarker for any aspects of surgical outcome remains to be assessed. miRNAs can be found in plasma and serum as well as brain tissue, where they are associated with proteins or are encapsulated in extracellular vesicles. These circulating miRNAs might represent a form of intercellular communication, because they can act as signalling molecules.\(^9\)\(^,\)\(^8\) miRNA-containing exosomes can be isolated from brain tissue and probably cross the blood–brain barrier, thus providing a possible peripheral blood source of a biomarker that is representative of a pathophysiological CNS process.\(^7\) Analysis of circulating miRNAs could be a non-invasive approach that could be used at any stage of the disease process.

Wang and colleagues\(^11\) measured serum miRNA concentrations in 30 people with epilepsy and 30 controls, and validated the selected miRNAs in a larger cohort of cases. Six miRNAs were differentially expressed in people with epilepsy compared with controls, and receiver operating characteristic (ROC) analysis revealed that hsa-miR-106b-5p had the highest sensitivity and specificity for a diagnosis of epilepsy of the miRNAs assessed. In another study, Wang and colleagues\(^12\) measured miRNA concentrations in serum from 77 drug-resistant and 81 drug-responsive people with epilepsy and 85 healthy controls. Five miRNAs were deregulated in drug-resistant patients compared with the drug-responsive and healthy control groups. Based on ROC analysis, hsa-miR-301a-3p had the best diagnostic value for drug-resistant epilepsy (table). As the investigators of these two studies acknowledged,\(^12\) prospective studies are needed to confirm the biomarker value of miRNAs in diagnosis of epilepsy and prediction of drug refractoriness.

Can measurement of circulating miRNAs be used to identify individuals who will develop epilepsy after brain injury? Attempts have been made to try and answer this question using animal models of status epilepticus.\(^3\)\(^8\)\(^,\)\(^9\) Roncon and colleagues\(^5\) analysed miRNAs using microarrays of plasma samples collected during epileptogenesis after systemic pilocarpine-induced status epilepticus. Concentrations of 27 miRNAs differed between the control and pilocarpine-treated animals. The miRNAs that had altered concentrations before the appearance of the first epileptic seizure, such as mno-miR-9a-3p, are potential biomarker candidates for epileptogenesis. However, the pilocarpine model is not optimum for biomarker studies because almost all animals will eventually develop epilepsy and the progression of epileptogenesis is fast, occurring within 1–2 weeks after status epilepticus, thus it does not accurately represent the human epilepsies. Therefore, further studies are needed to confirm the role of miR-9a-3p as a biomarker for epileptogenesis, both in animal models and in samples from patients.

Even though data are promising, a detailed validation of the analysis of miRNAs will be essential to bring these potential biomarkers to clinical practice. The main issues yet to be fully resolved relate to establishment of
standardised sampling (eg, elimination of platelet contamination), RNA extraction, and miRNA analysis protocols.  

**Structural biomarkers**

Structural changes are a notable feature of many epilepsies, particularly in patients with chronic TLE with hippocampal sclerosis, which shows characteristic patterns of damage, with segmental neuronal loss, gliosis, and axonal reorganisation. Whether more subtle forms of structural damage can be detected early in the course of disease and predict the future characteristics of epilepsy is unclear. T2-weighted MRI of the amygdala and thalamus has shown promise in the search for biomarkers for epileptogenesis in an experimental rat model of febrile seizures; reduced amygdala T2 relaxation times predicted development of TLE.  

In another imaging study, structural abnormalities in the thalamus and cerebral cortex, as shown by quantitative relaxation and diffusion MRI, were linked to increased seizure susceptibility in a model of post-traumatic epilepsy induced by lateral fluid-percussion injury.  

In the ongoing FEBSTAT study, MRI data from children with febrile convulsions are being collected. Over the next decade, we will learn whether pathological changes, some of which will eventually present as hippocampal sclerosis (eg, T2 hyperintensity), are predictive of the development of epilepsy.

Other advanced structural imaging parameters have not yet been assessed for their value as biomarkers of epileptogenesis. For example, in animal models, diffusion tensor imaging with high spatial resolution can be used to visualise the progression of structural changes (eg, mossy fibre sprouting in dentate gyrus) in different hippocampal subfields in the months after status epilepticus induced by systemically administered kainic acid or pilocarpine (figures 1A and B). Diffusion tensor imaging and more advanced diffusion MRI techniques, such as high-angular-resolution diffusion imaging (figure 1C), will probably provide even more specific information than conventional diffusion tensor imaging about microstructural changes related to cell death, reactive gliosis, and structural axonal plasticity. Furthermore, microstructural imaging techniques that assess local magnetic susceptibility differences, such as phase imaging and quantitative susceptibility mapping, will aid sensitive detection of microhaemorrhages, calcification, and white matter damage (figure 1D), which might have promise as biomarkers.

**Functional and electrophysiological biomarkers**

Electrophysiological parameters have been explored as both diagnostic and prognostic biomarkers. In established epilepsy, recurrent seizures constitute the defining disease symptom.  

An epileptic seizure is a transient occurrence of signs or symptoms, or both, caused by abnormal excessive or synchronous neuronal activity in the brain.  

In the context of epileptogenesis, the conceptual questions remaining to be answered are how networks transform from a healthy state to generate unprovoked recurrent seizures, and whether early excitability signatures exist that are less conspicuous than seizures. Such signatures could potentially constitute prognostic biomarkers for epileptogenesis or might represent biomarkers of seizure characteristics such as severity or drug response.

**Pathological high-frequency oscillations**

Arguably one of the strongest candidates for a prognostic biomarker of neuronal hyperexcitability are pathological high-frequency oscillations (HFOs).  

No uniform definition of pathological HFOs exists. However, most of the work on HFOs in both animal models and human studies has focused on brief events in the spectral range of 80–600 Hz, with different terms being used, depending on the frequency range (ie, ripples 80–250 Hz and fast ripples 250–500 Hz).

In people with chronic epilepsy, HFOs constitute an important interictal marker of focal epileptogenic zones and have been used clinically to localise such areas. More importantly, surgical removal of HFO-generating areas led to improvement in functional outcomes. Thus, HFOs are probably good indicators of the epileptogenic zone. However, few studies have assessed whether occurrence of HFOs is predictive of later development of epilepsy.

In the kainate-induced status epilepticus model of epileptogenesis, two different types of HFOs can be measured before the onset of generalised seizures, and their occurrence is predictive of the development of spontaneous seizures. Region-specific changes in HFOs have also been reported in the pilocarpine model of epilepsy, but their potential as predictive markers of the severity of the chronic seizure disorder is not clear. In the pilocarpine model of epilepsy, and in people with epilepsy, HFOs have been analysed in conjunction with interictal spikes. Findings from these studies have shown a complex temporal and spatial pattern of interictal spikes with and without superimposed HFO patterns.  

Together, these findings suggest that HFOs seem to constitute promising candidates for an excitability-based biomarker for epileptogenesis.

One of the most notable features of almost all studies on the relation between HFOs and the epileptogenic zone has been that these studies were done using invasive electroencephalography (EEG) techniques. These data have been extremely useful for delineation of the epileptogenic zone. However, to provide broadly applicable, clinically useful predictors of the disease course, non-invasive recording techniques would need to be established. These techniques would need to be capable of measuring the incidence and spectral properties of pathological HFOs, probably at the cost of anatomical resolution. Studies combining scalp EEG,
invasive EEG, and magnetoencephalography (MEG) will be needed to validate such non-invasive measures. High-sampling-rate MEG systems constitute a promising candidate method to test the validity of HFOs as biomarkers in human epilepsy.75–77 However, any high-resolution technology will have to overcome substantial problems. First, the management and analysis of large amounts of data generated by high sampling rates are challenging. Second, criteria for distinguishing HFO signals reliably, and preferentially automatically, from background noise need to be developed and validated.78,79 This task is far from trivial; however, presurgical assessment with the opportunity to identify intracranial local field potential signals and MEG signals in parallel might be valuable for this purpose. Finally, the use of HFO biomarkers in conjunction with behavioural state or other EEG markers such as interictal spikes, or both, might prove useful to optimise the predictive efficacy of HFOs.79 Solving these issues will pave the way for adequately powered clinical studies that can repeatedly and non-invasively assess cohorts of patients at risk to establish the validity of excitability biomarkers.

Future work should establish more precisely the spectrum of pathological HFOs in human beings and whether they constitute several classes of excitability signatures rather than a single entity;71 to identify which excitability signatures can be accurately distinguished from normal high-frequency activity; and to develop and validate methods suitable for non-invasive detection of these forms of activity in human beings.

Seizure susceptibility

Biomarkers for diagnosis of epileptogenesis would need to identify the presence of lowered seizure threshold without the occurrence of seizures. Seizure threshold, which is thought to determine the propensity or likelihood of seizure occurrence, is decreased after different types of epileptogenic brain injuries in experimental models, including those of genetic mutations, status epilepticus, stroke, or TBI. Investigators have assessed the seizure threshold in different animal models of TLE after status epilepticus during the course of epileptogenesis. They found some overlap in seizure susceptibility between the animals that developed and those that did not develop epilepsy, which might have been related to variability in the rate of progression of epileptogenesis within the follow-up time. The investigators expanded their findings by combining the data on seizure threshold and findings of behavioural alterations during the first 3 weeks after status epilepticus. By generating a combined seizure threshold and behavioural score, they were able to predict epileptogenesis with high sensitivity and specificity (figure 2A). Whether these data can be generalised to models of post-traumatic epilepsy and post-stroke epilepsy, and eventually to human beings, remains to be explored.

In patients assessed for epilepsy surgery, the area of ictogenic focus has a lower threshold for induced
electrographic epileptiform or imaging changes after administration of convulsants (eg, pentetrazol or bicuculline) or electrical stimulation of the ictogenic focus, when compared with non-focal areas. These findings provide some support to the idea of using seizure susceptibility testing to diagnose epileptogenesis,89–92 even though electrical stimulation was of little use at identifying focus lateralisation.92

Metabolic and activity biomarkers

In addition to electrophysiological measurements of function, several imaging methods can be used to establish the functional state of brain tissue by assessing metabolic parameters non-invasively. The findings from a preclinical longitudinal analysis88 of 1H-magnetic resonance spectroscopy of the hippocampus of rats after drug-induced status epilepticus showed a progressive increase in myo-inositol and glutathione concentrations before the development of spontaneous seizures. A negative association was found between the concentrations of these metabolites during epileptogenesis and the extent of neurodegeneration in chronically epileptic rats. Moreover, glutathione concentrations during epileptogenesis were inversely associated with the frequency of spontaneous seizures. ROC analyses showed that the concentrations of these two metabolites during epileptogenesis are good discriminators for differentiating control rats from rats that will develop epilepsy (figures 2B and 2C).

¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) PET has been used in two preclinical studies93,94 to assess the association between glucose metabolism and epileptogenesis after TBI or status epilepticus. In both studies, abnormalities in ¹⁸F-FDG PET were identified during epileptogenesis, but the sensitivity and specificity of the changes remain to be established. Furthermore, radioisotope imaging studies have assessed binding of GABA, and NMDA receptors in experimental and human epilepsy.95,96 However, the potential of receptor labelling imaging as epileptogenesis biomarkers remains to be shown.

Finally, large-scale network alterations have gained momentum as a key concept in epilepsy, mainly as a result of advances in non-invasive imaging techniques, which provide maps of functional and structural connectivity in the brain by exploiting resting-state functional MRI and diffusion-MRI-based tractography. Although there is evidence of functional connectivity changes in people with epilepsy, few studies have assessed connectivity changes during epileptogenesis, before the appearance of the first unprovoked seizure. In a preclinical study, resting-state connectivity was measured in rats 4 months after lateral-fluid-percussion-induced TBI and compared with hyperexcitability of the brain induced by pentetrazol in a seizure-susceptibility test. No significant association was found between functional connectivity and seizure susceptibility; however, the number of animals used in this study was small (12 injured vs eight controls).85 Nevertheless, adequately powered network analyses in animal models might produce clinically testable hypotheses in the search for biomarkers.

Biomarkers of neuroinflammation

Neuroinflammation is a prominent feature of most types of epilepsies. Neuroinflammation is characterised by the synthesis of cytokines, chemokines, danger signals mediated by endogenous molecules that are released upon tissue damage, and downstream effector molecules
from microglia, astrocytes, neurons, and the microvessel endothelium, as a result of activation of the innate immune system. Clinical evidence has shown that neuroinflammation is a hallmark of the epileptic focus in drug-resistant epilepsies. Moreover, neuroinflammatory mechanisms contribute to seizure generation (ie, ictogenesis) in animal models of epilepsy. Evidence also exists of rapid onset and persistent neuroinflammation during epileptogenesis in rodents. These findings, together with evidence of disease modifications mediated by drugs targeting the inflammatory cascade, suggest that neuroinflammation plays a part in the development of epilepsy. Even though separation of the changes in inflammatory markers that relate to injury from those that suggest the development of epilepsy will be crucial, neuroinflammation is not only a promising target for novel treatments, but also a potential mechanistic biomarker of epileptogenesis and ictogenesis.

Molecular imaging techniques that allow visualisation of inflammatory cells (figure 3A) and measurement of inflammatory mediators in the blood hold promise for monitoring neuroinflammation in people with epilepsy. Imaging techniques with high translational potential include PET and MRI. In a preclinical study, Choy and colleagues attempted to validate an imaging biomarker of epileptogenesis in a rat model of prolonged febrile seizures, in which only a subpopulation of animals developed epilepsy. ROC analysis showed that a decrease in amygdaloid and medial thalamic T2 relaxation times measured 2 h after febrile seizures predicted epileptogenesis. The reduction was associated with enhanced oxygen use, an increase in venous blood deoxyhaemoglobin concentration, and release of the neuroinflammatory protein HMGB1 from amygdaloid neurons. HMGB1 is bound to nuclei and its release is an energy-demanding process that increases oxygen demand. Moreover, the temporal evolution of HMGB1 translocation during epileptogenesis was consistent with the decrease in T2 signal. An early decrease in T2 signal in the amygdala and the dorsal hippocampus was also associated with the development of learning disabilities 2–3 months after febrile seizures, although the sensitivity and specificity of T2 as a biomarker for cognitive decline were not assessed.

So far, findings from only one proof-of-concept clinical study have shown that brain imaging can be used to detect neuroinflammation in people with epilepsy. Binding of TSPO was increased in the region of seizure origin and generalisation in patients with pharmaco resistant focal-onset epilepsy. Further studies are needed to assess whether TSPO-PET, other PET ligands, or MRI methods designed to detect neuroinflammation can be used to stratify patients at high risk of developing epilepsy, surgical candidates, or patients who will benefit from anti-inflammatory treatments.

Suitable blood biomarkers of neuroinflammation are molecules that are stable in biological fluids and can be rapidly measured in an affordable way. The short half-life of some neuroinflammatory molecules implicated in epilepsy, such as interleukin 1β, has hampered investigations of their biomarker value and produced discordant results. Circulating molecules that reflect neuroinflammation can originate by CSF-to-blood diffusion; however, they can also be derived from activated peripheral leucocytes, and thus do not always accurately represent neuroinflammation. Ideally, the inflammatory molecules measurable in blood should be CNS specific and their analysis should not be confounded by non-specific release from peripheral sources (eg, complement factors that are also released by the liver). In a proof-of-principle study, plasma concentrations of soluble intercellular adhesion molecule 5 (sICAM5, also

![Figure 3: Non-invasive detection of inflammation and neurovascular biomarkers](image-url)

(A) Translocator protein PET with the novel ligand (99m)Tc-fluticiclamide can be used for longitudinal assessment of neuroinflammation and has revealed microglia activation (arrows) 7 days after pilocarpine-induced status epilepticus in a rat. (B) Severity of BBB leakage after TBI as a prognostic biomarker for post-traumatic epileptogenesis. Within anatomically defined blood vessels, injection of gadolinium-based contrast agent resulted in a rapid increase in MR T1 signal due to arterial input followed by a signal decrease (washout, blue line). When signal dynamics within voxels of brain tissue with an intact BBB were analysed, the T1 signal followed the arterial input, but to a smaller magnitude (green line). After TBI, when the BBB became dysfunctional, the leakage of contrast agent resulted in a slow increase in T1 signal (red line). The signal change over time can be measured as the slope of increased signal (dashed line) using a linear regression analysis or by measuring the total change in signal intensity compared with the pre-injection scan (ΔT1). (C) Dynamic contrast-enhanced MRIs from a patient after mild TBI. The slope of the T1 signal change is increased in the injured right temporal lobe. (D) ROC analysis revealed that BBB dysfunction in the injury area was a prognostic biomarker for epileptogenesis after TBI with an AUC of 0.85 (data from Tomkins and colleagues). ROC=receiver operating characteristic. TBI=traumatic brain injury.
known as telencephalin), which originates in the CNS, was assessed. sICAM5 is expressed specifically by glutamatergic neurons; it plays an active part in synapse formation and dendritic spine plasticity and has anti-inflammatory effects when it binds to receptors on microglia and lymphocytes. sICAM5, together with an array of inflammatory mediators, was measured in blood samples from people with drug-resistant focal epilepsy during the interictal phase, disregarding timing of the last seizure, and compared with healthy controls. A five times reduction of sICAM5 was detected in patients, with a concomitant significant increase in concentrations of several pro-inflammatory cytokines, the highest of which was the pro-ictogenic cytokine interleukin 1β. Pollard and colleagues showed that none of the tested analytes, including interleukin 1, discriminated between people with epilepsy and controls. However, the ROC analysis showed that the plasma ratio between CCL17 (thymus and activation regulated chemokine) and sICAM5 was predictive of seizure activity. Together with TSPO-PET, these data support the idea that markers of neuroinflammatory changes can serve as biomarkers of the epileptogenic process.

Prospective studies in large cohorts of well-phenotyped patients with diverse causes of epilepsy are needed to validate the ability of inflammatory and other markers to predict outcomes of the epileptogenic process arising because of genetic abnormalities or brain injuries. Three ongoing studies, FEBSTAT, SANAD II, and the Human Epilepsy Project, might soon provide valuable information on this subject.

Microvascular injury biomarkers

Another functional system that is interlinked with neuronal function and is altered in many forms of epilepsy is the brain microvasculature. Microvascular injury can mediate delayed and long-lasting changes in the local neurovascular network (figure 3B–D). Specifically, blood–brain barrier dysfunction, and the associated leakage of serum proteins (eg, albumin), that occurs after epileptogenic injuries initiates glial activation and an inflammatory response, which are hallmark pathological abnormalities of the epileptogenic brain. Serum albumin activates specific signalling pathways in astrocytes, leading to a transcriptional response that mediates a reduction in buffering of extracellular potassium and glutamate, an increase in local neuroinflammatory responses, and selective excitatory synaptogenesis. These astrocyte-mediated responses lead to hyperexcitability and hyperconnectivity within the neuronal network.

Microvasculopathy and damage to the blood–brain barrier are prominent features of many animal models of epilepsy induced by TBI, stroke, or status epilepticus. Findings from animal studies suggest that the presence of microvascular injury in particular brain regions is suggestive of vascular-mediated epileptogenesis. In MRI studies of people with TBI or stroke, microvascular pathological abnormalities have been consistently noted, and these findings have been confirmed neuropathologically. Histological analysis of resected epileptic tissue from patients with drug-resistant epilepsy has also shown evidence of vascular damage and leakage of serum albumin into the neuropil. Moreover, cerebrovascular dysfunction is associated with comorbidities, such as behavioural and cognitive deficits, many of which occur in people with epilepsy. Finally, established and emerging neuroimaging technologies enable quantitative measurement of microvascular functional integrity (including blood–brain barrier permeability, cerebral blood flow, cerebral blood volume, angiogenesis, and cerebrovascular reactivity) in animals and human beings, and underscores the potential of microvasculopathy-linked biomarkers of epileptogenesis after injury.

However, so far, no prospective studies have tested the predictive value of microvascular dysfunction either in animal models or in the clinical setting. In a retrospective study, Tomkins and colleagues used contrast-enhanced MRI and quantitative EEG analysis in 37 patients after mild-to-moderate TBI, of whom 19 had post-traumatic epilepsy. The 18 patients without epilepsy had headache, cognitive impairment, mild motor dysphasia, or acute stress reaction. All patients with TBI showed some EEG slowing compared with controls (ie, higher delta and theta, and lower alpha bands), and no differences were noted between patients with and those without post-traumatic epilepsy. By contrast, people with post-traumatic epilepsy were more likely to have a blood–brain barrier lesion than those without post-traumatic epilepsy. Moreover, in most patients with post-traumatic epilepsy, the blood–brain barrier lesion was co-localised with the suspected epileptic region (ROC analysis resulted in an area under the ROC curve of 0·85; p=0·032; figure 3D). The volume of brain tissue with abnormal permeability was about six times higher in patients with than in those without post-traumatic epilepsy, suggesting that sensitivity and specificity would have been higher if a volume threshold had been applied. Although this study confirmed that quantification of vascular permeability is feasible in patients with TBI, it is limited by the small number of patients included and selection bias. Therefore, based on these findings, the conclusion that vascular functions can predict epilepsy, or in other words serve as a biomarker for epileptogenesis, cannot be made. However, findings from several reports have shown that blood–brain barrier dysfunction is co-localised to the site of the epileptic focus, further supporting the notion that microvasculopathy is likely to represent a relevant aspect of the epileptogenic network.

Current challenges

In the past 10 years, promising developments have been made in the biomarker specialty, but key challenges remain in the design of epilepsy biomarker studies.
Key challenges related to animal models
Animal models, in particular those in which an initial injury causes epilepsy, have been used successfully to identify and validate biomarkers in the preclinical setting. However, although stroke and TBI models might be expected to closely match the disease course in human beings, this is not always the case. Models of epilepsy caused by status epilepticus, for example, are phenotypically similar to TLE with hippocampal sclerosis, but to what extent they mimic processes of epileptogenesis in people with TLE without hippocampal sclerosis, or other type of epilepsy, is not clear. Research into biomarkers has mainly focused on the above-mentioned models. Which biomarkers are generally applicable across models, and which are specific to one model, is unclear.

A further challenge in models of disease after status epilepticus is the proportion of animals that ultimately develop chronic epilepsy. In rodent models of post-stroke epilepsy or post-traumatic epilepsy, only a subpopulation of animals develops epilepsy. However, in status epilepticus models, which are the most commonly used models of epileptogenesis, almost all animals develop epilepsy. In the past decade, efforts to refine status epilepticus models have been made. By using a drug cocktail that irreversibly terminates pilocarpine-induced status epilepticus after 60, 90, or 120 min, Brandt and colleagues were able to modify the outcome in such a way that only a subpopulation of animals developed epilepsy. Similar findings have been obtained by terminating chemically or electrically induced status epilepticus with diazepam122,123 or by using young animals.124 Another aspect of model development is that of high-throughput in-vitro models to predict the efficacy of new antiepileptogenic treatments.125

Key challenges related to heterogeneity of the epilepsies
Genetic heterogeneity, ethnicity, and sex probably have large effects on biomarker expression. Moreover, that drugs (anti-seizure or otherwise) or comorbidities can affect biomarker profiles cannot be excluded. One further important source of variability is the age of patients. So far, studies have focused on biomarker identification in adults. However, the onset of epilepsy is often in childhood, which will need to be taken into account in epilepsy biomarker studies in the future. Large-scale and more thorough genetic association studies (eg, using next-generation sequencing) will enable identification of mechanisms that are shared across different types of epilepsy and might help predict risk after brain injury. Such studies are underway.126,127

Key challenges related to accessibility of biomarkers
Although basic research has led to identification of several potential biomarkers, for translation into clinical practice, these biomarkers need to be accessible to physicians. That is, specimens to be analysed need to be accessible; brain tissue is accessible only from a small patient population at a single timepoint, and usually only in patients with severe or chronic disease or both. Biological fluids, including blood and, to a lesser extent, CSF, are more readily available than brain tissue, and can be sampled at several timepoints. Identifying the usefulness of biomarkers measured from accessible specimens will be crucial, as will be the validation of technologies to assess brain function non-invasively (ie, MEG or functional imaging techniques). The cost of biomarker analyses will be a factor in how extensively these biomarkers could be used in future trials and clinical practice.

Key challenges related to identification of patterns
An underestimated challenge in the assessment of biomarkers is the development of biomathematical analysis strategies for management of the complex datasets that are increasingly available from people with epilepsy. These datasets are likely to include many of the categories of data mentioned earlier (eg, from imaging studies). There will be a high demand on resources for mathematical modelling and pattern mining, as well as the development of novel analysis methods to define patterns of relevant biomarkers from large datasets.

Conclusions and future directions
What, then, is the way forward? Valuable lessons can be learned from other specialties. Probably the specialty most advanced in terms of biomarker discovery and their clinical use is oncology. This specialty has been driven by both technological advances in so-called omics technologies, which enable identification of molecular pathways, and by the wide availability of tumour tissue after resective surgery. As a consequence, molecular platforms for stratification of treatment of people with glioma are already a clinical reality.126 From the lessons learned in oncology, and the key challenges we have identified, we suggest that future research should focus on the following areas.

Animal models
Epilepsy is one of the few neurological disorders in which tissue is available from patients for validation of biomarker expression. Moreover, various animal models of epilepsy are available and have led to successful development of anti-seizure treatments. Still, many of the clinically prevalent epilepsy syndromes have no or few incompletely validated animal models (eg, post-stroke epilepsy, cortical dysplasia, and paediatric epilepsies). Development of animal models that reproduce the key features of human epilepsy syndromes requires accurate phenotyping of a human epilepsy syndrome. Examples of studies that will provide such information are the large studies on TBI in Europe and the USA (TRACK-TBI and CENTER-TBI).
Multimodal biomarker panels

Findings in oncology have shown the need to combine biomarkers to achieve higher clinical usefulness. First, pathological changes in cancer are diverse, and assessment of a panel of biomarkers of different pathogenic mechanisms progressing in parallel at any specific timepoint is likely to allow a clearer separation of different disease entities than assessment of individual biomarkers. Second, multimodal markers might offer an opportunity for staging of disease progression. Third, multimodal markers might enable better delineation of specific comorbidities (figure 4). Understanding the timing of biomarker expression during the disease process is important for the choice of a biomarker as well as the analysis platform (eg, blood vs imaging). Thus, we suggest that combinatorial molecular approaches will be needed both to be sensitive to a specific type of epilepsy and to stage the evolution of the disease.

Sensitivity of analysis platforms

In addition to model refinement, the sensitivity of analysis platforms needs to be improved. Progress in automated detection of various soluble biomarkers in body fluids, simultaneous pattern recognition of molecular combinations, and multiplexed analysis of single cell surface and intracellular macromolecules with next-generation flow cytometry techniques will undoubtedly advance biomarker identification, not only in preclinical studies, but also in patient samples. Animal studies provide only small amounts of blood, CSF, saliva, eye secretion fluid, microbiota, or urine samples for analysis, which is a factor to be considered when designing preclinical biomarker studies. High-field clinical MRI (≥3 T) with advanced parallel imaging is already commonly available for both human beings and animals, making translation of findings from preclinical MRI to clinical settings feasible. Technical improvements in gradient hardware and pulse sequences will aid more precise and faster mapping of brain networks.

Statistical approaches

The availability of datasets from high-throughput technologies, as well as increasingly complex clinico-pathological datasets, provides great potential for the identification of biomarkers. However, substantial biomathematical challenges exist in assessing these complex, multidimensional datasets and identifying combinatorial biomarker signatures. Further development of systems

Figure 4: Time dependence of epileptogenesis related to a set of biomarkers

Depending on the analysis timepoint after injury (x axis), the list of candidate biomarkers varies. Also, the magnitude of abnormality (y axis) can differ between people who will eventually develop epilepsy and those who will not. For example, at early timepoints, markers are probably released by damaged neurons, glial cells, or vasculature, whereas markers of cellular plasticity will probably become apparent later on. In MRI, the values of diffusion traces and T2 relaxation time also depend on the analysis timepoint (eg, early decrease in MRI diffusion trace followed by a long-lasting increase), and can be affected by recurrent seizures (asterisk). The purple triangle represents a gradual increase in seizure susceptibility before epilepsy diagnosis; purple bars show the fluctuation in number of seizures.
biology approaches that incorporate data-driven assessment of complex datasets, but also integrate expert knowledge about the relevant underlying biological function, will be necessary.12

Collaborative research approaches
Addressing the heterogeneity of epilepsies in human beings and developing and analysing animal models are huge tasks that need collaborative efforts. A start has been made by several large consortia that will be able to implement appropriately powered analyses in many animal models and large patient groups (eg, EPITARGET).

Validation and clinical translation
The key final goal of biomarker identification is the validation of candidate biomarkers in larger prospective preclinical and clinical studies across different cohorts and underlying pathological abnormalities.10–13 So far, study cohorts have been small, challenging the statistical power of the analyses, and phenotyping of epilepsy and comorbidities has been scarce. Finally, validation cohorts for candidate biomarkers have been used in only two studies of people with epilepsy.11,12 An additional challenge that will need to be faced is the absence of standardised preclinical and clinical designs for epilepsy biomarker studies. However, novel, multistage, adaptive clinical study designs are being developed for other indications.11

A cornerstone of any validation of biomarkers will be the recruitment of large numbers of patients and careful clinicopathological assessment. This issue has been recognised, and Common Data Elements for harmonisation of epilepsy studies between centres are available for clinical studies. The first Common Data Elements specifically designed for harmonisation of biomarker and proof-of-concept preclinical studies across laboratories are available through EPITARGET. The work ongoing in the International League Against Epilepsy and American Epilepsy Society translational task force for harmonisation of preclinical methods will extend the EPITARGET Common Data Elements to a wider spectrum of models and methods. The coming 10 years will show if these and related efforts will form a basis for the consistent collection of genetic, pathological, functional, and behavioural epilepsy phenotypes that can be used in clinical studies. The identified phenotypes might then, in turn, be used to develop models of epilepsy and identify preclinical biomarkers of the disease.

Conclusions
The development of epilepsy biomarkers, especially those capable of reliably predicting clinically significant epileptogenesis, would be of huge value for testing antiepileptogenic treatments. Whatever final common pathways might be shared across epilepsy syndromes in human beings in the clinical manifestation of seizures, the process of getting to those final common pathways, that is, the processes of epileptogenesis, is likely to be cause and disease specific. As our knowledge, especially from genetics, of the different disorders that cause epilepsy in human beings increases, we will need to consider how biomarkers will best reveal such diversity. Considering the cost and the efforts of developing disease-specific biomarkers for each individual epilepsy syndrome, data from animal models will be crucial in ensuring that properly designed and adequately powered clinical validation studies can be done. Combinatorial platforms would seem most likely to be successful in this context. Although the challenges ahead should not be underestimated, biomarkers would be a major advance in the management of people with epilepsy. Biomarker discovery could be regarded as an advance in the personalisation of medicine, and could allow prevention in the right person at the right time, rather than just symptomatic treatment.

The promising proof-of-concept data on putative epilepsy biomarkers summarised in the table will undoubtedly lead to the design of adequately powered studies to maximise the accuracy of the biomarkers assessed. Furthermore, inclusion of biomarker sample collection and analysis into ongoing and planned trials will enable possible prediction of treatment efficacy. These tasks need regulatory standards for biomarker development and use. The final challenge will be to compare the biomarker platforms between the clinical study cohorts and corresponding animal models, which has not been done thoroughly as yet in any of the brain diseases. In this regard, epilepsy research, with its various animal models to recapitulate epilepsy syndromes, provides a unique opportunity to assess preclinical biomarkers in the clinical setting and vice versa.

Search strategy and selection criteria
We searched PubMed for articles published between Jan 1, 2011, and Dec 31, 2015, with the terms “biomarker”, “epilepsy”, “seizure”, “seizure threshold”, “epileptogenesis”, “antiepileptogenesis”, “imaging biomarker”, “co-morbidity”, and their combinations. Data from experimental and clinical studies were included. For genetics, we did searches using the terms “polymorphism and epilepsy” and “polymorphism and epilepsy and outcome”. We also identified articles through searches of the authors’ own files. Only articles published in English were reviewed.

Contributors
This Review was envisioned and planned as a collaborative activity within the EU consortium EPITARGET, of which all authors are members. The general framework of the article was conceived by AP and HB. Individual sections of the article were drafted by AP, WL, AV, AJB, MS, KL, OG, JPB, AF, EA, JAG, TR, SMS, MK, and HB. A comprehensive manuscript draft was assembled and written by AP and HB. Revision of the manuscript was done by all authors. All authors approved the final version of the Review.
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