The search for circulating epilepsy biomarkers

Few would experience greater benefit from the development of biomarkers than those who suffer from epilepsy. Both the timing of individual seizures and the overall course of the disease are highly unpredictable, and the associated morbidity is considerable. Thus, there is an urgent need to develop biomarkers that can predict the progression of epilepsy and treatment response. Doing so may also shed light on the mechanisms of epileptogenesis and pharmacoresistance, which remain elusive despite decades of study. However, recent advances suggest the possible identification of circulating epilepsy biomarkers – accessible in blood, cerebrospinal fluid or urine. In this review, we focus on advances in several areas: neuroimmunology and inflammation; neurological viral infection; exemplary pediatric syndromes; and the genetics of pharmacoresistance, as relevant to epilepsy. These are fertile areas of study with great potential to yield accessible epilepsy biomarkers.

Keywords: autoantibodies • biomarkers • blood • cerebrospinal fluid • epilepsy • epileptogenesis • inflammation • pharmacoresistance

Background

For decades, the care of epilepsy patients has been limited by a paucity of biomarkers. There have been few indicators of disease progression or remission, aside from the rates of seizures themselves. Even seizure frequency has proven suboptimal, as patients may be amnesic for their seizures, or seizure manifestations may be subclinical. In fact, until recently, the primary tool used for prognostication in newly diagnosed epilepsy has been the electroencephalogram – a venerable test first used clinically by the pioneering German neurophysiologist Hans Berger in 1924. Since then, advances in imaging have allowed identification of lesions that confer an increased risk of seizures, such as mesial temporal sclerosis, vascular malformations and focal cortical dysplasia. The advent of 3T MRI promises to improve our sensitivity in this arena even further. However, these advances are insufficient to meet the level of uncertainty that face both clinician and patient during counseling after a first unprovoked seizure.

In patients for whom no epileptogenic lesion is identified or whose EEG demonstrates no evidence of epileptiform activity, biomarkers are elusive. Indeed, our ability to predict seizure recurrence after a first seizure – or predict development of epilepsy after diagnosis of a potentially epileptogenic neurological insult (e.g., traumatic brain injury and intracerebral hemorrhage) – is quite limited if the MRI and EEG are unrevealing. We currently lack measurable surrogates for the process of epileptogenesis, disease progression or remission. There are few predictors of treatment response, risk of adverse effects of anticonvulsants, quality of surgical candidacy or development of common comorbidities.

However, considerable progress has been made in identifying markers that may soon prove worthy of routine clinical use. As described previously [1,2], we are entering an era of improved neurophysiological and imaging tools that may provide new epilepsy biomarkers in the coming years. In this article, we

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Table 1. Other exploratory epilepsy biomarkers.

Molecule	Modality	Notes
Bcl-2	Serum	Increased serum levels in children with TLE versus controls; serum levels correlated with epilepsy duration, severity, seizure frequency and were negatively correlated with IQ [89]
HSP70	Serum	Significantly increased serum levels in TLE patients versus controls; HSP level correlated inversely with memory scores and hippocampal volume [67]
NCAM-1	CSF	Significantly lower levels in epilepsy patients versus controls; pharmacoresistant patients also had significantly lower NCAM-1 levels than treatment responsive group [90]
Superoxide dismutase 1	CSF	Significantly lower levels in epilepsy patients versus controls; pharmacoresistant patients also had significantly lower SOD-1 levels than treatment responsive group [91]
CD-133-enriched membrane particles	CSF	Significantly elevated in CSF of epilepsy patients versus controls; significantly elevated in TLE patients versus extratemporal epilepsy patients [92]
Gelsolin	CSF	An actin-binding protein assisting in cytoskeletal rearrangements; significantly reduced levels in 70 epilepsy patients versus 60 controls; gelsolin expression was also decreased in resected temporal neocortical tissue from TLE patients, assessed by immunohistochemistry and western blot [93]
IL-17	Resected FCD tissue	IL-17, IL-17 receptor and downstream modulators NF- κ B activator 1 and p65 were all significantly elevated compared with control cortex; IL-17 and IL-17R levels correlate with seizure frequency [94]
Nrf-2 mRNA	Resected human TLE tissue; rat hippocampal tissue (pilocarpine model)	This transcription factor triggers expression of a host of anti-inflammatory genes; Nrf-2 mRNA significantly increased in resected hippocampal tissue from humans with TLE; expression also found to peak shortly after SE in rats treated with pilocarpine [95]
miR-146a	Ganglioglioma and FCD resected tissue	Significantly increased expression in lesions compared with control tissue; negative feedback regulator of inflammatory processes in human glial cell cultures [96,97]. Increased levels during latent period following SE in pilocarpine model [42]. Unclear if miR-146a is detectable peripherally
miR-134	TLE resected tissue	Increased in CA1 and CA3 hippocampal subfields of mice using kainate epilepsy model; miR-134 inhibition reduces acute and chronic seizures in this model. Increased expression in resected tissue from humans with TLE [98]. Unclear if miR-134 is detectable peripherally
miR-155 and TNF- α	TLE resected tissue	Significantly higher TNF- α and miR-155 expression in resected hippocampal tissue from children with TLE versus controls [99]. Unclear if miR-155 is detectable peripherally

CSF: Cerebrospinal fluid; FCD: Focal cortical dysplasia; HSP: Heat shock protein; SE: Status epilepticus; TLE: Temporal lobe epilepsy.

focus on the concept of circulating biomarkers – factors that may be measured in the cerebrospinal fluid, blood or urine – that are of promise in patients with epilepsy. Such factors may reflect activation of inflammatory cascades specific to the process of epileptogenesis, products of differential gene expression or metabolic changes in

response to seizures or anticonvulsant medication usage. An overview of the state of epilepsy biomarkers was published in a series of articles in this *Biomarkers in Medicine* in 2011 [1]; here, we largely focus on more recent developments, with particular attention paid to candidate biomarkers that show the most promise in humans,

rather than in animal models alone. We also report on several pathways of increasingly understood relevancy to epilepsy that have the potential to yield a circulating biomarker, although none may presently exist. Our review therefore covers: antibodies to neuronal antigens; infectious markers; inflammatory markers; white blood cells and associated cell adhesion molecules; pediatric syndromes; and treatment-related biomarkers.

Antibodies to neuronal antigens

The past decade has seen the identification of several syndromes mediated by autoantibodies to neuronal antigens [3]. Several limbic encephalitides (LE) have been attributed to such autoantibodies, and some of these syndromes have seizures as a prominent and often presenting component. In fact, a prior history of LE may be an under-recognized cause of TLE. In a retrospective analysis, Bien *et al.* found that roughly half of patients with temporal lobe epilepsy (TLE) and hippocampal sclerosis had either a prior diagnosis of LE, or such typical MRI findings as hippocampal swelling, subsequent atrophy and T2 or FLAIR signal abnormality [4]. The full range of these autoantibody-mediated phenotypes is likely not yet appreciated. Cryptogenic focal epilepsy – in the absence of fever, encephalopathy, mood/personality disturbance, or other symptoms typically seen in LE – may prove to be such a phenotype. Four targets in particular have received attention: the *N*-methyl-D-aspartate (NMDA) glutamate receptor; the voltage-gated potassium channel complex (VGKCC); glutamic acid decarboxylase (GAD), which catalyzes the synthesis of the inhibitory neurotransmitter gamma aminobutyric acid (GABA); and the metabotropic G-protein-coupled GABA_B receptor. GAD is intracellular; the remaining targets are neuronal cell surface antigens [5]. The prevalence of these autoantibodies in cryptogenic focal epilepsy is an area of active investigation. In a study of 67 patients with medically refractory epilepsy, McKnight and colleagues found that two patients had antibodies to the VGKCC; three had antibodies to GAD; and one had antibodies to the ganglioside GM1 [6]. No patients had antibodies to glutamate receptor subunit 3 (GluR3) or voltage-gated calcium channels (VGCCs). Bien and Scheffer subsequently described unpublished data from a mixed sample of roughly 400 patients from different clinics in the UK [7]. All had epilepsy, but the duration of disease was heterogeneous. VGKCC antibodies were detected in 10% (vs 0.5% controls) of the overall sample. NMDA receptor antibodies were present in 7% of newly diagnosed patients, and in 2.5% of the established epilepsy cohort. GAD antibodies were present in 1.6–1.7% of the overall sample.

More recently, larger series have supported the hypothesis that autoantibodies are pathogenic in some cases of

focal epilepsy. Brenner *et al.* studied cohorts with newly diagnosed and chronic focal epilepsy [8]. Of the 416 participants with epilepsy, 46 tested positive for serum antibodies to VGKCC, GAD or receptors for NMDA or glycine using either radioimmuno- or cell-based assays. Prevalence for all autoantibodies was significantly higher in the epilepsy groups than for the controls. VGKCC was again the most common antibody encountered, found in 5% of the sample population; glycine receptor antibodies were found in 3%, while antibodies to GAD (1.7%) or NMDA receptors (1.7%) accounted for the remainder of the positive results. Titers were significantly higher in patients without a known structural or metabolic etiology for their epilepsy, furthering the argument that these antibodies are pathogenic rather than an epiphenomenon. The autoantibody groups also had a nonsignificant trend towards poor initial anticonvulsant response. Finally, there was no significant difference in autoantibody prevalence between those with longstanding epilepsy versus those who were recently diagnosed, arguing against the antibodies being an epiphenomenon or effect of epilepsy (rather than a possible cause) in these patients. Limited data suggests that this phenomenon may be present in children as well. In a small series of children (*n* = 12) with suspected autoimmune epilepsy, three had NMDA receptor antibodies, three had VGKCC antibodies, and one had antibodies to GAD. All five of the five autoantibody-positive children improved with immunomodulatory therapy [9]. We discuss data specific to the most common antigens encountered in adults and children below.

VGKCC

The importance of voltage-gated potassium channels in epilepsy has long been known; VGKCC mutations are associated with benign familial neonatal seizures [10]. In addition to the prevalence data described above, two distinct VGKCC epitopes have recently been identified that may be associated with different phenotypes. Mutations in the *LGI1* gene, which encodes an extracellular matrix protein associated with the presynaptic potassium channel, are observed in autosomal dominant partial lobe epilepsy with auditory features, or autosomal dominant lateral TLE [11]. LGI1 appears to play a role in dendritic pruning, seizure threshold and excitatory neurotransmission by regulating the fast inactivation of presynaptic potassium channels, as well as the subunit composition of postsynaptic NMDA receptors [12]. Antibodies to LGI1 have also been associated with LE [13]. More recently, a novel seizure phenotype has been associated with this condition: faciobrachial dystonic seizures, which may herald onset of LE [14]. Antibodies may also be found to another protein in the VGKCC complex, Caspr2. A

small subset of patients with LE and seizures have these antibodies present, but are more likely to present with Morvan's syndrome or neuromyotonia, which are more closely associated with Caspr2 antibodies [6,7].

NMDA

Anti-NMDA receptor encephalitis is an acute condition characterized by seizures, neuropsychiatric disturbances, dysautonomia and choreoathetosis. Recent work has identified a potential electrographic signature, the extreme delta brush, based on review of continuous EEGs from these patients [19]. Previously, this syndrome was thought to be a paraneoplastic phenomenon restricted to young women with ovarian teratomas. While that phenotype remains predominant, it appears that both genders may be affected in the absence of a detectable tumor [20]. This broadening of the phenotype has led to the recognition that explosive onset of epilepsy alone may warrant suspicion for presence of anti-NMDA receptor antibodies. In one series of 19 women (aged 15–45 years) with unexplained new-onset epilepsy, five had anti-NMDA receptor antibodies [21]. Prolonged nonconvulsive status epilepticus has also been reported in patients with anti-NMDA encephalitis [22]. Given the increasing recognition of this condition, as well as its range of presentations, its relevance to new-onset epilepsy (in the absence of other LE symptoms) needs to be determined. In addition, while a recent large series demonstrated a good response to immunomodulatory therapies in anti-NMDA receptor encephalitis patients as assessed by the Rankin scale, the long-term data from Titulaer *et al.* on seizure outcomes will be useful in determining whether these treatments are generalizable to the seizure-only NMDA phenotype [23].

GAD

Antibodies to glutamic acid decarboxylase have been associated with insulin-dependent (Type 1) diabetes mellitus, as well as the rare neurological disorder, stiff-person syndrome. However, anti-GAD antibodies have been found in a subset of epilepsy patients, independent of these comorbidities. In addition to McKnight *et al.* [6] above, Liimatainen *et al.* [15] found anti-GAD antibodies in 5.9% of 253 epilepsy patients compared with 1.5% of 200 controls. TLE patients were more likely to have anti-GAD antibodies, and at higher titers [15]. In another series, anti-GAD antibodies were seen in 2.58% of 233 patients (two with idiopathic generalized epilepsy [IGE]; four with cryptogenic TLE) [16]. One small series evaluated four patients with high anti-GAD serum titers and epilepsy using magnetic resonance spectroscopy (MRS). All were found to have significantly lower cortical GABA levels when compared

with ten controls, suggesting a potential pathogenic mechanism for reduced seizure thresholds (cortical disinhibition) [17]. Furthermore, a case report described a patient with DM1, Hashimoto's thyroiditis, vitamin B₁₂ deficiency and subacute onset of pharmacoresistant generalized seizures, ataxia and eye movement abnormalities. She was found to have anti-GAD antibodies, and improved with immunosuppressive therapy [18]. However, in contrast to the other autoantibodies discussed here, GAD is an intracellular molecule, and antibodies directed toward this target are therefore more difficult to implicate in epileptogenesis.

GABA_B receptor

The relevance of GABA_B receptor pathology to epilepsy has not been definitively established. The G1465A single nucleotide polymorphism was associated with TLE in one study [24], but subsequent studies failed to replicate this finding [25]. Data regarding antibodies to the GABA_B receptor are similarly scant, but promising. A series from Lancaster and colleagues described 15 cases of autoimmune LE who presented predominantly with seizures, and were found to have antibodies to extracellular GABA_B receptor epitopes [26].

While the prevalence of neuronal autoantibodies in epilepsy is now becoming understood, the relevance of antibodies to treatment remains murky. As of yet, there are no published prospective studies to help determine whether patients with these autoantibodies are more likely to prove pharmacoresistant to traditional anticonvulsants. Such data will be essential in determining whether the autoimmune epilepsy phenotype is in fact distinct and warrants alternative treatment approaches to the typical course of anticonvulsants, surgical resection or palliative procedures offered to other medically refractory epilepsy patients. However, a small retrospective series published by Quek and colleagues suggests that immunomodulatory therapies may be beneficial in patients with suspected autoimmune epilepsy. 91% of the 32 patients included in the study had a demonstrated neuronal autoantibody (most commonly VGKCC LGI1). 27 patients received some form of immunotherapy – intravenous methylprednisolone, intravenous immunoglobulin, cyclophosphamide, plasma exchange or a combination of the above. 81% had improved by a median follow-up time of 17 months; 18 of the 27 were seizure-free [27]. This small series suggests that a prospective trial of immunotherapy for autoimmune epilepsy may be warranted, assuming that neuronal autoantibodies can be used as a biomarker for a subpopulation of epilepsy patients.

In summation, autoantibodies to neuronal antigens are an increasingly recognized cause for a number of neurological phenotypes. The next challenge will be

to understand the full range of these phenotypes, and determine which are epilepsy-predominant. It will also be critical to determine whether any of these antibodies impact such epilepsy outcomes as seizure severity, pharmacoresistance and comorbidities (e.g., mood or cognitive disorders).

Infectious markers

Human herpesvirus 6 & 7

Early childhood infection with human herpesvirus (HHV)-6 is often asymptomatic, but latent infection is associated with febrile seizures, and possibly with later development of mesial temporal sclerosis (MTS). Small series have found HHV-6 present in 10–30% of resected hippocampal tissue with MTS, often with variable history of febrile seizures or encephalitis. Mechanisms include reactivation of latent virus; impaired glutamate reuptake (or enhanced release) with subsequent excitotoxicity; or activation of inflammatory cascades [28,29]. However, a subsequent study found HHV-6 DNA in only one of 33 TLE patients' resected tissue, casting doubts about the generalizability of these findings [30]. More recently, a large prospective study examined the association between HHV-6, HHV-7 and febrile status epilepticus in children. The FEBSTAT study enrolled children aged 1–60 months presenting with febrile status epilepticus, and examined blood (and occasionally CSF) samples for presence of HHV DNA and RNA using quantitative PCR. HHV-6B viremia was found in 32% of participants, while HHV-7 viremia was seen in 7.1%. Of the 58 patients with viremia, 14 had specific antibodies to HHV-6 or HHV-7, indicating reactivation of a latent infection. No significant differences in MRI or EEG findings were observed when the viremia group was compared with those with aseptic febrile status epilepticus (FSE). Comparison groups still need to be established, including infected patients without seizures. In addition, it is unclear whether infection with HHV-6B and/or HHV-7 confers additional risk when compared with those presenting with aseptic FSE alone; the same researchers are engaged in longitudinal prospective studies to answer this question. Finally, it is not clear whether direct cytotoxic injury from these viruses is epileptogenic, or whether the inflammatory response to infection is the culprit. Should HHV-6B- or HHV-7-associated FSE prove to predict later development of TLE, PCR for these viruses could become a useful biomarker for epileptogenesis.

Human papillomavirus

Human papilloma virus (HPV) has been implicated in both cervical and oropharyngeal cancers, and its role in other malignancies is under active investigation [31].

HPV 16 expresses the oncoprotein E6, which activates the mTORC1 second-messenger cascade. In turn, the mTORC1 pathway appears constitutively activated in the balloon cells that characterize focal cortical dysplasia type IIB, an epileptogenic lesion seen frequently in pediatric cases of focal epilepsy. Chen and colleagues analyzed biopsy tissue from patients undergoing surgical resections for medically refractory epilepsy [32]. Immunohistochemistry demonstrated E6 expression in 100% of specimens classified as focal cortical dysplasia type IIB (FCDIIB) (n = 50), and in 79% of balloon-cell neurons. By contrast, E6 was not present in any of the control samples, including tissue resected from patients with TLE, tuberous sclerosis, or nonepileptic postmortem samples. Furthermore, analyses of resected FCDIIB tissue identified the presence of HPV 16 DNA, E6 full-length mRNA transcripts and colocalization of E6 with phosphorylated mTORC1 substrates. Finally, the authors demonstrated that E6 expression in developing mouse brain results in disorganization of cortical laminae, including an E6-positive cortical malformation.

It remains to be seen whether a causal link truly exists between HPV infection and the development of focal cortical dysplasia. Furthermore, it is unclear if HPV plays a role in the development of the dysplastic lesion, the symptomatic seizures that result, or both. Therefore, it will be necessary to determine whether there are differential effects on seizure reduction in tissue that is HPV-positive versus HPV-negative. Should resection of HPV-positive FCDs prove less effective in ameliorating seizures than resections of HPV-negative FCDs (or should the presence of HPV predict a more medically refractory course of epilepsy), one might postulate that the infection itself is epileptogenic, rather than simply being associated with a symptomatic epileptogenic lesion. This question remains open until further research is completed.

These findings raise the intriguing possibility that HPV infection may be a biomarker for epileptogenesis, given the association of HPV 16 with an epileptogenic brain lesion. Furthermore, it is possible that the increasingly widespread use of HPV vaccine – and the subsequent reduction in infection rates in girls and young women [31] – has the potential to reduce incidence of FCDIIB, should a causal link exist.

Inflammatory markers

The idea that inflammation contributes to epileptogenesis has waxed and waned in popularity over several decades. Efforts to study this area are frequently confounded by issues of false causation (effect–cause) or spurious correlations with epiphenomena. However, as our understanding of inflammatory pathways

and neuroimmunology deepens, the links to epilepsy become clearer. Resected tissue from medically refractory focal epilepsy patients shows evidence of chronic inflammation [33]. In focal cortical dysplasia, hallmarks of adaptive immunity are seen, such as T- and dendritic cell infiltrates. By contrast, resected TLE tissue demonstrates a more localized inflammatory response consisting of activated microglia, reactive astrocytes and endothelial cells [34–36]. An inflammatory role has been postulated in several pediatric epilepsy syndromes as well, including Rasmussen's encephalitis, febrile infection-related epilepsy syndrome and idiopathic hemiconvulsion-hemiplegia syndrome, although the pathophysiology is largely unclear in all three conditions [37].

Vezzani and colleagues reviewed brain inflammation as a potential biomarker in 2011 [38]. A number of potentially epileptogenic events – such as traumatic brain injury, infection or a first episode of status epilepticus – may raise inflammatory markers preceding onset of chronic spontaneous seizures, suggesting a role in epileptogenesis. Determining which markers are pathogenic, and which markers can be detected in the periphery, has been an ongoing challenge. Some candidate markers and pathways are discussed here.

Toll-like receptor pathway

Toll-like receptor (TLR) expression is increased in FCD and tuberous sclerosis (TS) complexes, and two TLR agonists have been studied in these tissues: HMGB1 and IL-1 β . HMGB1 is released by neurons, activated astrocytes and microglia in response to physiological stress – including, perhaps, a first seizure – with subsequent binding to RAGE and TLR4 [39]. TLR4 activation, in turn, appears to increase neuronal excitability via modification of the NMDA receptor through aberrant subunit expression or post-translational phosphorylation [40,41]. As for IL-1 β , most epilepsy research has been limited to the kindling model of epilepsy in rats. IL-1 β levels rise in the acute phase during and shortly after SE; they fall during the latent period between a brain insult and chronic epilepsy onset, and then rise again once epilepsy develops. Interestingly, levels of miR-146a, a post-transcriptional inflammatory modulator, are inversely associated with IL-1 β during the acute seizure and latent phases [42]. In the latent period in these animals, IL-1 receptor antagonist synthesis is delayed or reduced. The relative dearth of this anti-inflammatory peptide results in unchecked IL-1 β , and likely contributes to the IL-1 β rise at the start of chronic seizures, as well as a potentially detrimental inflammatory cascade. Conversely, IL-1 β antagonism stops or delays progression to stage 5 (generalized) Racine scale seizures in rats, and increases after discharge threshold, further lend-

ing credence to an epileptogenic role for this cytokine [38]. In addition, IL-1 β is released from microglia and astrocytes within the epileptic focus, triggering more widespread inflammation and blood–brain barrier compromise shortly after seizures [43]. In a unilateral mesial temporal kainate injection mouse model of epilepsy, differential activation of IL-1 β and IL-1 receptor antagonist was observed when comparing ipsilateral (side of later seizure onset) and contralateral mesial temporal lobes, suggesting this is a localized cytokine response specific to the site of epileptogenesis [44]. The variables affecting the degree of inflammatory activation in the latent period have not been identified; genetic background and the nature and severity of the insult are possible candidates.

mTOR pathway

mTOR has been linked with a handful of epilepsy syndromes, raising the possibility that second messengers in this signaling cascade may be useful biomarkers of epilepsy. At this point, that possibility remains unrealized; none of the mTOR second messengers has been studied peripherally (e.g. in blood, CSF or urine) in the context of epilepsy. However, the myriad actions of this pathway – as well as its attractiveness as a therapeutic target in some epilepsies – make it a logical target for identifying a circulating epilepsy biomarker.

Constitutive activation of the mTOR pathway is seen in conditions with epileptogenic lesions, including tuberous sclerosis and focal cortical dysplasia; less definitive associations have been observed in nongenetic cases of infantile spasms and TLE [45]. Downstream targets of mTORC1 include: VEGF, which is important for angiogenesis; S6, a ribosomal kinase believed to play a role in cell growth and proliferation; and EIF4E, a protein that binds mRNA and facilitates its delivery to ribosomes. Galanopoulou *et al.* reviewed the role of mTOR in specific epilepsies in detail [45]. In the case of tuberous sclerosis, loss-of-function mutations in the mTOR inhibitors TSC1 or TSC2 result in the growth of tubers and subependymal giant cell astrocytomas, which are associated with epileptic seizures and cognitive impairment. Type II cortical dysplasia demonstrates pathological hallmarks also seen in TS, including the enlarged dysmorphic cells of varying neuronal or glial lineage known as balloon cells. These cells, in both tubers and FCDs, demonstrate aberrant glutamate receptor expression patterns, likely resulting in hyperexcitability and, ultimately, seizures [46]. As a result, mTOR inhibitors are being studied actively for their therapeutic potential in preventing seizures – or possibly epileptogenesis itself.

Inhibitors of the mTOR pathway have shown some promise in clinical trials. Everolimus was recently

found to reduce the size of subependymal giant cell astrocytomas, although the effects on seizure burden (a secondary outcome) were inconclusive [47]. However, in a small subsequent prospective open-label trial, 12 out of 20 children with TS and refractory epilepsy experienced a greater than 50% reduction in seizure frequency after treatment with everolimus [48].

The importance of the mTOR pathway to other epilepsies is not yet known. If pathogenic in other, more common phenotypes (i.e., TLE), then measuring endogenous mTOR inhibitors or downstream targets may serve as an epilepsy biomarker. Such findings might suggest a larger therapeutic role for existing or future pharmaceutical mTOR inhibitors, which work through novel mechanisms not employed by existing traditional anticonvulsants. For now, this remains an aspirational goal, as measurable peripheral mTOR pathway proxies have not been successfully identified. However, the mTOR pathway appears important in other diseases – including breast, neuroendocrine and other cancers – ensuring that researchers in multiple disciplines will be searching for biomarkers relevant to this signaling cascade. Epilepsy investigators may learn from these efforts, and contribute their own discoveries as well.

Cyclooxygenase-2

Cyclooxygenase (COX)-2 is another potential inflammatory mediator, although data to this point are mixed. COX-2 expression is increased in neurons after such insults as traumatic brain injury and status epilepticus in animal models, and prevents CA1 pyramidal and interneuron excitotoxicity, suggesting a neuroprotective role [49]. However, COX-2 activation may lead to delayed neurodegeneration after SE, likely due to secondary inflammatory mechanisms. For example, in a COX-2 conditional knockout mouse treated with pilocarpine, delayed mortality and cognitive performance (Morris water maze) were significantly improved after SE, compared with controls [50]. COX-2 therefore appears to play a dual role in neuroprotection and neurotoxicity, and in different time points during the process of epileptogenesis [40]. Elucidating the temporal course of COX-2's shifting role, as well as determining if blood or CSF measurements of this molecule are feasible, reliable and indicative of this role, will be important to determining its viability as an epilepsy biomarker.

IL-6

IL-6 levels are elevated in resected tissue within malformation of cortical development (MCD)/FCDs in TS patients, suggesting this epileptogenic pathology may trigger a robust inflammatory response [51]. Serum IL-6 levels were also significantly elevated in patients with intellectual disability who had a very high frequency of

seizures [52]. In a mouse model of maternal immune activation, kindling progressed more rapidly in offspring mice whose mothers were sham-treated. Furthermore, administration of antibodies to IL-6 abolished this effect [53]. However, a recent study found significantly increased IL-6 levels with pilocarpine-induced SE in rats, but not after electrically kindled status [54]. The authors cited prior findings that IL-6 may rise during intense muscle activity (i.e., convulsions), making its validity as an epilepsy biomarker unclear.

In summary, inflammatory markers are appealing as potential biomarkers, given the large number of studies demonstrating statistically significant changes in expression in people with epilepsy. However, circulating inflammatory mediators have short half-lives, may not reflect regional brain differences, and are released nonspecifically in response to physiological stresses, including seizures themselves. Anticonvulsant medications may also affect levels of these inflammatory mediators, further confounding their use as biomarkers. More study will be needed to determine the specificity of inflammatory markers as markers of seizures, epileptogenesis, both or neither.

White blood cells & cell adhesion molecules

Given the growing recognition that inflammation occurs in epilepsy, it is not surprising that leukocytes and lymphocytes are now postulated to play a role in epileptogenesis. Lymphocyte expression patterns and distribution appear altered in the rat pilocarpine epilepsy model. Specifically, increased CD3⁺ T cells are seen in the spleen, and increased percentages of NK 1.1 and CD8⁺ cells are seen peripherally. Interestingly, brain inflammatory cells are unchanged. Knockouts of perforin (a downstream NK/cytotoxic lymphocyte (CTL) effector) or splenectomy reduced seizure mortality and status onset in rats [55]. In humans, circulating levels of CD8⁺ and NK cells are elevated postictally [56,57]. Such findings suggest that analyses of T-cell activation peripherally may provide information about seizure activity, and possibly epileptogenesis.

There is increasing evidence from animal models that cerebrovascular endothelial cells respond to SE by upregulating different cell adhesion molecules. In turn, this triggers leukocyte infiltration and may facilitate the blood-brain barrier compromise and inflammatory response, described elsewhere in the present article [58]. In a kainate mouse model of epilepsy, increased leukocyte rolling and arrest in brain vasculature was found, mediated by PSGL-1 (P-selectin) and leukocyte integrins VLA-4 and LFA-1. Treatment with monoclonal antibodies to either PSGL-1 or the leukocyte integrins slowed the development of chronic seizures after SE in this model [59]. Other cell adhesion molecules (VCAM,

ICAM and E-selectin) are also upregulated in the kainate, pilocarpine and bicuculline models of epilepsy [59–61]. Expression of the leukocyte chemokine receptor CCR5 also increases after kainate seizure induction, as does its ligands (CCL3, CCL5, MIP-1 α and RANTES) on cerebrovascular endothelium. Pretreatment with CCR5 RNAi reduced CCR5 expression, and reduced seizures in these animals [62].

Finally, there are tantalizing data regarding at least one cell adhesion molecule in humans. The ratio of thymus and activation-regulated chemokine (TARC; an inflammatory mediator) to soluble ICAM5 (sICAM5 or telencephalin, an anti-inflammatory molecule from the brain also found in blood) was measured in the plasma of a cohort of ten focal epilepsy patients. sICAM5 was significantly decreased in plasma, while TARC was elevated when compared with controls. The TARC/sICAM5 ratio was significantly elevated (13x, $p = 0.034$) as well [63]. While it is possible the finding is indicative of recent seizure activity alone (rather than a true epileptogenic process), the results are encouraging given the accessibility of the marker (plasma), the small error values reported and the use of a robust ratio that takes two candidate biomarkers into account simultaneously. Such an approach – in which a panel of mediators is assessed rather than a single protein – may represent a promising avenue for epilepsy biomarker investigators.

Pediatric syndromes

Febrile status epilepticus

The importance of febrile status epilepticus (FSE) in those who later develop TLE remains unclear. A large prospective longitudinal study of children with febrile status epilepticus has the potential to clarify this association while also identifying biomarkers of epileptogenesis. The aforementioned FEBSTAT study has enrolled roughly 200 children (at five hospitals) who experienced prolonged febrile seizures. Of the 136 enrolled participants who had nontraumatic lumbar punctures, 135 had seven or fewer leukocytes per mm³ in their CSF, and only three had a protein concentration >60 mg/dl [64]. These findings indicate the difficulty of finding evidence of inflammation or circulating biomarkers in CSF, even from patients recently experiencing a form of status epilepticus. FSE is also associated with the release of inflammatory cytokines, principally IL- β . Efforts to measure IL- β in CSF of children with FSE have yielded mixed results, although elevated levels of this cytokine have indicated increased risk of progression to epilepsy in rodent models. IL- β expression also increases significantly when leukocytes from children with FSE are stimulated with a Toll-like receptor agonist, when compared with con-

trol subjects' leukocytes [65]. It therefore appears that additional study of the inflammatory sequelae of FSE is required to determine its consequences for epileptogenesis. IL- β in particular may emerge as a viable biomarker of epileptogenesis from FEBSTAT or other studies of FSE.

The S100B protein is an acute phase reactant that has been associated with brain injury in a number of different clinical settings, including traumatic brain injury, encephalopathy secondary to sepsis, and ischemic stroke [66]. As such, S100B may lack the specificity needed of a biomarker of epileptogenesis in cases with a symptomatic proximate cause. Two recent studies of children with FSE have failed to support the use of S100B as a marker of seizure-specific stress, much less a biomarker of epileptogenesis. Mikkonen *et al.* found no significant difference in serum levels of S100B between 103 children with FSE and 33 controls with acute infection in the absence of seizures [66]. Likewise, Atici and colleagues found that the serum levels of S100B were no different for children with simple febrile seizures when compared with febrile and afebrile control subjects without seizures. These results have been important in ruling S100B out as a potential biomarker in children with FSE. However, S100B may be useful in other forms of epilepsy; a recent study demonstrated significantly higher serum levels of S100B in 34 TLE patients when compared with 34 sex- and age-matched controls [67].

Glut1 deficiency syndrome

Glut1 deficiency syndrome (Glut-1 DS) is an under-recognized cause of epilepsy in children. Glut1 is a protein that transports D-glucose into the brain via endothelial cells; the protein is also expressed in human erythrocytes. Glut-1 DS is characterized by epilepsy beginning in infancy, global developmental delay and movement disorders. Measuring glucose in CSF typically makes the diagnosis. However, the degree of hypoglycorrachia necessary to make a diagnosis is uncertain. In addition, a lumbar puncture is an invasive (although routine) procedure, and a definitive reference range for normal CSF glucose has not been established. As a result, Yang and colleagues have advanced the use of the erythrocyte-based 3-O-methyl-D-glucose (3-OMG) assay as an alternate method of diagnosis [68]. Uptake was reduced in 74 out of 109 participants with suspected Glut-1 DS, as defined by clinical appearance and hypoglycorrachia. 70 out of the 74 with reduced 3-OMG uptake were subsequently found to have a pathogenic mutation in the *GLUT1* gene. When the criteria for 'low uptake' were adjusted to 74% of normal, assay sensitivity increased to 99% and specificity to 100%. To validate the 3-OMG uptake

assay as an epilepsy biomarker, investigators will need to determine whether differential uptake levels correspond with seizure frequency, severity, pharmacoresistance or related outcomes. Nonetheless, the compelling results to date suggest that the 3-OMG uptake assay is a potential epilepsy biomarker, and is already becoming a viable biomarker of a specific syndrome in which epilepsy plays a prominent role.

Pyridoxine-dependent epilepsy

Pyridoxine-dependent epilepsy (PDE) is an inborn error of metabolism with autosomal recessive inheritance. Clinically, the disorder presents with refractory epilepsy in the neonatal period, as well as encephalopathy and, at times, gastrointestinal and respiratory dysfunction. High-dose pyridoxine is the only effective treatment, and empiric treatment (based on clinical suspicion) has long been the mainstay of clinical care for these patients. The diagnosis was made previously by brief pyridoxine withdrawal, with seizure recurrence taken as proof of the diagnosis [69]. However, such an approach places the infant at risk of additional seizure-associated morbidity. Furthermore, the diagnosis may remain unclear in cases with a partial improvement (rather than complete resolution) in seizure frequency. EEG changes following pyridoxine administration are also often ambiguous and nondiagnostic [70]. Since the turn of this century, biomarkers have been developed for this condition, aiding more rapid and safer diagnoses. In 2000, increased levels of pipercolic acid in blood and CSF were found to be associated with PDE [71]; elevated levels of α -aminoadipic semialdehyde in urine and serum were linked with the condition thereafter [72]. In 2006, mutations in the *ALDH7A1* gene (which codes for the protein antiquitin) were found to be associated with PDE cases [73]. Antiquitin is an aldehyde dehydrogenase necessary for lysine degradation; pipercolic acid and aminoadipic semialdehyde are intermediates that accumulate when antiquitin is deficient or absent [74]. Furthermore, the application of these biomarkers in combination led to the recognition that PDE and folinic acid-responsive seizures are in fact the same disorder. As a result, adding folinic acid supplementation to pyridoxine may improve treatment efficacy [75]. Recent work has characterized the spectrum of phenotypes associated with antiquitin deficiency, further underscoring the utility of a biomarker-based approach to test clinical hypotheses [76]. As with the 3-OMG assay in Glut-1 DS, the next step is determining which *ALDH7A1* mutations (or to what degree of antiquitin deficiency) confers differential seizure outcomes for affected patients. Doing so will further PDE as a model for the advances in diagnosis and treatment that are possible following the identification of easily interpretable biomarkers.

Treatment-related biomarkers

While this paper has largely focused on biomarkers of epileptogenesis, there is an urgent need for pharmacogenomic biomarkers that will predict treatment response, treatment resistance or the development of adverse effects of anticonvulsants. At present, anticonvulsant choice is based on broad guidelines derived from postulated seizure type, as well as side-effect profiles that rely on generalizations about different patient populations. The development of treatment-related biomarkers may allow for more precise anticonvulsant selection, and a more efficient progression to effective treatments with minimal adverse effects.

Human leukocyte antigen alleles

The human leukocyte antigen (HLA)-B*1502 allele variant represents the first definitive circulating biomarker to be validated in epilepsy. The allele confers 92% sensitivity and 98% specificity in predicting development of Stevens–Johnson syndrome (SJS) in response to carbamazepine in high-risk populations: Chinese, Thai and Malaysians [77,78]. SJS is a severe immune-mediated drug hypersensitivity reaction that causes a rash over the skin and mucous membranes, and is further characterized by fever, blistering and epidermal necrosis and sloughing. Prevalence of HLA-B*1502 in high-risk populations exceeds 10%, and clinicians are advised to test at-risk patients before initiating carbamazepine treatment. Widespread testing for this biomarker in populations with high pretest probability has the potential to significantly reduce rates of SJS in Chinese, Thai or Malaysian patients. Smaller studies have associated HLA-B*1502 with increased risk of SJS in Asian populations taking other aromatic anticonvulsants (oxcarbazepine, phenytoin and lamotrigine) [79–81]. HLA-B*1502 has also been implicated in milder cutaneous reactions to oxcarbazepine in a Han Chinese population [82].

More recently, a genome-wide association study of participants with Northern European ancestry identified an allele that confers risk of carbamazepine hypersensitivity reactions. Sixty five participants were compared with nearly 4000 controls. Presence of the HLA-A*3101 allele was significantly associated with the hypersensitivity syndrome. Allele presence raised the risk from 5 to 26%. The findings of increased risk were later confirmed with genotyping [83]. Glauser extensively reviewed other probable (but not validated) treatment response biomarkers in 2011 [84].

ABCB1, ABCC2 & drug resistance

Given that roughly 30% of patients with epilepsy are refractory to medication, it has been speculated that differential activity of ATP-binding drug efflux pro-

teins may be observed within the epilepsy population. This hypothesis posits that increased expression of these proteins results in reduced brain anticonvulsant levels and, in turn, treatment resistance. Two different transporters have been implicated in anticonvulsant resistance: ABCB1 and ABCC2. ABCB1 expression was at one time thought at least partially responsible for 30–40% of treatment failures in epilepsy [85]. However it is unclear to what degree multidrug resistance in epilepsy is due to polymorphisms in the *ABCB1* or *ABCC2* gene, different levels of P-gp expression or post-translational modification [86]. Encouraging early results regarding ABCB1 have not been replicated in larger more recent studies, calling its relevance in epilepsy into doubt [84]. More recent studies of ABCC2 have also yielded mixed results. Ufer and colleagues genotyped roughly 200 consecutive Caucasian children with epilepsy, searching for 3 polymorphisms thought to be associated with anticonvulsant pharmacoresistance [87]. Two variants were significantly associated with anticonvulsant response (the -24C>T polymorphism in the 5'-untranslated region; and for oxcarbazepine and carbamazepine specifically, 1249G>A), while two other variants lacked any association. However, Hilger and colleagues studied genotypes of 381 caucasians with epilepsy, stratified by degrees of pharmacoresistance, and compared with 247 healthy controls [88]. None of the alleles, haplotypes, or genotypes studied – including 1249G>A – demonstrated any significant degree of association with any of the categories of pharmacoresistance studied. The populations were not completely comparable; the latter study enrolled adults rather than children. However, the authors point out that such discrepancies are not uncommon; gene polymorphism associations may actually be due to many different rare variants that share a common SNP, rather than the single polymorphism that is initially identified. As such, replication studies may fail to support the hypotheses initially proposed. The end result is that a biomarker for anticonvulsant pharmacoresistance remains elusive.

Future perspective

Numerous hurdles lie ahead for the development of epilepsy biomarkers that are detectable in blood, urine or CSF. First, potential biomarkers may be the result of seizures, rather than a cause. It can be difficult to determine whether a candidate biomarker is pathogenic, or even an accurate surrogate of the underlying process of epileptogenesis. The presence of a candidate biomarker may represent a snapshot in time rather than a surrogate for an overarching disease process. Second, the process of epileptogenesis, particularly in focal epilepsy, may be localized to a single brain region, or even

a single microscopic zone that may not communicate with the periphery. This finding is further complicated by the recent discovery of somatic mutation mosaicism in neurodevelopmental disorders, suggesting regional genetic differences that might cause focal dysfunction [88]. Therefore, identifying circulating surrogates for a small and isolated epileptic focus within the brain may be challenging. Third, biomarkers within the CNS may be sequestered in CSF by the blood–brain barrier. Therefore, obtaining CSF, perhaps in serial fashion, may be needed to detect meaningful epilepsy biomarkers. However, lumbar punctures are generally not part of standard clinical evaluation of epilepsy, and studies requiring frequent CSF collection may not be feasible from a recruitment perspective.

Identifying the correct control group for use in biomarker validation is another unsolved challenge. One might envision three types of control groups, each with unique merits. First, a control group could be composed of people who have never had a seizure. Such a control group might be most useful when studying biomarkers that indicate neuronal hyperexcitability, or other biomarkers that might predict recurrence after a patient presents with their first seizure. The counterargument to using such a control group lies in the fact that epilepsy is a clinical diagnosis. The clinical value of an epilepsy biomarker lies in predicting disease course, not indicating presence of the disease itself. Therefore, a biomarker that distinguishes between a control group without epilepsy and those with seizures may not add sufficiently to the clinical evaluation to prove useful. A second type of control group might consist of patients with neurological disease other than epilepsy. Such a group would help distinguish nonspecific (i.e., inflammatory) epiphenomena from biomarkers that actually contribute to epileptogenesis. The final type of control group would include participants with well-controlled epilepsy, who would be used to evaluate biomarkers of epilepsy pharmacoresistance. In such studies, experimental groups should be composed of participants with similar epilepsy types, ideally distinguished solely by presence or absence of the biomarker of interest being studied.

Given that the process of epileptogenesis may unfold over months, years or even decades, it is difficult to envision a control group robust enough to account for the myriad variables that might impact disease progression over time. Perhaps the best way to solve this conundrum is to phenotype epilepsy patients in as great detail as possible, and restrict biomarker studies to narrow phenotypes. For example, to determine the significance of autoantibodies in focal epilepsy pharmacoresistance, the experimental group should be a group of antibody-positive partici-

pants with similar types of seizures, similar localization (e.g., mesial temporal lobe onset), and similar age of onset, epilepsy duration and imaging findings; the control group would also have epilepsy with extremely similar clinical and demographic characteristics, distinguished only by the absence of the antibody in question.

However in spite of these hurdles, we now have some models for the development of circulating epilepsy biomarkers. The discoveries of HLA-B*1502 in carbamazepine-associated SJS, the pathophysiology of anti-uitin deficiency in PDE, evidence of HPV infection in epileptogenic lesions, and the range of phenotypes caused by antibodies to neuronal antigens are promising and provide examples for future investigators. Additional aspirational biomarkers are described in **Table 1**. The combination of precise epilepsy phenotyping, prospective longitudinal study, and advanced cellular and molecular biology techniques – with particular focus on genetics and immunology – will likely yield a handful of biomarkers that will assist in the prognostica-

tion of epilepsy progression and pharmacoresistance. Discovery of the relevant pathways may also pave the way for new metabolic and immunomodulatory treatments that might broaden the armamentarium of the practicing neurologist.

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Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Background

- A paucity of biomarkers contributes to the diagnostic uncertainty in determining risk of epilepsy after a first seizure. Few biomarkers exist to determine disease progression or treatment response.
- Despite this fact, there is progress toward the development of circulating biomarkers in epilepsy – detectable in plasma, urine or cerebrospinal fluid.

Antibodies to neuronal antigens

- Autoantibodies to the voltage-gated potassium channel complex, NMDA receptor, GABA_B receptor and GAD have been implicated in numerous neurological syndromes in recent years, and may contribute to focal epilepsy as well. Circulating levels of these antibodies have the potential to serve as epilepsy biomarkers.

Infectious markers

- Infection with human papillomavirus 16 appears to be associated with focal cortical dysplasia. A less definitive association has been documented between human herpes virus-6 or -7 and mesial temporal sclerosis.

Inflammatory markers

- Several inflammatory second-messenger pathways appear important to epileptogenesis in animal models, including the toll-like receptor pathway, and the mTOR pathway. Cell-adhesion molecule expression in cerebrovascular endothelium and altered patterns of lymphocyte expression and distribution may also serve as immune or inflammatory biomarkers of epileptogenesis.

Pediatric syndromes

- Recent and ongoing advances in our understanding of pediatric epilepsy syndromes may lead to the development of new circulating biomarkers. Glut-1-deficiency syndrome and pyridoxine-dependent epilepsy provide encouraging models.

Treatment-related biomarkers

- HLA-B*1502, an allele that confers risks of Stevens–Johnson syndrome in high-risk populations treated with the anticonvulsant carbamazepine, is one of the few treatment-related biomarkers that exists for epilepsy today.

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