Epilepsia Open® The Open Access Journal of the International League Against Epilepsy

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CRITICAL REVIEW AND INVITED COMMENTARY

Discovery and validation of blood microRNAs as molecular biomarkers of epilepsy: Ways to close current knowledge gaps

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Epilepsia Open, 3(4):427–436, 2018
doi: 10.1002/epi4.12275

Summary

There is a major unmet need for biomarkers of epilepsy. Biofluids such as blood offer a potential source of molecular biomarkers. MicroRNAs (miRNAs) fulfill several key requirements for a blood-based molecular biomarker being enriched in the brain and dysregulated in epileptic brain tissue, and manipulation of miRNAs can have seizure-suppressive and disease-modifying effects in preclinical models. Biofluid miRNAs also possess qualities that are favorable for translation, including stability and easy and cheap assay techniques. Herein we review findings from both clinical and animal models. Studies have featured a mix of unbiased profiling and hypothesis-driven efforts. Blood levels of several brain-enriched miRNAs are altered in patients with epilepsy and in patients with drug-resistant compared to drug-responsive seizures, with encouraging receiver-operating characteristic (ROC) curve analyses, both in terms of sensitivity and specificity. Both focal and generalized epilepsies are associated with altered blood miRNA profiles, and associations with clinical parameters including seizure burden have been reported. Results remain preliminary, however. There is a need for continued discovery and validation efforts that include multicenter studies and attention to study design, sample collection methodology, and quality control. Studies focused on epileptogenesis as well as associations with covariables such as sex, etiology, and timing of sampling remain limited. We identify 10 knowledge gaps and propose experiments to close these. If adequately addressed, biofluid miRNAs may be an important future source of diagnostic biomarkers that could also support forthcoming trials of antiepileptogenesis or disease-modifying therapies.

KEY WORDS: **Noncoding RNA, Hippocampus, Status epilepticus, Biomarker, Epileptogenesis.**

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Epilepsy is a common, chronic neurologic disease that is characterized by recurring seizures, which affects about 65 million people worldwide. The causes of epilepsy are varied, ranging from genetic mutations to acquired forms that follow earlier brain injuries.[1] Seizures are controlled in approximately two-thirds of patients using antiseizure drugs, although these provide only symptomatic relief. A number of priorities exist to improve the lives of patients with epilepsy, including the need to discover and trial antiepileptogenic and disease-modifying therapies.[2,3] However, any future clinical trial of an antiepileptogenic treatment would require identification of at-risk patients.[4] In addition, diagnosis of epilepsy remains principally based on clinical history and examination. Electroencephalography

Accepted September 25, 2018.

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Key Points

- MicroRNAs (miRNAs) offer various promising qualities as molecular biomarkers
- We review all clinical and animal studies published to date on blood miRNAs as biomarkers of epilepsy
- Results are promising, with, in some cases, strong diagnostic value of certain miRNAs in blood
- We identify a set of 10 knowledge gaps that remain and suggest research strategies that will move the field forward

(EEG) recording, brain imaging, and genetic testing provide important supports, but misdiagnosis rates remain unacceptably high.[5,6] The identification of biomarkers of epilepsy and the epileptogenic process would transform the discovery of disease-modifying therapies and the diagnosis of epilepsy in the future.[4]

A biomarker is defined as "a defined characteristic that is measured as an indicator of normal biologic processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions."[7] Several categories and types of biomarkers are recognized. Categories are susceptibility/risk, monitoring, diagnostic, prognostic, predictive, pharmacodynamic/response, and safety biomarkers.[8] The main types of biomarkers of relevance to epilepsy are electrophysiologic (i.e., EEG), imaging, and molecular.[7] The studies reviewed in this article relate primarily to the categories of diagnostic and prognostic biomarkers. Among the different types, molecular biomarkers are particularly attractive because they offer the possibility of rapid, simple, and inexpensive bedside tests where a set of one or more molecules are measured in a convenient biofluid such as blood.[4,7] Several potential molecule classes could be suitable sources of biomarkers, including brain proteins and protein-coding messenger RNAs (mRNAs).[7] Progress has been relatively modest, however, due to the lack of specificity and technical complexity of their assay.[9]

In 2008, a new class of molecule emerged as a potential biomarker: microRNAs (or miRNAs).[10,11] These are small, noncoding RNAs that function to control protein levels in all cells.[12] miRNAs have offered various advantages as biomarkers over other molecules. They have been expressed in tissue-specific patterns, amenable to assay using polymerase chain reaction (PCR)–type kits, and have been relatively stable.[10,13,14] Mechanisms also existed for their intercellular transfer.[15,16] The first animal study to look at the effects of seizures on miRNA levels in blood appeared in 2010,[17] and in 2015, the first clinical studies were published on blood-based miRNAs for the diagnosis of epilepsy.[18,19] There have been more than 20 studies published on this topic to date. Herein, we review the rationale behind their use as biomarkers and the published studies to date, focusing attention on those that include statistical assessment of biomarker potential. Overall, the findings suggest that blood-based miRNAs could provide suitable biomarkers of epilepsy and the epileptogenic process. However, key gaps and challenges remain. Therefore, we finish by identifying 10 remaining questions in this nascent field and propose experiments to answer them.

Why microRNAs as Molecular Biomarkers of Epilepsy?

miRNAs are small noncoding RNAs, the main function of which is to negatively regulate protein levels in cells.[12] They are critical components of the posttranscriptional control of gene expression and impart precision to cellular protein noise.[20] The human genome contains nearly 600 distinct and well-annotated miRNA genes,[21] and many miRNA gene families show strong structural and functional conservation between species.[12] The biogenesis of miRNAs begins with generation of a primary transcript that contains a hairpin loop structure.[22] Two stages of processing occur, beginning in the nucleus and then later in the cytoplasm, resulting in generation of a miRNA duplex structure. One strand of the ~22 nucleotide mature miRNA is selected and uploaded to an Argonaute (AGO) protein, whereas the passenger strand is usually degraded.[23] Once AGO-loaded, the so-called RNA-induced silencing complex (RISC) locates and then traffics along the length of potential target mRNAs until it finds a region of sufficient complementarity.[24] Base-pairing occurs between the miRNA and target, often within the 3′ untranslated region (UTR) of the mRNA, resulting in recruitment of additional factors that lead to either degradation of the mRNA or inhibition of translation.[23,25] miRNAs have been demonstrated to be involved in virtually all cellular processes,[26,27] although their effects are strongest on mRNAs with longer 3′UTRs, and low-expressed miRNAs are not thought to exert important biologic effects.[12]

Why might miRNAs be suitable biomarkers of epilepsy? The first factor is their enrichment in the brain.[28] More than half of all known miRNAs are expressed in the brain, with several miRNAs such as miR-124 highly abundant in brain cells but scarcely detectable in other tissues.[29–32] Within the brain, specific cell types express specific miRNAs that are required for the establishment and maintenance of physiologic properties and cell structure. miRNAs have been found enriched or uniquely expressed in excitatory neurons, inhibitory neurons, astrocytes, microglia, and oligodendrocytes.[33,34] Loss of key miRNA biogenesis enzymes results in profound changes to brain structure and function, and thereby in neurodegeneration and the occurrence of recurrent seizures (i.e., epilepsy).[35,36] Loss of individual miRNAs can also be sufficient to produce profound central nervous system (CNS) phenotypes. Loss of miR-9 results in brain development defects,[37] loss of miR-124 produces

neurodegeneration within the hippocampus,[38] and postnatal deletion of miR-128 from dopaminergic neurons results in epilepsy.[39]

Second, the presence of brain-enriched miRNAs in a biofluid such as blood would be highly suggestive of a brain injury or neurologic disease. Unique pathophysiologic mechanisms—differing contributions of neuronal injury, glial activation, and other cellular responses—would be predicted to produce unique patterns of such molecules in biofluids. Various mechanisms exist that could facilitate transfer of miRNAs from brain cells into biofluids. Direct physical injury to the brain parenchyma would result in lytic release of miRNAs into the circulation, after which their detection could be achieved. Studies in models of intracerebral hemorrhage and stroke confirm that various brain-enriched miRNAs enter the circulation within hours of the event.[17] Cerebrospinal fluid contains neurologic disease-specific differences in levels of various miRNAs.[40] In addition, there is growing evidence of controlled release and paracrine signaling that is mediated via exosomes, which occur under both physiologic and pathophysiologic conditions.[15,41] These exosomes may reach the blood, because there is evidence that a population of circulating exosomes has CNS origins.[42,43] Thus, various mechanisms exist by which brain-based miRNAs could end up in the circulation in a disease-specific manner. Therefore, these circulating miRNAs may have diagnostic value.

Third, multiple studies have reported that miRNA expression is altered in brain tissue in experimental and human epilepsy.[44] This includes changes within sclerotic and non-sclerotic hippocampus and in neighboring neocortical structures. Animal models have shown that epileptogenic insults such as status epilepticus cause time-dependent changes in expression of various miRNAs,[45] and demonstrated unique as well as shared miRNA expressional responses in the CA1, CA3, and dentate gyrus subfields of the hippocampus.[46] Some of the same mechanisms of miRNA transfer from brain to biofluids would therefore be expected to reflect any expression changes of miRNAs, particularly those that are very abundant.

Fourth, experimental studies have shown that manipulating miRNAs can have strong effects on seizures, attendant neuropathology, and epileptogenesis. More than a dozen miRNAs have now been targeted in experimental models and have been reported to produce changes to evoked and spontaneous seizures.[47] Although coexpression of clusters of miRNAs targeting common pathways has been shown during epileptogenesis,[45] some miRNAs may even act individually as master regulators of this phenomenon. Inhibition of a brain-enriched miRNA, miR-134, has been shown to alter levels of proteins that control dendritic morphology and transcription, and to potently suppress the occurrence of spontaneous recurrent seizures following status epilepticus in rat and mouse models.[48,49] Selective deletion of miR-128 in dopaminergic neurons produces fatal epilepsy in mice, likely through its control of the extracellular signal–regulated kinase (ERK) pathway.[39] Neuroinflammatory signaling is in part controlled by miR-146a, and overexpression of miR-146a following status epilepticus potently suppresses spontaneous recurrent seizures in mice.[50] In addition, reduced levels of miR-124 have been proposed to promote epileptogenesis through inflammatory and epigenetic targets in models of status epilepticus[51] and traumatic brain injury.[52] Thus, miRNAs also provide direct targets for antiepileptogenesis and disease-modifying therapy development.

Finally, there are biochemical characteristics that support the biomarker potential of miRNAs. Unlike protein coding mRNAs and proteins themselves, miRNAs appear to be stable in biofluids such as blood. This stability has been attributed to their being enclosed in microvesicles such as exosomes,[41] or circulating bound and protected in protein complexes containing AGO.[53] Once plasma or serum is prepared, miRNA levels remain stable despite freeze-thaw cycles and can be extracted and assayed from formalin-fixed tissues,[13,54,55] even if caution should be used when comparing tissue from biopsies with that from autopsies.[56] Nevertheless, various technical issues including extraction technique and preparation and storage methods introduce variability into miRNA assays from biofluids, which was recently reviewed.[57] Once extracted, miRNAs can be measured relatively simply using modified versions of PCR-based assays, although their profiling is now commonly performed using RNA-sequencing protocols adapted for their small size.[58] Various efforts are underway to simplify and speed up their detection further, including direct, nonamplified detection of miRNAs in biofluids.[59–61]

The Emergence of microRNAs as Blood-Based Biomarkers of Epilepsy

Research on miRNAs as biomarkers of epilepsy appeared in 2010. Liu et al.[17] showed that intracerebral hemorrhage, stroke, and status epilepticus in rats produced unique as well as common changes to small numbers of miRNAs in whole blood. This study established the proof-of-concept that brain injuries generate unique patterns of miRNAs in biofluids that could have diagnostic or prognostic value. Several other animal studies have now investigated miRNA changes following status epilepticus during the acute, latent, and chronic epilepsy phases.[46,62,63] (Table 1) The first human studies on blood miRNAs appeared in 2015.[18,19,60] There have since been more than a dozen studies reporting individual or patterns of miRNAs in blood samples from patients with epilepsy.[61,64–75] Table 2 details a subset of these studies in which the authors performed a formal assessment of biomarker potential using receiver-operating characteristic (ROC) curve analysis.

Table 1. Preclinical animal studies on blood miRNA biomarkers of epileptogenesis

Model (SE)	Time point	Biofluid	Study design	Findings	References
Systemic KA in rats	24 h	Whole blood	Profiling only	10 miRNAs upregulated and 21 downregulated by ≥2-fold in blood and brain (none passed FDR)	Liu et al. (2010)[17]
Systemic PILO in rats	24 h	Whole blood	Individual miRNA assays	Increased miR-34a Increased miR-125a Increased miR-22 Decreased miR-21	Hu et al. (2011)[62]
Electrical stimulation in rats	D, W, M	Plasma	Individual miRNA assays	Increased miR-21-5p (W) Increased miR-146a-5p (M) Increased miR-142-5p (D)	Gorter et al. (2014)[46]
Systemic PILO in rats	L, C	Plasma	Profiling followed by validation of miRNAs	Increased miR-9a-3p (L) Decreased miR-598-5p (L) Decreased miR-300-3p (L) Decreased miR-142-3p (C)	Roncon et al. (2015)[63]

C, chronic (epilepsy); D, day; W, week (latency), M, month; L, latency; SE, status epilepticus; FDR, false discovery rate; KA, kainic acid; PILO, pilocarpine.

MicroRNAs as Biomarkers of Epilepsy and Epileptogenesis—Animal Model Findings

In the first published study on blood miRNAs in an epilepsy model and using a criterion of coregulation in the brain and a greater than or equal to twofold change threshold, Liu et al.[17] reported that 10 miRNAs were upregulated in blood samples obtained 24 hours after kainate-induced status epilepticus in rats with 21 miRNAs downregulated. They noted that many were also dysregulated following other known epileptogenic injuries. However, small group sizes meant that none of the reported miRNA changes in blood survived correction for multiple comparisons. Nevertheless, this seminal paper indicated the potential of blood miRNAs as biomarkers of epileptogenic injuries. Shortly after, Hu et al.[62] reported levels of a set of miRNAs in blood collected 24 hours after status epilepticus induced in rats using pilocarpine. They found that brain and blood changes occurred in the same direction, strongly supporting blood sampling reflecting central changes. However, the study did not perfuse animals at the time of brain tissue collection, thereby confounding interpretation of these results.

Two other studies have analyzed blood miRNA levels in animal models of status epilepticus. Both studies included additional time points corresponding to the latent phase after status epilepticus and sampling in chronically epileptic rodents. Gorter et al.[46] found phase-specific changes in plasma levels of 3 miRNAs that were dysregulated in the hippocampus in rats that developed epilepsy. This included changes at early, latent, and chronic phases, with each miRNA showing time-specific changes in the blood. Notably, levels of miR-146a were selectively altered in chronic epilepsy, and recent studies show that supplementation of this mRNA during epileptogenesis has disease-modifying effects in mice.[50] Roncon et al.[63] included the most time points between status epilepticus and chronic epilepsy and assayed different miRNAs including brain-specific miR-9 and miR-598. Unexpectedly, the brain-specific miRNAs showed divergent responses in the model, with miR-9 showing the expected spike in plasma levels shortly after status epilepticus, whereas miR-598 showed an initial drop in plasma levels followed by restoration to normal levels in epileptic animals. These findings provide encouraging support for a complex mechanism by which brain-expressed miRNAs change within biofluids. They also further support miRNAs as being dysregulated in blood during all phases of the epileptogenesis process.

MicroRNAs as Biomarkers of Epilepsy—Human Studies

Table 2 provides summary details on the clinical studies on blood miRNA findings in patients with epilepsy. The

Table 2. Clinical studies on blood miRNA biomarkers of epilepsy

miRNA	Biofluid	Patient type	No. of patients	Validation cohort?	ROC curve	Other findings	References
let-7d-5p	Serum	Mixed	n = 147	Yes	AUC = 0.79	No correlations found with clinical variables including sz frequency	Wang, Yu et al. (2015)[19]
miR-106b-5p					AUC = 0.88		
miR-130a-3p					AUC = 0.78		
miR-146a-5p					AUC = 0.78		
miR-15a-5p					AUC = 0.84		
miR-194-5p					AUC = 0.81		
miR-301a-3p	Serum	Mixed	n = 218	Yes	AUC = 0.89	Serum miR-301a-3p correlated with sz frequency (NHS3 score)	Wang, Tan et al. (2015)[18]
miR-30b-5p					AUC = 0.68		
miR-342-5p					AUC = 0.72		
miR-4446-3p					AUC = 0.70		
miR-194-5p					AUC = 0.74		
miR-106b	Serum	Mixed	n = 90	No	AUC = 0.79	Correlated with seizure severity score	An et al. (2016)[65]
miR-146a					AUC = 0.77		
miR-129-2-3p	Plasma	TLE	n = 25	No	AUC = 0.78		Sun et al. (2016)[67]
miR-134-5p	Plasma	mTLE	n = 65	Yes	AUC = 0.67	Levels differed in drug-responsive and drug-resistant patients but no relation to seizure frequency	Avansini et al. (2017)[72]
miR-323a-5p	Plasma	FCD	n = 30	No	AUC = 0.74	Correlated with duration of epilepsy and seizure frequency	Che et al. (2017)[74]
miR-4521	Serum	FCD	n = 30	No	AUC = 0.72		Wang et al. (2016)[64]
miR-3613-5p	Plasma	mTLE	n = 43	Yes	AUC = 0.84		Yan et al. (2017)[73]
miR-4668-5p	(exosomes)				AUC = 0.79		
miR-8071					AUC = 0.93		
miR-197-5p					AUC = 0.80		

-3p and -5p strands were not specified in some studies.
AUC, area under the curve; FCD, Focal cortical dysplasia; mTLE, mesial temporal lobe epilepsy; ROC, receiver operating characteristic.

studies fall broadly into those that began with an unbiased profiling experiment before moving on to focus on a subset of miRNAs and those that took a hypothesis-driven approach, selecting miRNAs based on known links to epilepsy. Both plasma and serum have been used in studies. An additional discriminating factor is whether studies included an appropriate statistical assessment of biomarker potential (ROC curve analysis). The value of an ROC curve analysis for assessing biomarker potential was recently reviewed.[7] Briefly, ROC curve analysis allows an assessment of the sensitivity and specificity of the measured analyte for distinguishing 2 groups (e.g., patients and controls). Eight studies published to date have included ROC curve assessments of biomarker potential. However, several studies performed the ROC curve analyses on the original samples, which would exaggerate biomarker potential. Thus studies that feature ROC curve analysis on the validation cohort samples provide the more realistic assessment of biomarker potential.

The first human study on miRNAs as diagnostic biomarkers of epilepsy, by Wang et al.,[19] used RNA sequencing to profile miRNAs in serum from patients with epilepsy. The study included a mixed population of patients with different phenotypes and pooled samples for the purposes of profiling. During validation, they reported ROC curve data on 6 miRNAs, and several had promising ROC curve results with areas under the curve (AUCs) above 0.8.[19] Among those validated as diagnostic biomarkers of epilepsy was let-7d-5p, a miRNA enriched in the brain. The miRNA with the best ROC curve results—miR-106b-5p—was one widely expressed outside the brain. Together, the study provided proof-of-principle that blood miRNAs could be useful for the diagnosis of epilepsy. The study did not, however, explore the influence of factors including epilepsy syndrome, etiology, sex, or clinical variables relating to seizure activity. In addition, medication is an obvious confounder that could not be controlled for.

Only one other clinical study has included an unbiased profiling study as the starting point. The Wang group looked at differences between patients with drug-responsive versus drug-resistant epilepsy.[18] As before, they pooled samples and then ran RNA sequencing, and then prioritized hits for individual miRNA validations. They also included a separate patient validation cohort and ran healthy controls at that time. They selected 6 miRNAs for the validation stage and reported ROC curve analyses of the biomarker value to distinguish the 2 patient groups. Results showed AUCs as high as 0.89 for a single miRNA (miR-301a-3p) and that a combination of the miRNAs could reach an AUC of 0.902.[18] Overall, these results provide encouraging support that blood-based miRNAs could support a diagnosis of drug-

resistant epilepsy, although it is uncertain whether the same miRNAs could predict this outcome when patients first present with a seizure. Such a prognostic biomarker would be of great benefit to clinicians.

In addition to profiling studies, several studies have reported hypothesis-driven analysis of miRNA biomarkers in patients with epilepsy. The first such study looked at miR-134-5p, a miRNA enriched in brain tissue that had been reported previously to be upregulated in focal epilepsy and that has been shown to be a target for seizure suppression and antiepileptogenesis.[60] Plasma levels of miR-134 were increased in the majority of samples from patients with epilepsy including generalized as well as focal epilepsies.[60] However, this study did not include an ROC curve analysis. Two other clinical studies have investigated the same miRNA. Wang et al.[71] found that plasma levels of miR-134 were increased in patients, whereas Avansini and colleagues reported lower plasma levels of miR-134 compared to healthy controls.[72] This finding highlights the challenge of interstudy reproducibility.

With few exceptions, the miRNAs listed in Table 2, including miR-146a, have all been proposed as biomarkers for other diseases. This raises the concern of whether they can be used for epilepsy. In the case of miR-146a, its role in the control of inflammation would likely suggest that it is a broad responder to any systemic condition that features immune and inflammatory responses.[76] In addition, the high abundance of this miRNA in healthy plasma could rule it out as a suitable biomarker for epilepsy.[77] In contrast, some studies that included ROC curve analyses identified miRNAs not yet linked to other neurologic diseases as biomarkers. Foremost is the study by Sun et al.,[67] who reported increased miR-129 levels in patients with epilepsy. Recent work shows that this miRNA serves an important role in synaptic plasticity, is overexpressed in the hippocampus of patients with temporal lobe epilepsy, and that targeting the miRNA can potently protect against kainate seizures in mice.[78]

Associations Between Blood microRNA Levels and Clinical Variables

A number of the clinical studies included analysis of associations between blood miRNA levels and clinical variables (see Table 2). In their original study, Wang et al.[19] reported no associations between the blood miRNA levels and clinical variables including seizure frequency, although minimal details were included to appraise the quality of the reporting. In their article looking at miRNAs as drug-resistance biomarkers they found a strong positive association between NHS3 seizure burden scores (National Hospital Seizure Severity Scale 3; formerly Chalfont seizure severity scale) and plasma levels of miR-301a-3p.[18] Wang et al.,[71] in their study focusing specifically on miR-134, reported that plasma levels of miR-134 correlated with seizure burden in patients with severe epilepsy. Because miR-134 is upregulated in brain tissue from patients with epilepsy, this provides evidence that there are direct links between changes in the brain driven by the disease and the blood level of the miRNA. This study also reported that effective therapy using valproic acid reduced plasma levels of miR-134,[71] presumably as a consequence of reducing seizures in the patients, although this was not directly assessed. An et al.[65] found that serum levels of miR-106b and miR-146a both correlated with NHS3 scores based on seizure diaries. Thus, there is encouraging evidence that certain miRNAs in blood reflect seizure burden in patients.

The study by Surges et al.[66] was designed specifically to look for evidence of seizure-induced changes in blood miRNAs in human epilepsy. They serially sampled patients in the epilepsy monitoring unit and analyzed miRNAs in serum. The main finding was a large spike in miRNAs in samples collected 30 minutes after a generalized seizure. They did not find any miRNA changes in samples collected several hours later.[66] The spike in serum levels shortly after the seizure therefore probably reflects systemic factors and release from other tissue sources. Indeed, the list includes a heart-specific miRNA.[66] Another interpretation is that recent seizure activity is probably a confounder in biomarker studies.

Already we can see a number of miRNAs appearing in more than one study. This is encouraging support for a set of reproducible miRNA biomarkers, albeit with the caveat that some of the repeat appearances are due to their inclusion based on preexisting links to epilepsy (e.g., miR-146a and miR-134).

Exosome Analyses Reveal Additional Unique microRNA Populations

Around the same time that miRNAs were first being pursued as blood-based biomarkers of disease, researchers reported that secreted extracellular vesicles called exosomes carried various miRNAs.[15] This suggested a new mechanism of intercellular communication, whereby following engulfment by a recipient cell, the miRNA contents of the exosome would enter the cell and regulate gene expression. Although an attractive theory, there is scepticism as to whether the low copy numbers of miRNAs contained in these vesicles could produce biologically meaningful effects.[12,79] Regardless, if a disease process changed the quantity or composition of secreted exosomes then an analysis of this fraction alone, rather than the total circulating pool of miRNAs in blood, could provide better diagnostic yield. To date, only a single study has been published that looked at miRNA content within exosomes in patients with

epilepsy. Yan et al.[73] profiled exosomal miRNAs from a small set of patients with mesial temporal sclerosis, finding a set of miRNAs that were different from exosomes from healthy controls. Unexpectedly, the majority of the altered miRNA levels were lower in the patient exosomes. They validated a set of 6 miRNAs in a larger cohort and performed ROC curve analyses, with results ranging from AUCs of 0.71 to 0.93. However, several of the miRNAs are not likely to be bona fide miRNAs,[21] and their ultra-low abundance could make reproducing these findings or developing an assay based on these challenging. Nevertheless, this fractionation approach appears to be an important source of epilepsy biomarkers.

Blood microRNAs in Patients with Genetic Epilepsy

Although it makes intuitive sense that acquired and injury-induced epilepsies might carry an attendant blood miRNA profile, would genetic epilepsies produce a miRNA signature in blood? The answer is likely to be yes. Although there have been no studies designed specifically to determine whether blood miRNA profiles differ according to epilepsy syndrome, many patients with generalized epilepsies have a genetic etiology, and studies show that they too have altered blood miRNA profiles (see Table 2). However, to date there has been only a single study to look for blood miRNA levels in a specific population of patients with genetic epilepsy. Trelinska et al.[70] profiled miRNAs in serum from a set of 10 patients with tuberous sclerosis complex (TSC), detecting an average of 136 miRNAs in at least half the samples. Of these, 11 miRNAs were found to be differentially expressed. The list included several miRNAs that are relevant to epilepsy including changes to miR-142, which had also been identified in experimental epileptogenesis.[63] Next, they compared levels of the miRNAs before and after treatment with everolimus. This resulted in normalization of levels of 2 of the miRNAs.[70] This is a significant finding because everolimus may be disease-modifying in these patients. Thus, these findings may be the first evidence that blood-based miRNAs are responsive to disease-modifying therapies in epilepsy. This significantly increases the attractiveness of miRNAs for further development and suggests that they may have utility in future clinical trials of antiepileptogenic and disease-modifying therapies. Results from miRNA profiling planned within the EPISTOP project (www.epistop.eu) may yield further discoveries.

Knowledge Gaps and Experiments to Address Them

The studies reported to date offer encouraging proof-of-concept that a blood-based miRNA or panel of miRNAs could be a useful biomarker of epileptogenesis or epilepsy. However, a number of limitations exist in the studies to date. Here, we identify 10 gaps in our knowledge and propose experiments that can close these and ensure that the field progresses to deliver on the potential of blood-based miRNAs as biomarkers of epilepsy and epileptogenesis.

1. The evidence of blood miRNA biomarkers of epileptogenesis remains scarce. Despite being one of the most obvious applications where blood miRNAs could be useful, we have few data to go on. There have been several profiling studies using status epilepticus as the trigger for epilepsy, all in rats, and none of the studies performed an ROC curve analysis to determine biomarker performance. *Suggestion:* Efforts should continue to identify and validate miRNAs dysregulated during epileptogenesis in preclinical animal models. For these, priority should be given to non–hypothesis-driven (i.e., sequencing) studies.
2. Use experimental therapies to validate miRNA biomarkers of epileptogenesis. Biomarkers for treatment response are important for any clinical trials. There is a number of highly promising candidate molecules in the preclinical pipeline that show potent disease-modifying effects on epilepsy.[80,81] *Suggestion:* Include an experimental disease-modifying therapy in the validation phase.
3. Validating miRNA biomarkers of epileptogenesis in humans. Studies of patients with TSC and certain other groups offer opportunities to discovery miRNAs at presymptomatic stages and follow-up as epilepsy occurs, that is, we can validate blood-based miRNAs as biomarkers of human epileptogenesis. Moreover, there is evidence that early treatment of patients with TSC, for example, using vigabatrin, can permanently reduce epilepsy.[82] That is, have a disease-modifying effect. Thus, we have an opportunity to study miRNA responses to a disease-modifying therapy in a human population. *Suggestion:* Further biofluid collections should be encouraged where there are opportunities to collect samples during human epileptogenesis such as with TSC, severe traumatic brain injury (TBI), and hypoxic–ischemic encephalopathy.
4. We have limited mechanistic understanding of miRNAs as biomarkers of epilepsy. There is no evidence yet that circulating miRNAs in patients with epilepsy actually come from the brain. In addition, the presence of a change in the circulating levels of miRNA after a seizure may be mechanistically unrelated to epilepsy. *Suggestion:* Future experiments should focus on establishing the cellular origins of circulating miRNAs in epilepsy models and patients, for example, by labeling and tracking the release of miRNAs.
5. There is a need for independent validation of findings by other teams. So far, all the studies have been essentially single-center. *Suggestion:* Researchers interested in miRNAs as biomarkers of epilepsy should discuss

collaborative research, sample-sharing, and dual-analysis approaches that would significantly increase confidence in findings. Multicenter collaborative efforts could also generate important discovery and validation cohorts. Implementation of standard guidelines on sample preparation and analysis and common data elements recently developed by the International League Against Epilepsy (ILAE) and other groups could help in this regard.[57,83]

6. Missing control groups. Key control patient groups have been missing from much of the work to date, and there has been insufficient attention to whether miRNA levels differ between epilepsy syndromes or in different phases of the natural history of the disease. *Suggestion:* Future studies should include novel controls such as patients with psychogenic nonepileptic seizures, who represent an important group of patients for which a biomarker of seizures or epilepsy would provide a valuable diagnostic. Validation cohorts should be probed for associations between miRNA profiles and different epilepsy syndromes or endophenotypes (e.g., hippocampal sclerosis).
7. It is unclear whether different etiologies generate different miRNA biomarker profiles. Only one preclinical study has directly compared the miRNA profiles produced by different epileptogenic injuries.[17] This is important to know, as the first clinical trials of antiepileptogenic or disease-modifying therapies would probably include a narrow patient group (e.g., severe TBI). We may need a miRNA panel specific for TBI-induced epilepsy rather than, say, miRNAs discovered in models of status epilepticus. Similarly, only few clinical studies have queried whether structural lesions such as hippocampal sclerosis,[72] or different epilepsy syndromes,[65] influence the miRNA profile.[72] *Suggestion:* Discovery and/or validation phases of epileptogenesis biomarkers should include a range of etiologies beyond TBI such as genetic causes and insult-induced epilepsies (e.g., hypoxia, stroke, infection). Collaboration with teams outside the epilepsy field should help in this regard. Future studies should explore whether blood miRNA profiles can distinguish between patients with different epilepsy types (e.g., temporal lobe epilepsy [TLE] vs genetic generalized epilepsy [GGE].
8. What is the range of values or fold changes for a miRNA biomarker panel to be a clinically useful? Several of the miRNAs reported as differentially expressed show modest changes, often in the two- to threefold range. This may not be sufficient when it comes to the heterogenous clinical setting. *Suggestion:* Prioritization of miRNAs as biomarkers could focus on those showing the largest fold changes or signal-to-noise ratio, or miRNAs that are either present or absent in one or the other group (i.e., a dichotomous rather than continuous variable).
9. Additional factors that could influence biomarker performance. We do not know whether developmental age, sex, or other factors such as recent seizures influence miRNA biomarker profiles, or are significant confounders. Biomarker performance should be restricted to validation cohorts rather than be run on the discovery cohort. Medication (both for epilepsy or other diseases) is a problematic confounder. *Suggestion:* Future miRNA biomarker evaluation methods should interrogate associations with clinical variables. Combining categories of biomarker could increase sensitivity and specificity; for example, blood miRNAs in combination with EEG or imaging biomarker. Measurement of antiseizure medication effects on blood levels of miRNAs could address this confounder.
10. Several technical factors remain to be fully explored. Is serum or plasma the better source of biomarkers for epilepsy? Most studies selected one or the other but did not check later whether the same miRNA measured in the other biofluid preparation performed as well as a biomarker. Does the exosome fraction yield more sensitive and specific miRNA biomarkers? *Suggestion:* Future experiments to directly compare miRNA performance between serum and plasma and focus on the different circulating sources of miRNAs.

In summary, miRNAs show strong promise as blood-based biomarkers of epilepsy but there is now a need for progress in key areas. Foremost, we must increase our focus on discovery of miRNAs dysregulated during the phase of epileptogenesis in a range of experimental models that represent core etiologies relevant to epilepsy. These must then be validated in clinical studies. This is challenging in itself, but select patient groups including patients with TSC offer the means to achieve this in the future. Research should incorporate collaborative, cross-center preclinical and clinical studies to address the issue of reproducibility, whereas individual discovery and hypothesis-driven biomarker research remains vital where a focus on brain-enriched miRNA species may represent the best opportunity to discover specific and sensitive biomarkers. Attention to some of the variables and opportunities could provide important advances in this field in the near future.

Acknowledgments

The authors would like to thank colleagues who contributed to the National Institute of Neurological Disorders and Stroke (NINDS) workshop "Accelerating the development of therapies for anti-epileptogenesis and disease-modification," held on August 6–7, 2018. The authors also thank Mary D. King for support and advice. This publication and some of the research described herein have emanated from research conducted with the financial support of the European Union's "Seventh Framework" Programme (FP7) under Grant Agreement no. 602130 (EpimiRNA) and no. 602102 (EpiTarget). Additional support is from the Medical Research Charities Group and Epilepsy Ireland (2016-9), a research grant from

Science Foundation Ireland (SFI) under Grant Number 16/RC/3948 and co-funded under the European Regional Development Fund and by FutureNeuro industry partners and SFI grants SFI/13/IA/1891, SFI/14/ADV/RC2721, 12/COEN/18, and 12/RC/2272.

Disclosures

None of the authors has any conflicts of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

References

1. Devinsky O, Vezzani A, O'Brien TJ, et al. Epilepsy. *Nat Rev Dis Primers* 2018;4:18024.
2. Simonato M, Brooks-Kayal AR, Engel J Jr, et al. The challenge and promise of anti-epileptic therapy development in animal models. *Lancet Neurol* 2014;13:949–960.
3. Baulac M, de Boer H, Elger C, et al. Epilepsy priorities in Europe: a report of the ILAE-IBE Epilepsy Advocacy Europe Task Force. *Epilepsia* 2015;56:1687–1695.
4. Engel J Jr, Pitkanen A, Loeb JA, et al. Epilepsy biomarkers. *Epilepsia* 2013;54(Suppl 4):61–69.
5. van Donselaar CA, Stroink H, Arts WF, et al. How confident are we of the diagnosis of epilepsy? *Epilepsia* 2006;47(Suppl 1):9–13.
6. Chowdhury FA, Nashef L, Elwes RD. Misdiagnosis in epilepsy: a review and recognition of diagnostic uncertainty. *Eur J Neurol* 2008;15:1034–1042.
7. Pitkanen A, Ekolle Ndode-Ekane X, Lapinlampi N, et al. Epilepsy biomarkers – toward etiology and pathology specificity. *Neurobiol Dis* 2018. https://10.1016/j.nbd.2018.05.007.
8. FDA-Biomarker Working Group. *BEST (Biomarkers, EndpointS, and other Tools) resource*. Silver Spring, MD; Bethesda, MD: Food and Drug Administration; National Institutes of Health; 2016.
9. Hegde M, Lowenstein DH. The search for circulating epilepsy biomarkers. *Biomark Med* 2014;8:413–427.
10. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008;18:997–1006.
11. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA* 2008;105:10513–10518.
12. Bartel DP. Metazoan microRNAs. *Cell* 2018;173:20–51.
13. Siebolts U, Varnholt H, Drebber U, et al. Tissues from routine pathology archives are suitable for microRNA analyses by quantitative PCR. *J Clin Pathol* 2009;62:84–88.
14. Weber JA, Baxter DH, Zhang S, et al. The microRNA spectrum in 12 body fluids. *Clin Chem* 2010;56:1733–1741.
15. Valadi H, Ekstrom K, Bossios A, et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;9:654–659.
16. Kosaka N, Iguchi H, Yoshioka Y, et al. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem* 2010;285:17442–17452.
17. Liu DZ, Tian Y, Ander BP, et al. Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. *J Cereb Blood Flow Metab* 2010;30:92–101.
18. Wang J, Tan L, Tan L, et al. Circulating microRNAs are promising novel biomarkers for drug-resistant epilepsy. *Sci Rep* 2015;5:10201.
19. Wang J, Yu JT, Tan L, et al. Genome-wide circulating microRNA expression profiling indicates biomarkers for epilepsy. *Sci Rep* 2015;5:9522.
20. Schmiedel JM, Klemm SL, Zheng Y, et al. Gene expression. MicroRNA control of protein expression noise. *Science* 2015;348:128–132.
21. Fromm B, Billipp T, Peck LE, et al. A uniform system for the annotation of vertebrate microRNA genes and the evolution of the human microRNAome. *Annu Rev Genet* 2015;49:213–242.
22. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 2005;6:376–385.
23. Meister G. Argonaute proteins: functional insights and emerging roles. *Nat Rev Genet* 2013;14:447–459.
24. Chandradoss SD, Schirle NT, Szczepaniak M, et al. A dynamic search process underlies microRNA targeting. *Cell* 2015;162:96–107.
25. Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 2010;11:597–610.
26. Sood P, Krek A, Zavolan M, et al. Cell-type-specific signatures of microRNAs on target mRNA expression. *Proc Natl Acad Sci USA* 2006;103:2746–2751.
27. Friedman RC, Farh KK, Burge CB, et al. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009;19:92–105.
28. Rao P, Benito E, Fischer A. MicroRNAs as biomarkers for CNS disease. *Front Mol Neurosci* 2013;6:39.
29. Lagos-Quintana M, Rauhut R, Yalcin A, et al. Identification of tissue-specific microRNAs from mouse. *Curr Biol* 2002;12:735–739.
30. Miska EA, Alvarez-Saavedra E, Townsend M, et al. Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biol* 2004;5:R68.
31. Shao NY, Hu HY, Yan Z, et al. Comprehensive survey of human brain microRNA by deep sequencing. *BMC Genom* 2010;11:409.
32. Ludwig N, Leidinger P, Becker K, et al. Distribution of miRNA expression across human tissues. *Nucleic Acids Res* 2016;44:3865–3877.
33. He M, Liu Y, Wang X, et al. Cell-type-based analysis of microRNA profiles in the mouse brain. *Neuron* 2012;73:35–48.
34. Jovicic A, Roshan R, Moisoi N, et al. Comprehensive expression analyses of neural cell-type-specific miRNAs identify new determinants of the specification and maintenance of neuronal phenotypes. *J Neurosci* 2013;33:5127–5137.
35. Hebert SS, Papadopoulou AS, Smith P, et al. Genetic ablation of Dicer in adult forebrain neurons results in abnormal tau hyperphosphorylation and neurodegeneration. *Hum Mol Genet* 2010;19:3959–3969.
36. Fiorenza A, Lopez-Atalaya JP, Rovira V, et al. Blocking miRNA biogenesis in adult forebrain neurons enhances seizure susceptibility, fear memory, and food intake by increasing neuronal responsiveness. *Cereb Cortex* 2016;26:1619–1633.
37. Shibata M, Nakao H, Kiyonari H, et al. MicroRNA-9 regulates neurogenesis in mouse telencephalon by targeting multiple transcription factors. *J Neurosci* 2011;31:3407–3422.
38. Sanuki R, Onishi A, Koike C, et al. miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression. *Nat Neurosci* 2011;14:1125–1134.
39. Tan CL, Plotkin JL, Veno MT, et al. MicroRNA-128 governs neuronal excitability and motor behavior in mice. *Science* 2013;342:1254–1258.
40. Raoof R, Jimenez-Mateos EM, Bauer S, et al. Cerebrospinal fluid microRNAs are potential biomarkers of temporal lobe epilepsy and status epilepticus. *Sci Rep* 2017;7:3328.
41. Gallo A, Tandon M, Alevizos I, et al. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One* 2012;7:e30679.
42. Levy E. Exosomes in the diseased brain: first insights from in vivo studies. *Front Neurosci* 2017;11:142.
43. Perez-Gonzalez R, Gauthier SA, Kumar A, et al. A method for isolation of extracellular vesicles and characterization of exosomes from brain extracellular space. *Methods Mol Biol* 2017;1545:139–151.
44. Henshall DC, Hamer HM, Pasterkamp RJ, et al. MicroRNAs in epilepsy: pathophysiology and clinical utility. *Lancet Neurol* 2016;15:1368–1376.
45. Srivastava PK, Roncon P, Lukasiuk K, et al. Meta-analysis of microRNAs dysregulated in the hippocampal dentate gyrus of animal models of epilepsy. *eNeuro* 2017;4:6.
46. Gorter JA, Iyer A, White I, et al. Hippocampal subregion-specific microRNA expression during epileptogenesis in experimental temporal lobe epilepsy. *Neurobiol Dis* 2014;62:508–520.
47. Henshall DC. Manipulating microRNAs in murine models: targeting the multi-targeting in epilepsy. *Epilepsy Curr* 2017;17:43–47.
48. Jimenez-Mateos EM, Engel T, Merino-Serrais P, et al. Silencing microRNA-134 produces neuroprotective and prolonged seizure-suppressive effects. *Nat Med* 2012;18:1087–1094.

49. Reschke CR, Fernando L, Norwood BA, et al. Potent anti-seizure effects of locked nucleic acid antagomirs targeting miR-134 in multiple mouse and rat models of epilepsy. *Mol Ther* 2017;6:45–56.
50. Iori V, Iyer AM, Ravizza T, et al. Blockade of the IL-1R1/TLR4 pathway mediates disease-modification therapeutic effects in a model of acquired epilepsy. *Neurobiol Dis* 2017;99:12–23.
51. Brennan GP, Dey D, Chen Y, et al. Dual and opposing roles of microRNA-124 in epilepsy are mediated through inflammatory and NRSF-dependent gene networks. *Cell Rep* 2016;14:2402–2412.
52. Vuokila N, Lukasiuk K, Bot AM, et al. miR-124-3p is a chronic regulator of gene expression after brain injury. *Cell Mol Life Sci* 2018;75 (24):4557–4581.
53. Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci USA* 2011;108:5003–5008.
54. McDonald JS, Milosevic D, Reddi HV, et al. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem* 2011;57:833–840.
55. Zucchini S, Marucci G, Paradiso B, et al. Identification of miRNAs differentially expressed in human epilepsy with or without granule cell pathology. *PLoS One* 2014;9:e105521.
56. Roncon P, Zucchini S, Ferracin M, et al. Is autopsy tissue a valid control for epilepsy surgery tissue in microRNA studies? *Epilepsia Open* 2017;2:90–95.
57. van Vliet EA, Puhakka N, Mills JD, et al. Standardization procedure for plasma biomarker analysis in rat models of epileptogenesis: focus on circulating microRNAs. *Epilepsia* 2017;58:2013–2024.
58. Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. *Nat Rev Genet* 2012;13:358–369.
59. Hunt EA, Goulding AM, Deo SK. Direct detection and quantification of microRNAs. *Anal Biochem* 2009;387:1–12.
60. Spain E, Jimenez-Mateos EM, Raoof R, et al. Direct, non-amplified detection of microRNA-134 in plasma from epilepsy patients. *RSC Adv* 2015;5:90071–90078.
61. McArdle H, Jimenez-Mateos EM, Raoof R, et al. "TORNADO" – Theranostic One-Step RNA Detector; microfluidic disc for the direct detection of microRNA-134 in plasma and cerebrospinal fluid. *Sci Rep* 2017;7:1750.
62. Hu K, Zhang C, Long L, et al. Expression profile of microRNAs in rat hippocampus following lithium-pilocarpine-induced status epilepticus. *Neurosci Lett* 2011;488:252–257.
63. Roncon P, Soukupova M, Binaschi A, et al. MicroRNA profiles in hippocampal granule cells and plasma of rats with pilocarpine-induced epilepsy – comparison with human epileptic samples. *Sci Rep* 2015;5:14143.
64. Wang X, Sun Y, Tan Z, et al. Serum microRNA-4521 is a potential biomarker for focal cortical dysplasia with refractory epilepsy. *Neurochem Res* 2016;41:905–912.
65. An N, Zhao W, Liu Y, et al. Elevated serum miR-106b and miR-146a in patients with focal and generalized epilepsy. *Epilepsy Res* 2016;127:311–316.
66. Surges R, Kretschmann A, Abnaof K, et al. Changes in serum miRNAs following generalized convulsive seizures in human mesial temporal lobe epilepsy. *Biochem Biophys Res Commun* 2016;481:13–18.
67. Sun Y, Wang X, Wang Z, et al. Expression of microRNA-129-2-3p and microRNA-935 in plasma and brain tissue of human refractory epilepsy. *Epilepsy Res* 2016;127:276–283.
68. Sun J, Cheng W, Liu L, et al. Identification of serum miRNAs differentially expressed in human epilepsy at seizure onset and post-seizure. *Mol Med Rep* 2016;14:5318–5324.
69. Li Y, Huang C, Feng P, et al. Aberrant expression of miR-153 is associated with overexpression of hypoxia-inducible factor-1alpha in refractory epilepsy. *Sci Rep* 2016;6:32091.
70. Trelinska J, Fendler W, Dachowska I, et al. Abnormal serum microRNA profiles in tuberous sclerosis are normalized during treatment with everolimus: possible clinical implications. *Orphanet J Rare Dis* 2016;11:129.
71. Wang X, Luo Y, Liu S, et al. MicroRNA-134 plasma levels before and after treatment with valproic acid for epilepsy patients. *Oncotarget* 2017;8:72748–72754.
72. Avansini SH, de Sousa Lima BP, Secolin R, et al. MicroRNA hsa-miR-134 is a circulating biomarker for mesial temporal lobe epilepsy. *PLoS One* 2017;12:e0173060.
73. Yan S, Zhang H, Xie W, et al. Altered microRNA profiles in plasma exosomes from mesial temporal lobe epilepsy with hippocampal sclerosis. *Oncotarget* 2017;8:4136–4146.
74. Che N, Zu G, Zhou T, et al. Aberrant expression of miR-323a-5p in patients with refractory epilepsy caused by focal cortical dysplasia. *Genet Test Mol Biomarkers* 2017;21:3–9.
75. Gong GH, An FM, Wang Y, et al. MiR-153 regulates expression of hypoxia-inducible factor-1alpha in refractory epilepsy. *Oncotarget* 2018;9:8542–8547.
76. Rusca N, Monticelli S. MiR-146a in immunity and disease. *Mol Biol Int* 2011;2011:437301.
77. Mooney C, Raoof R, El-Naggar H, et al. High throughput qPCR expression profiling of circulating microRNAs reveals minimal sex- and sample timing-related variation in plasma of healthy volunteers. *PLoS One* 2015;10:e0145316.
78. Rajman M, Metge F, Fiore R, et al. A microRNA-129-5p/Rbfox crosstalk coordinates homeostatic downscaling of excitatory synapses. *EMBO J* 2017;36:1770–1787.
79. Chevillet JR, Kang Q, Ruf IK, et al. Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc Natl Acad Sci USA* 2014;111:14888–14893.
80. Pitkanen A, Lukasiuk K. Mechanisms of epileptogenesis and potential treatment targets. *Lancet Neurol* 2011;10:173–186.
81. Klein P, Dingledine R, Aronica E, et al. Commonalities in epileptogenic processes from different acute brain insults: do they translate? *Epilepsia* 2017;59:37–66.
82. Jozwiak S, Kotulska K, Domanska-Pakiela D, et al. Antiepileptic treatment before the onset of seizures reduces epilepsy severity and risk of mental retardation in infants with tuberous sclerosis complex. *Eur J Paediatr Neurol* 2011;15:424–431.
83. Harte-Hargrove LC, French JA, Pitkanen A, et al. Common data elements for preclinical epilepsy research: standards for data collection and reporting. A TASK3 report of the AES/ILAE Translational Task Force of the ILAE. *Epilepsia* 2017;58(Suppl 4):78–86.