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, blood miRNAs may be an important future source of diagnostic biomarkers that could also support forthcoming trials of antiepileptogenic or disease-modifying therapies.

KEY WORDS: Noncoding RNA, Hippocampus, Status epilepticus, Biomarker, Epileptogenesis.
(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.\(^1,\,^8,\,^19\) 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 **miRNA**s 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, and postnatal deletion of miR-128 from dopaminergic neurons results in epilepsy.

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. Cerebrospinal fluid contains neurologic disease-specific differences in levels of various miRNAs. 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. These exosomes may reach the blood, because there is evidence that a population of circulating exosomes has CNS origins. 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. 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, and demonstrated unique as well as shared miRNA expression responses in the CA1, CA3, and dentate gyrus subfields of the hippocampus. 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. Although coexpression of clusters of miRNAs targeting common pathways has been shown during epileptogenesis, 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. 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. Neuroinflammatory signaling is in part controlled by miR-146a, and overexpression of miR-146a following status epilepticus potently suppresses spontaneous recurrent seizures in mice. In addition, reduced levels of miR-124 have been proposed to promote epileptogenesis through inflammatory and epigenetic targets in models of status epilepticus and traumatic brain injury. 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 miRNAs 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, or circulating bound and protected in protein complexes containing AGO. Once plasma or serum is prepared, miRNA levels remain stable despite freeze-thaw cycles and can be extracted and assayed from formalin-fixed tissues, even if caution should be used when comparing tissue from biopsies with that from autopsies. Nevertheless, various technical issues including extraction technique and preparation and storage methods introduce variability into miRNA assays from biofluids, which was recently reviewed. 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. Various efforts are underway to simplify and speed up their detection further, including direct, nonamplified detection of miRNAs in biofluids.

**The Emergence of microRNAs as Blood-Based Biomarkers of Epilepsy**

Research on miRNAs as biomarkers of epilepsy appeared in 2010. Liu et al. 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. (Table 1) The first human studies on blood miRNAs appeared in 2015. There have since been more than a dozen studies reporting individual or patterns of miRNAs in blood samples from patients with epilepsy. 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.
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. 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. 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. 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. Roncon et al. 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.

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

| Model (SE) | Study design | Time point | Biofluid | Findings |
|------------|--------------|------------|----------|----------|
| Systemic KA in rats | Profiling only | 24 h | Whole blood | 10 miRNAs upregulated and 21 downregulated by ≥2-fold in blood and brain (none passed FDR) |
| Systemic PILO in rats | Individual miRNA assays | 24 h | Whole blood | Increased miR-34a |
| Electrical stimulation in rats | Plasma | D, W, M | Plasma | Increased miR-146a-5p (M), Increased miR-598-5p (L) |
| Systemic PILO in rats | Profiling followed by validation of miRNAs | L, C | Plasma | Decreased miR-9a-3p (L) |

| Model (SE) | Study design | Time point | Biofluid | Findings |
|------------|--------------|------------|----------|----------|
| Systemic KA in rats | Individual miRNA assays | 24 h | Whole blood | Increased miR-125a |
| Systemic PILO in rats | Individual miRNA assays | L, C | Plasma | Decreased miR-21 |
| Electrical stimulation in rats | Plasma | D, W, M | Plasma | Increased miR-21-5p (W), Increased miR-146a-5p (M), Increased miR-598-5p (L) |

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

### 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 | Serum    | Mixed        | n = 90          | No                 | AUC = 0.79 | Correlated with seizure severity score            | An et al. (2016)65      |
| miR-129-2-3p | Plasma   | TLE          | n = 25          | No                 | AUC = 0.78 | Levels differed in drug-responsive and drug-resistant patients but no relation to seizure frequency | Sun et al. (2016)67     |
| miR-134-5p | Plasma   | mTLE         | n = 65          | Yes                | AUC = 0.67 | Correlated with duration of epilepsy and seizure frequency | Avanesini et al. (2017)72 |
| miR-323a-5p | Plasma   | FCD          | n = 30          | No                 | AUC = 0.74 |                                                    | 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) | Serum | FCD | n = 90 | No | AUC = 0.79 |
| miR-8071  | Plasma   | mTLE         | n = 65          | Yes                | AUC = 0.79 |
| miR-197-5p | Plasma   | mTLE         | n = 65          | Yes                | AUC = 0.93 |

-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.
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. Plasma levels of miR-134 were increased in the majority of samples from patients with epilepsy including generalized as well as focal epilepsies. However, this study did not include an ROC curve analysis. Two other clinical studies have investigated the same miRNA. Wang et al. 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. 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. In addition, the high abundance of this miRNA in healthy plasma could rule it out as a suitable biomarker for epilepsy. 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. 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.

**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. 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. Wang et al., 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, presumably as a consequence of reducing seizures in the patients, although this was not directly assessed. An et al. 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. 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. 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. 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. 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. 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. profiles 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, 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. 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. Next, they compared levels of the miRNAs before and after treatment with everolimus. This resulted in normalization of levels of 2 of the miRNAs. 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. **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. **Suggestion:** 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 etologies 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 anti-seizure 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 prepared 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
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Science Foundation Ireland (SFI) under Grant Number 16/R/3948 and co-founded under the European Regional Development Fund and by Future-Neuro industry partners and SFI grants SFI/13/IA/1891, SFI/14/ADV/R2271, 12/COEN/18, and 12/R/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.

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