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Genome-wide microRNA profiling of plasma from three different animal models identifies biomarkers of temporal lobe epilepsy

Gary P. Brennan[a,b,c,*], Sebastian Bauer[d,g,1], Tobias Engel[b,c,1], Eva M. Jimenez-Mateos[e,1], Federico Del Gallo[f,1], Thomas D.M. Hill[b,c], Niamh M.C. Connolly[b], Lara S. Costard[g,h], Valentin Neubert[g,i], Beatrice Salvetti[f], Amaya Sanz-Rodriguez[b,c], Mona Heiland[b,c], Omar Mamad[b,c], Elizabeth Brindley[b], Braxton Norwood[j,k], Aasia Batool[b], Rana Raoof[b], Hany El-Naggar[a], Cristina R. Reschke[b,c], Norman Delanty[c,l,m], Jochen H.M. Prehn[b,c], Paolo Fabene[f], Catherine Mooney[c,n], Felix Rosenow[d,g], David C. Henshall[b,c]

[a] School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland
[b] Department of Physiology and Medical Physics, Royal College of Surgeons Ireland, Dublin D02 YN77, Ireland
[c] FutureNeuro SFI Research Center, Royal College of Surgeons Ireland, Dublin D02 YN77, Ireland
[d] Epilepsy Center Frankfurt Rhine-Main, Department of Neurology, University Hospital Frankfurt and Center for Personalized Translational Epilepsy Research (CePTER), Goethe University, Frankfurt, Germany
[e] Discipline of Physiology, School of Medicine, Trinity College Dublin, Dublin, Ireland
[f] Department of Neurosciences, Biomedicine, and Movement Sciences, University of Verona, Verona, Italy
[g] Department of Neurology, Phillips University, Marburg, Germany
[h] Department of Regenerative Medicine, Royal College of Surgeons Ireland, Dublin D02 YN77, Ireland
[i] Oscar-Langendorff Institute of Physiology, Rostock University Medical Center, Germany
[j] Expesicor Inc, Kalispell, MT, USA
[k] FYR Diagnostics, Missoula, MT, USA
[l] Department of Neurology, Beaumont Hospital, Dublin, Ireland
[m] Department of Molecular and Cellular Therapeutics, Royal College of Surgeons Ireland, Dublin D02 YN77, Ireland
[n] School of Computer Science, University College Dublin, Ireland

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ABSTRACT

Epilepsy diagnosis is complex, requires a team of specialists and relies on in-depth patient and family history, MRI-imaging and EEG monitoring. There is therefore an unmet clinical need for a non-invasive, molecular-based, biomarker to either predict the development of epilepsy or diagnose a patient with epilepsy who may not have had a witnessed seizure. Recent studies have demonstrated a role for microRNAs in the pathogenesis of epilepsy. MicroRNAs are short non-coding RNA molecules which negatively regulate gene expression, exerting profound influence on target pathways and cellular processes. The presence of microRNAs in bio-fluids, ease of detection, resistance to degradation and functional role in epilepsy render them excellent candidate biomarkers.

Here we performed the first multi-model, genome-wide profiling of plasma microRNAs during epileptogenesis and in chronic temporal lobe epilepsy animals. From video-EEG monitored rats and mice we serially sampled blood samples and identified a set of dysregulated microRNAs comprising increased miR-93-5p, miR-142-5p, miR-182-5p, miR-199a-3p and decreased miR-574-3p during one or both phases. Validation studies found miR-93-5p, miR-199a-3p and miR-574-3p were also dysregulated in plasma from patients with intractable temporal lobe epilepsy. Treatment of mice with common anti-epileptic drugs did not alter the expression levels of any of the five miRNAs identified, however administration of an anti-epileptogenic microRNA treatment prevented dysregulation of several of these miRNAs. The miRNAs were detected within the Argonuate2-RISC complex from

* Corresponding author at: School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland.
E-mail address: gary.brennan@ucd.ie (G.P. Brennan).
[1] These authors contributed equally to the study.

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both neurons and microglia indicating these miRNA biomarker candidates can likely be traced back to specific brain cell types.

The current studies identify additional circulating microRNA biomarkers of experimental and human epilepsy which may support diagnosis of temporal lobe epilepsy via a quick, cost-effective rapid molecular-based test.

1. Introduction

Temporal lobe epilepsy (TLE) is the most common form of drug resistant epilepsy in adults. TLE is characterised by focal seizures that originate from structures such as the hippocampus. Diagnosis of TLE is complex and relies on a combination of clinical examination, patient history, electroencephalographic (EEG) recordings and brain imaging which require a range of technical expertise and are resource intensive (Amin and Benbadis, 2019; Galovic et al., 2019; Sidhu et al., 2018). Misdiagnosis, or delay in diagnosis is common (Amin and Benbadis, 2019; Mathias and Bensalem-Owen, 2019). This includes patients who exhibit non-epileptic attack disorder (NEAD or psychogenic non-epileptic seizures) which can result in patients being treated with ineffective anti-seizure drugs instead of appropriate psychiatric care (Dickson et al., 2017; Goldstein et al., 2019). It is also not yet possible to identify patients who are at risk of developing TLE following a potential epilepsy-inciting event such as traumatic brain injury (TBI), stroke or status epilepticus (SE) (Engel Jr. and Pitkanen, 2020; Garner et al., 2019; Klein and Tyrlikova, 2019; Loscher, 2020). As such, there is a major unmet need for biomarkers of epilepsy which might aid clinicians with diagnosis, prognosis and inform treatment decisions. This is particularly important in resource-limited countries (Caraballo and Fejerman, 2015; Engel Jr. et al., 2013).

Circulating molecule(s) in biofluids such as blood, if mechanistically linked to pathomechanisms of epilepsy, offer a minimally invasive, simple and cheap source of potential biomarker. Previous efforts to identify circulating biomarkers of epileptogenesis or chronic epilepsy have focussed on proteins in peripheral blood such as S100β and pro/anti-inflammatory cytokines and related molecules (Choy et al., 2014; Lu et al., 2010; Ravizza et al., 2018; Walker et al., 2016). While cytokines and related markers may correlate with epileptogenicity and seizures, they probably lack sufficient specificity to distinguish between epilepsy and other neurological or systemic conditions in which inflammation is a pathomechanism (Paudel et al., 2018). Protein coding transcripts offer potential specificity, as the brain expresses unique RNA species, but their detection and stability presents a challenge towards their utility as informative biomarkers. In contrast, small noncoding RNAs offer tissue and cell type-specificity, stable detection in biofluids and mechanistic links to epilepsy development and seizure susceptibility (Hogg et al., 2019; Raoof et al., 2018; Raoof et al., 2017). MicroRNA (miRNA) are the leading class in this regard, being a conserved class of small noncoding RNA that negatively regulates gene expression, they are abundant in peripheral biofluids, relatively stable, amenable to various methods of detection and display dynamic expression changes in response to disease development or environmental stimulus. Many miRNA are significantly dysregulated in the brain during both epilepsy development and in chronic epilepsy as detected in both rodent models and in surgically resected tissue from patients with intractable TLE (Bot et al., 2013; Brennan et al., 2016; Jimenez-Mateos et al., 2011; Jimenez-Mateos et al., 2012; Risbud and Porter, 2013; Schouten et al., 2016). Brain-enriched miRNAs probably gain access to the circulation via paracrine release of vesicle-enclosed miRNAs (e.g. exosome-enclosed microRNAs) or via disturbance to the blood brain barrier (BBB) following epilepsy-inciting events including TBI, stroke or SE or acute seizures (Batool et al., 2019; Raoof et al., 2018; Ruber et al., 2018).

Thus, measurement of circulating brain-enriched miRNAs may provide a by-proxy measurement of critical stages of disease development and pathomechanisms involved in seizure development and initiation (Dadas and Janigro, 2019; Gorter et al., 2015; Prager et al., 2019).

Here, for the first time we search for peripheral miRNA signatures of epileptogenesis and chronic epilepsy using three clinically relevant rodent models of epilepsy. Video-EEG monitored animals were serial sampled to follow miRNA level changes over the course of the disease (from baseline to epileptogenesis to chronic epilepsy) and we performed genome-wide discovery followed by extensive post-hoc validation. This included analysis of the differentially expressed miRNAs in human plasma from patients with TLE, testing effects of common anti-epileptic drugs and a miRNA-based experimental disease-modifying therapy on circulating miRNA profiles and, finally, confirming the presence of these epilepsy-related miRNA within the functional silencing complex in specific brain cell types. Together, these results extend the evidence that circulating miRNAs may be an important new class of biomarker for the diagnosis of epilepsy.

2. Materials and methods

2.1. Animals

All procedures were performed in accordance with the principles of European Union Directive (2010/63/EU) and were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (REC # 842) under license from the Health Products Regulatory Authority (AE19127/I084, AE19127/I089, AE19127/I152), Dublin, Ireland. Animal experiments performed at the University of Verona were approved by the University of Verona ethics committee under license from the Italian Ministry of Health (27/2014/PR) and carried out in accordance with local relevant guidelines and regulations. Perforant Pathway Stimulation (PPS) was performed at the Philipps University Marburg and was approved by the local regulation authority (Regierungspraesidium Giessen) according to Directive 2010/63/EU. All efforts were made to minimise animal suffering and the number of animals used. For the intraamygdala kainic acid (IAKA) model we used 6–9 weeks old male C57BL/6JOlaHsd mice, originally from Harlan Laboratories (UK) and inbred at Biomedical Research Facility RCSI. For the pilocarpine model (Pilo) we used male NMRI mice (University of Verona). All mice were housed with a 12 h light-dark cycle at 21-23° C and humidity of 40–60% with ad libitum access to food and water. Adult male Sprague-Dawley rats weighing between 320 and 450 g were used for the PPS model. Rats were single housed to prevent damage to implants by cage mates. Rats had unlimited access to food and water and were subjected to a 12 h light-dark cycle.

2.2. IAKA model of TLE in mice

Induction of SE using the IAKA technique was performed as previously described (Diviney et al., 2015). Briefly, mice (C57BL/6JOlaHsd) were anesthetized (isoflurane; 5% induction, 1–2% maintenance) and equipped for continuous EEG and video recordings using implantable EEG telemetry devices (Data Systems International). Transmitters (model F20-EET) which record bilateral EEG from the skull were implanted in a subcutaneous pocket at the time of cannula placement (on the dura mater following coordinates from Bregma; IA: AP = −0.95 mm, L = +2.85 mm, V = 3.1 mm). The behaviour of the animals was recorded using a video camera placed next to the cage. Continuous video-EEG data were acquired for each animal. After

transmitter-cannula fitting, mice underwent intra-amygdala microinjection of KA (0.3 μg in 0.2 μL; Sigma-Aldrich, Ireland) to induce SE, followed after 40 min by intraperitoneal (IP) lorazepam (8 mg/kg) to reduce morbidity and mortality. Mice were placed in an incubator at 26˚C to recover. Serial blood samples were obtained from mice at baseline (BL), 48 h (epileptogenesis) and 2 weeks (chronic epilepsy) post KA or PBS injection via submandibular blood draw.

2.3. Pilocarpine model in mice

The pilocarpine model of SE was performed as previously described (Engel et al., 2013). Adult male NMRI mice were fitted for DSI telemetry as above. Briefly they were anesthetised (isoflurane 5% induction followed by 2% maintenance) and equipped with the DSI F20-EET telemetry device. During surgery, transmitters were implanted into a subcutaneous pocket at the level of the peritoneum. Leads run subcutaneously to the head of the animal, two to the right nuchal muscle which serve as EMG electrodes. The other two were implanted to the surface of the dura mater via a small craniotomy with the following coordinates relative to Bregma, AP: +1.7, L: −1.5 and AP: -2.5, L: +2.0 mm. Following surgery, animals were placed in Phenotyper cages to recover. Following recovery, animals were given methylscopolamine (1 mg/kg) to block peripheral cholinergic actions and then after 30 min, injected with pilocarpine IP (300 mg/kg). The development of SE was recorded and, as above, blood draws were taken via submandibular bleed at BL, 48 h (epileptogenesis) and 4 w (chronic epilepsy) and PBS time-matched controls.

2.4. Perforant pathway stimulation model in rats

The perforant pathway stimulation (PPS) model was performed in rats as described (Costard et al., 2019). Animals were first anesthetized (isoflurane; 5% induction, ~3% maintenance) and then equipped with bipolar stainless-steel stimulating electrodes positioned in the angular bundles of the perforant pathway, and custom unipolar recording electrodes were lowered into the dorsal dentate gyrus. Electrode locations were determined by optimizing the potentials evoked by low-frequency PPS. Electrodes and ground screws were connected to miniature wireless transmitters (A3028R-FB, Open Source Instruments Inc., Watertown, MA, USA) that were implanted subcutaneously on the animal's flank. Plastic connectors (Ms363, Plastics One, Roanoke, VA, USA) joined the electrodes with stimulation/recording equipment. The PPS protocol utilized a paradigm designed to evoke and maintain hippocampal seizure activity throughout the stimulation, but not convulsive status epilepticus, which consisted of continuous, bilateral 2 Hz paired-pulse stimuli, with a 40 ms interpulse interval, plus a 10 s train of 20 Hz single-pulse stimuli delivered once per minute, generated by an S88 stimulator (Grass Instruments, West Warwick, USA). All pulses (0.1 ms duration) were delivered at 20–24 V with associated current typically between 15 and 30 μA. As described previously, stimulation for 30 min (on two consecutive days) required only isoflurane to terminate seizures (Norwood et al., 2010). Eight-hour stimulation on the third day did not induce status epilepticus and, therefore, did not require pharmacological termination. Blood samples were drawn as described above at BL, 24 h (epileptogenesis), 72 h (epileptogenesis), 10 d (epileptogenesis) and day of first spontaneous seizure (chronic epilepsy).

2.5. Patient samples

Patient and control samples were collected from the Epilepsy Monitoring Unit (EMU), at the Epilepsy Centre Hessen/Department of Neurology, Philipps University of Marburg, Germany. Ethical approval was obtained from the local medical ethics committee (MAR17/14). All patients consented to the study and signed patient consent forms according to the Declaration of Helsinki. All patients were on poly drug therapy. The reasons for admission were mainly to confirm diagnosis of refractory focal epilepsy and assess patients for surgical resection. Continuous video EEG monitoring was performed using a standard EEG electrode placement. From each patient a baseline blood sample was taken and a second blood sample was taken 24 h post seizure. In addition non-fasting healthy volunteer blood samples were also collected at this site. Patient information is listed in the supplementary excel file 1.

2.6. Blood collection and processing

Blood from rodents was collected from submandibular blood draws into a 0.5 mL tube coated with K2 EDTA (0.5 M Sigma-Aldrich UK). The superficial temporal vein runs diagonally across the face of the rodent, under the eye towards the ear. Mice or rats were lightly anesthetized using isoflurane and ~ 150–250 μL of blood was collected at baseline and at experimental time-points before the animal was sacrificed.

Within 30 mins of collection, blood samples collected from both rodent and human was centrifuged at 1300 $\times g$ for 10 mins at 4 ˚C to separate the plasma from blood cells. The plasma supernatant was removed and placed in a fresh tube and immediately placed on ice. Within 30 mins of centrifugation, haemolysis levels of samples were evaluated using a Nanodrop 2000 spectrophotometer. Only samples with a reading of < 0.25Abs at 414 nm were used in experiments.

2.7. Anti-epileptic drug administration

Male mice (C57BL/6JOlaHsd; 23–30 g) were anesthetized with isoflurane and placed in a mouse-adapted stereotaxic frame. Three electrodes were implanted for surface EEG along with a guide cannula for induction of SE by the IAKA method. Subsequently, KA (KA; 0.3 μg/0.2 μL) or PBS was injected into the right basolateral amygdaloid nucleus to induce SE. After 40 min, all mice received lorazepam (8 mg/kg; IP.). The mice were then returned to their home cages. Mice were video-recorded for 2 weeks post-SE (or sham) to monitor epilepsy development. Blood was then taken from the submandibular vein and processed to produce plasma (Epileptic BL). The mice were then assigned to a vehicle group (4% tween) in water, carbamazepine (CBZ; 40 mg/kg/day, i.p.), or diazepam (DZP; 5 mg/kg/day, i.p.) and treated for 3 days. The day after the final dose the animals underwent a further submandibular bleed (Epileptic AED).

2.8. Anti-epileptogenesis treatment with oligonucleotide targeting miR-134 (Ant-134)

Male C57BL/6JOlaHsd mice (23–30 g) were implanted with skull electrodes and a cannula for IAKA as well as a cannula for intracerebroventricular (ICV) drug administration. SE and subsequent epileptogenesis was induced via IAKA as before. Animals then received antagomir oligonucleotides targeting miR-134 (Ant-134; 120 pM; ICV) or a scrambled oligonucleotide, 1 h post-SE to inhibit epileptogenesis and reduce the development of chronic epilepsy (Jimenez-Mateos et al., 2015; Jimenez-Mateos et al., 2012). Bloods were taken both pre-SE and 2 weeks post-SE and processed for plasma as described.

2.9. RNA isolation and genome-wide profiling of plasma miRNAs

RNA was isolated from both rodent and human plasma using a starting volume of 200 μL with the miRCURY RNA isolation kit for biofluids from Exiqon which enriches for small RNA molecules including miRNA. Manufacturer's instructions were followed except the final RNA was eluted in 25 μL. RNA integrity and miRNA concentration and percentage was measured using the Fragment Analyser from Agilent formerly Advanced Analytical (AATI) using the small RNA kit and protocol.

For miRNA expression profiling of individual samples we used the

QuantStudio™ 12 K Flex OpenArray system (ThermoFisher Scientific). OpenArray technology is a high-throughput, real-time PCR–based method for miRNA detection that enables simultaneous amplification of 750 rodent miRNAs in each sample. Samples are initially reverse transcribed to cDNA before undergoing a pre-amplification step and finally PCR amplification as previously described (Raoof et al., 2018).

2.10. Real Time-qPCR (Individual miRNA expression analysis)

Individual miRNA Taqman assays were performed as previously described (18) using miRNA specific RT primers (miR-93-5p (ID: 001090), miR-142-5p (ID: 002248), miR-199a-3p (ID: 002304), miR-182-5p (ID mmu + rno: 002599, hsa: 002334), miR-574-3p (ID: 002349) (All Thermo Fisher assays). Reverse transcription was carried out using equal volumes of RNA from each sample. cDNA was diluted and qPCRs for each sample were performed in triplicate on the Quantstudio, 12 K Flex. A negative control was included on all plates using water as a template. MiR-16, a plasma enriched miRNA was used for all normalisation.

2.11. Blood brain barrier analysis in chronically epileptic mice

To study the disruption of the BBB in the chronic epilepsy phase, Biotin dextran amine (BDA, 70 kDA) was systematically administered to epileptic mice followed by staining for the presence of BDA in the brain. Briefly, animals underwent IAKA-induced epileptogenesis as described. BDA at a concentration of 90 mg/kg was then delivered intraperitoneally 11 days after KA injection. Mice were then transcardially perfused with PBS 72 h following BDA injection and brains were immediately frozen in 2-methylbutane on dry ice and kept at −80 °C until use. Brains were then sectioned coronally at 12 μm thickness on a Leica cryostat and directly mounted on slides.

To detect BDA presence in the brain, sections were post-fixed in formalin for 30 min, and then rinsed twice for 5 min in 0.01 M PBS. Afterwards, slides were blocked for 1.5 h in 1% bovine serum (BSA) in PBS containing 0.3% Triton X-100. Sections were then incubated with a primary antibody against NeuN (1:400; Millipore) diluted in blocking solution and incubated overnight at 4 °C. Slices were then washed in PBS, before being incubated with secondary antibody (1:500), diluted in 3% BSA in PBS containing 0.3% Triton X-100. Streptavidin-Alexa-Fluor 594 (1:500; Thermo) was added to the secondary antibody and incubated for 2 h at room temperature. Finally, slides were washed three times for 10 min in 0.01 M PBS, dried and mounted with FluorSave reagent (Merck-Millipore). Sections were then analysed using a Leica DM4000 fluorescent microscope.

2.12. Brain cell origins of circulating miRNAs

To explore the cell types expressing the identified miRNAs, we generated mice which express FLAG-Ago2 in either neurons or glia by crossing Rosa-Stop$^{fl/fl}$-Flag-Ago2 mice (a gift from Dr. A Schaefer (Schaefer et al., 2010; Tan et al., 2013)) with inducible cre-recombinase mouse lines driven by brain cell type restricted promoters for neurons (Thy1, Jax Stock 012708) or microglia (Cx3cr1, Stock 021160). Treatment of the resultant transgenic lines (*Thy1-cre*$^{tg/+}$*;Rosa-Stop*$^{fl/fl}$*-Flag-Ago2;* TxA and *Cx3cr1-cre*$^{tg/tg}$*;Rosa-Stop*$^{fl/fl}$*-Flag-Ago2;* CxA) with tamoxifen (10 mg/mL, Sigma-Aldrich, Ireland) administered via IP injection once a day for 5 days followed by 2 days rest and a second period of once a day dosing for 5 days resulted in Flag-Ago2 expressed in either neurons or microglia.

Mice were euthanised and hippocampi collected on dry ice and then transferred to a − 80 °C freezer. Hippocampi were lysed in 300 μL RNase free IP buffer (0.1%-NP40; 50 mM Tris_HCl, pH 7.4; 300 mM NaCl; 5 mM $MgCl_2$; Protease inhibitors), centrifuged and supernatant was transferred to RNase free tubes. Protein concentration was determined using a BCA assay and 1000 μg of protein was made up to 500 μL with IP buffer and diluted a further 500 μL with RNase-free PBS. Lysate was also retained and used as an input sample. 10 μg of α-FLAG antibody (Abcam, ab1162) was added to each sample and incubated overnight at 4 °C followed by overnight incubation with protein A:G agarose beads (SantaCruz Biotechnology). Samples were then centrifuged, beads were washed twice and trizol added. RNA extraction was carried out using a standard phenol-chloroform extraction protocol. Isolated RNA was resuspended in 20 μL of RNase free water and qPCR was performed as above. RNA was isolated from 100 μL of "input" samples, using phenol-chloroform extraction and quantified and analysed for impurities using a nanodrop spectrophotometer 2000. 250 ng of RNA was used for reverse transcription and individual qPCR was performed and used to normalise values from IP using DDCT method.

2.13. miRNA-mRNA target identification and pathway enrichment analysis

We identified predicted (miRDIP V4.1 (Tokar et al., 2018)) and experimentally validated (miRTarBase V7 (Chou et al., 2018) TarBase V7.0 (Karagkouni et al., 2018) mRNA targets of hsa-miR-93-5p, hsa-miR-199a-3p and hsa-miR-574-3p. We prioritised targets by filtering for human miRNA-mRNA interactions that were experimentally validated with strong low-throughput evidence (Western blot, PCR or reporter assay) or predicted with very high confidence. Reactome pathway enrichment analysis was performed using the ReactomePA and clusterProfiler packages (Jassal et al., 2020; Yu and He, 2016; Yu et al., 2012) in R version 3.6.3 (Gu et al., 2014) with an adjusted *p*-value (Benjamini-Hochberg) of 0.05 considered significant. Significantly enriched pathways were manually grouped into their respective parent Reactome pathways. For visualisation, we identified those mRNA targets in at least one MTI with strong experimental evidence, and generated a circos plot of all MTIs targeting these mRNAs, using the R 'circlize' package (Gu et al., 2014). mRNAs implicated in epilepsy were identified from CARPEDB [http://carpedb.ua.edu/], epiGAD (Tan and Berkovic, 2010), Wang et al (Wang et al., 2017) and curated epilepsy-genes from the Comparative Toxicogenomics Database (CTD) (Davis et al., 2019). All R codes are available on request.

2.14. Open array analysis and statistics

Filtering and statistical analysis of the OA microRNA profiling data were performed in R/Bioconductor (Huber et al., 2015). Data were first filtered according to amplification score (AmpScore > /= 1.24) and quantification cycle confidence (Cqconf > /= 0.8) provided by the ExpressionSuite software (ThermoFisher Scientific). A miRNA was considered "present" in a set of samples if the cycle threshold (Ct) was > 10 and < 28. Only bona fide miRNAs were considered (Fromm et al., 2015). Samples with < 50 miRNA detected were removed. Missing data points were imputed (Bioconductor package "non-detects") and the data was Quantile normalised as implemented in Bioconductor package "HTqPCR" (Dvinge and Bertone, 2009). For the comparison of the baseline samples across the three models, miRNA were included if they were present in at least 50% of the samples (i.e. 3 of the 6 mouse samples, and 4 of the 8 rat samples). For the individual analysis of the three models only miRNAs expressed in > 80% of samples were included in the study.

Differential expression analysis was performed using the limma package (Ritchie et al., 2015). *p*-Values were adjusted for multiple testing by controlling the false discovery rate (FDR) according to the method of Benjamini and Hochberg (Benjamini and Hochberg, 2000). A miRNA was considered to be differentially expressed if the adjusted *p*-value was < 0.05. Fold changes (FC) were calculated as $FC = 2^{-\Delta Ct}$. Receiver operating characteristic (ROC) curve analyses were performed using the R pROC package (Robin et al., 2011). Logistic regression analysis of the combined miRNA was carried out with the R glm

package using the normalised expression of miRNAs from qRT-PCR experiments as independent variable and epilepsy status as the dependent variable.

qPCR was analysed using the DDCt method with all miRNA analysis normalised to miR-16 levels. Statistical analysis on individual qPCR was performed using standard *t*-test for direct comparison and ANOVA where more than two groups are present followed by Dunnet test. Significance was assigned as $p < 0.05$.

3. Results

3.1. Shared circulating miRNA profiles across animal models of epilepsy

To identify circulating miRNAs associated with epileptogenesis or chronic epilepsy, we profiled peripheral blood plasma isolated from three different animal models of epilepsy. An overview of the study design is shown in Fig. 1. Three well established rodent models of epilepsy were used, IAKA in C57BL/6 mice ($n = 3$/group) (Mouri et al., 2008), Pilo in NMRI mice ($n = 3$/group) (Becker, 2018; Turski et al., 1989) and PPS using Sprague Dawley rats ($n = 6$/group) (Kienzler et al., 2009; Norwood et al., 2010). Each model was performed in a different laboratory whose expertise is aligned with that specific model and to increase robustness of biomarker identification. We used a genome-wide profiling platform and analysed serially sampled plasma collected from the same animal during both epileptogenesis and in chronic epilepsy compared to control animals at matching time-points.

We first explored miRNA profiles in baseline (BL) samples to identify the general miRNA composition of plasma in rodents. BL samples contained between 50 and 150 miRNA present in all samples (Fig. 2A). Principal component analysis (PCA) of BL samples revealed clustering consistent with highly similar miRNA profiles between the two mouse strains (C57BL/6, NMRI) and rats (Sprague Dawley) (Fig. 2B). As expected, the highest similarities were between C57BL6 and NMRI mice when compared to the Sprague Dawley rat. We also observed closer clustering of plasma miRNA profiles among mice consistent with the inbred nature of the strains whilst data clustering was less compact in the outbred Sprague Dawley rat (Fig. 2B).

We next performed differential expression analysis across all groups, focusing on differences between controls and animals at both an epileptogenic time point and at the stage of chronic epilepsy (Fig. 2C). Substantial changes in plasma miRNA composition were detected at each stage of disease and in all 3 models. We identified numerous miRNA present in peripheral blood plasma from epileptic rodents which were not detected in naïve animals while also detecting numerous miRNA whose expression was altered in epileptic animals compared to control in multiple models (Fig. 2D) (adj p value < 0.05). Despite extensive compositional changes in blood plasma miRNA levels during epileptogenesis, we were unable to detect a single miRNA whose expression was only dysregulated in the epileptogenesis stage of disease. Indeed, most miRNA whose expression was altered during epileptogenesis remained altered in chronically epileptic mice. Five miRNA were identified with epilepsy biomarker potential which warranted further investigation based upon consistency of change across models, known brain enrichment, previous functional links to epilepsy and novelty. These were miR-93-5p (elevated in epileptogenesis and epilepsy), miR-574-3p (reduced during epileptogenesis and epilepsy), miR-182-5p (present in epilepsy only), miR-142-5p (elevated in epilepsy and implicated in pathogenesis of epilepsy (Venø et al., 2020) and miR-199a-3p (present in epilepsy only). We next ran individual Taqman assays for each miRNA from rodent plasma to validate the OpenArray data. In line with OpenArray profiling data, miR-93-5p, miR-182-5p and miR-574-3p all showed the same expression profile in each model. MiR-199a-3p was upregulated in the IAKA model at all timepoints but was not significantly different in the Pilo or PPS models and miR-142-5p failed to validate using the individual Taqman assays (Fig. 2F–H).

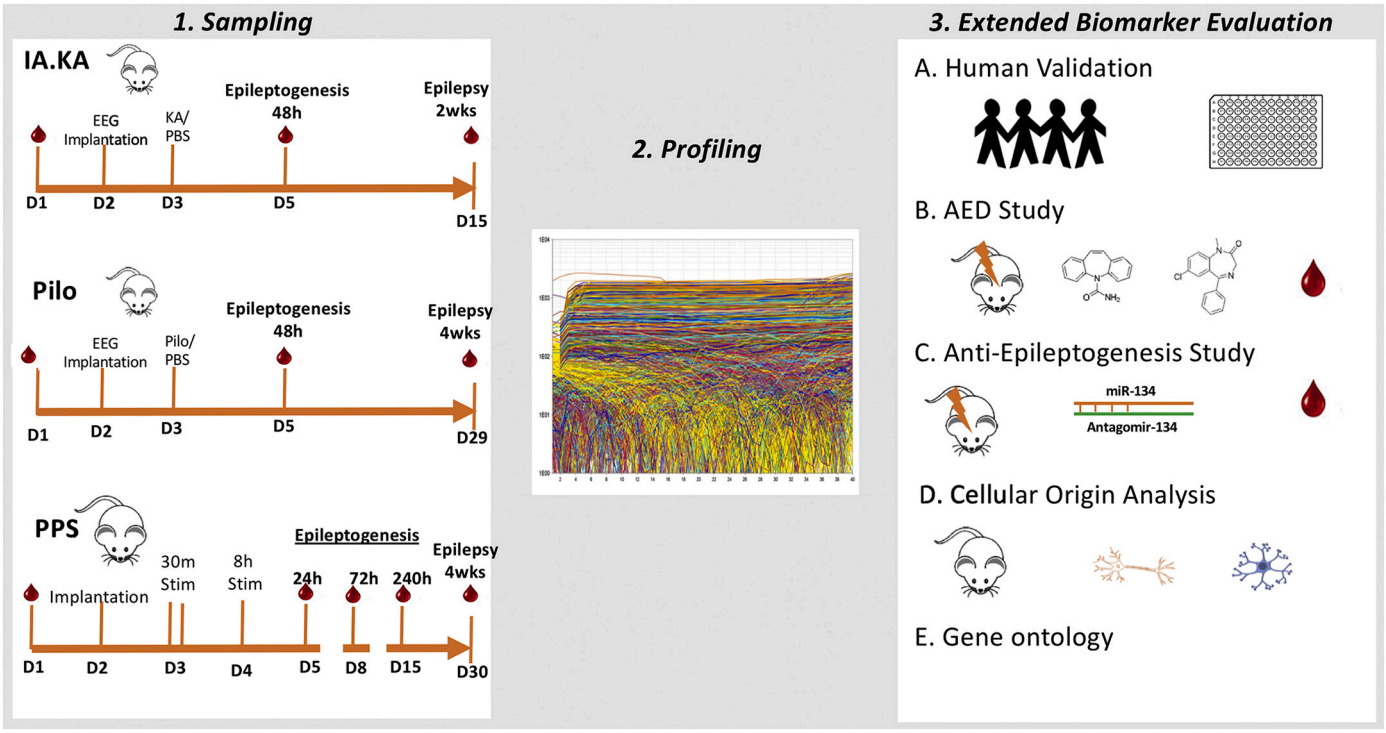

Fig. 1. Study design and biomarker identification in rodent models of epilepsy. (A) Schematic of overall study design. Plasma was collected from rodents before any procedures to measure baseline miRNA levels, plasma was also collected at specific time-points both during epileptogenesis and following the emergence of spontaneous seizures. MiRNA were then profiled using the OpenArray platform. MiRNA with biomarker potential were then validated as legitimate biomarkers of epilepsy by testing in human TLE plasma and a series of post-hoc validations in mice.

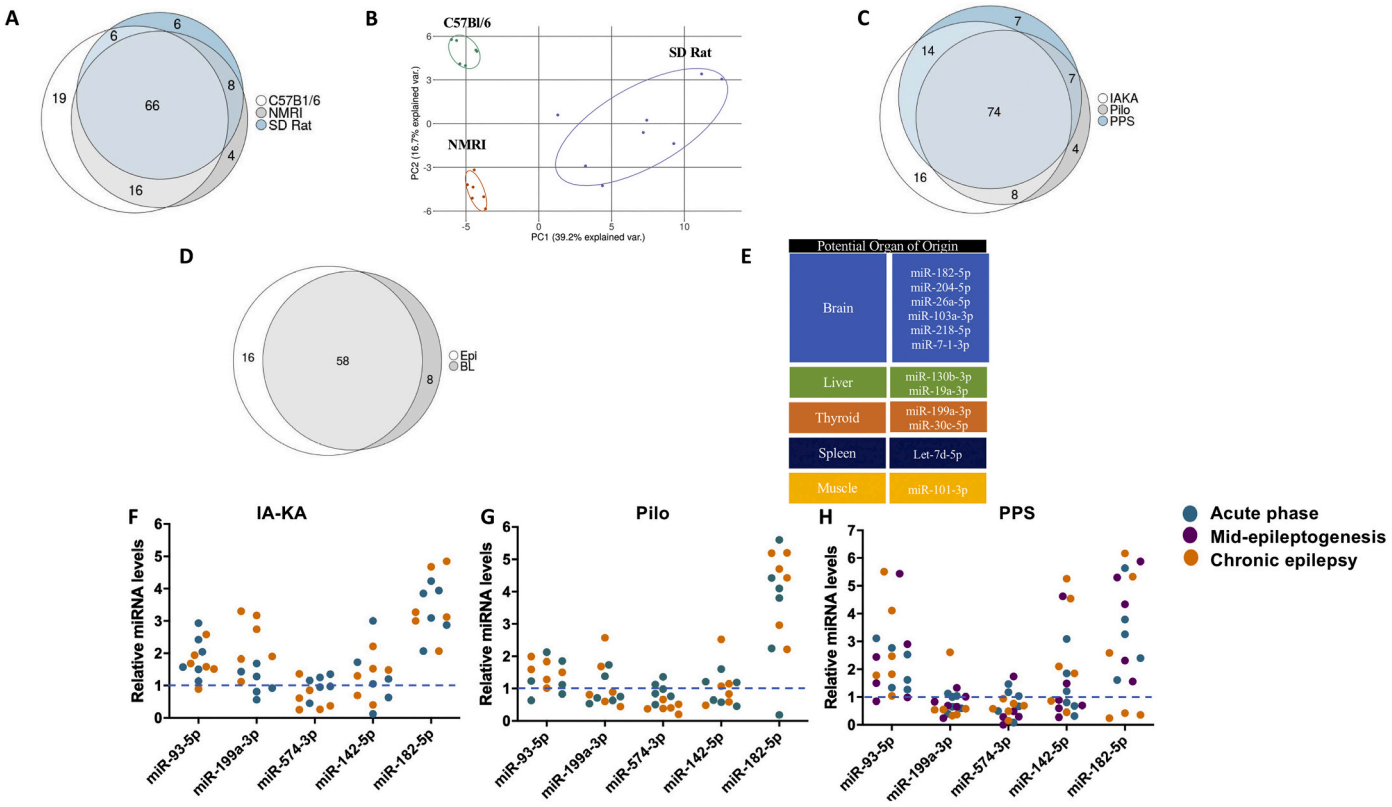

Fig. 2. OpenArray profiling identifies 5 microRNA with biomarker potential (OA: IAKA, $n = 3$ per group; Pilo, $n = 3$ per group; PPS $n = 8$ per group). (A) Venn diagram showing numbers of miRNA common across baseline plasma samples among rodents demonstrating strong overlap among species. (B) PCA plot to identify clustering of plasma among species revealed strong clustering between mouse strain and rat. Tight clustering was observed for inbred rodents C57Bl/6 and NMRI while the outbred rat strain Sprague Dawley showed larger variance in miRNA profile. (C) Venn diagram representation of the marked differences in plasma miRNA composition during disease development. (D) A number of miRNA unique to plasma isolated from epileptic rodents was detected. (E) Potential organ of origin of several differentially expressed miRNA in epilepsy as determined by miRNA expression atlas. (F–H) RT-qPCR validation of differentially expressed miRNAs with biomarker potential in each model. qPCR assays for individual miRNA showed strong correlation with OpenArray data with differential expression detected for each of the five miRNA tested at specific timepoints. ($^*p < 0.05$). Dashed line represents baseline levels of miRNA when normalised to miR-16. (RT-qPCR Validations: IAKA $n = 6$/group; Pilo $n = 6$/group; PPS $n = 6$/group).

3.2. Differentially expressed miRNA identified in rodents are also dysregulated in human TLE

We next sought to validate the exploratory rodent model findings in plasma collected from human patients with TLE (patient information Supplementary File 1). Here, we obtained plasma from patients 24 h following admittance to the EMU (during a seizure-free period) and again 24 h post seizure (PS; video-EEG confirmed). Any miRNA showing dysregulation in both patient samples (pre- and post-seizure) would be a biomarker of epilepsy whereas miRNAs altered only in PS samples would be potential seizure biomarkers. Additionally, we analysed the miRNA in plasma before and after seizure-like behaviour in patients later diagnosed with NEAD (Fig. 3A–E).

Of the five rodent-identified miRNA, the levels of three were significantly elevated or reduced in the BL plasma samples from patients with TLE compared to controls. Notably, we detected increased levels of miR-93-5p and miR-199a-3p and decreased levels of miR-574-3p, matching the direction of change observed in rodents (Fig. 3A, B, C). Levels of miR-142-5p and miR-182-5p did not differ between controls and TLE (Fig. 3D, E). Levels of most of the miRNAs did not differ in PS samples compared to BL indicating they represent potential biomarkers of epilepsy rather than acute seizures. Interestingly, levels of miR-142-5p were higher in PS samples compared to BL suggesting this miRNA may be transiently affected by acute seizure activity (Fig. 3D). Plasma levels of miR-93-5p, miR-199a-3p and miR-574-3p were not different from controls in samples from patients diagnosed with NEAD (Fig. 3A–E).

ROC curve analysis of each individual miRNA from both BL samples (Fig. 3F–H) and PS samples (Fig. 3J–L), plus combinations of the three (Fig. 3I and M), revealed strong predictive value of these miRNA as biomarkers. All three miRNA, when analysed individually gave an area under the curve of close to 0.8. A combination of all three miRNA however raised the value of the area under the curve to 0.88 using BL samples (Fig. 3I) and 0.86 in PS samples (Fig. 3M) providing further discrimination between control and epilepsy patients.

3.3. Effects of common anti-epileptic drugs (AEDs) on miRNA profiles

Changes to AEDs including medication tapering are common during diagnostic video-EEG monitoring. This is an unlikely confounder in the present study because levels of the three validated miRNAs (miR-93-5p, miR-199a-3p and miR-574-3p) were similar in PS and BL seizure samples despite being obtained at different times. Nevertheless, to exclude an effect of AEDs on miRNA levels, we generated additional epileptic mice (IAKA model in mice) and dosed mice with either the anti-seizure drug Carbamazepine (Cbz) or anticonvulsant Diazepam (Dzp). Plasma was obtained before and after 3 days of dosing with clinically-relevant doses of Cbz or Dzp or vehicle (Veh) (Fig. 4A) (Klein et al., 2015; Loscher and Honack, 1997). The levels of miR-93-5p, miR-199a-3p and miR-574-3p as well as miR-142-5p and miR-182-5p were then analysed comparing before and after drug dosing. Neither drug had a significant effect on plasma levels of the five miRNAs (Fig. 4B–F).

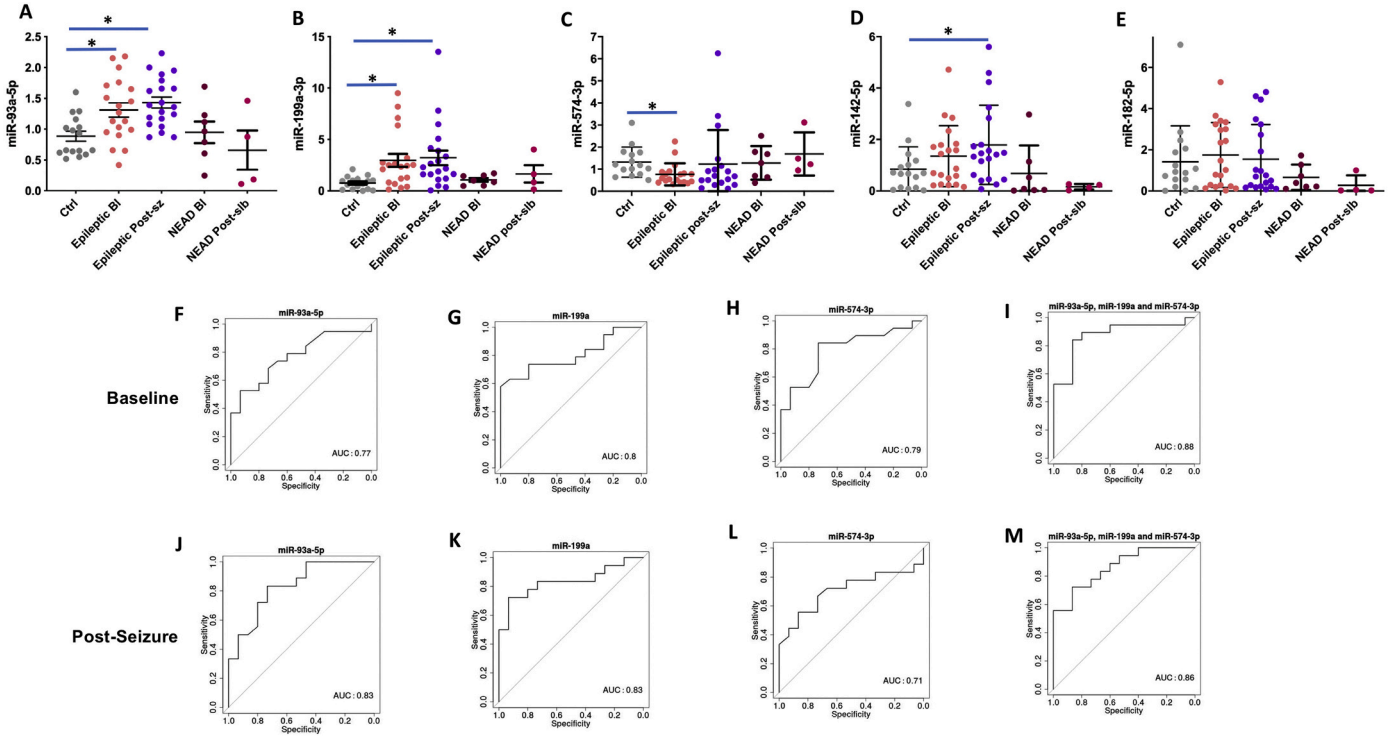

Fig. 3. Validation of rodent biomarkers in human TLE patients. (A - E) RT-qPCR assays for individual miRNA in plasma collected from healthy controls (n = 16), TLE patient baseline samples (24 h seizure free) (n = 20), TLE patient post-seizure sample (24 h post seizure) (n = 20) and NEAD patients baseline (n = 7) and 24 h post seizure-like behaviour (slb) (n = 4). Analysis revealed miR-93-5p, miR-199a-3p and miR-574-3p were all differentially expressed in patients with TLE compared to controls with no differences between pre- and post-seizure samples. Patients with confirmed NEAD did not have differentially expressed miRNAs compared to control patients. (F – I) ROC curve analysis of miRNA levels in baseline samples showed that all three miRNA differentially expressed in human TLE compared to controls had good predictive value, however combination analysis was most predictive. (J – M) ROC analysis of post-seizure samples reveals strong predictive value for miR-93-5p and miR-199a-3p but less predictive value for miR-574-3p. As such combination analysis was less predictive in post-seizure samples compared to baseline samples. (*p < 0.05).

3.4. Disease-modifying therapy alters plasma miRNA profiles

To extend evidence that the miRNAs are biomarkers of epilepsy rather than seizures per se (or other systemic factors), we explored effects of an experimental disease-modifying therapy on plasma levels of miR-93-5p, miR-199a-3p and miR-574-3p. For this we suppressed epileptogenesis by administering antisense oligonucleotide inhibitors (antagomirs) targeting miR-134 (Ant-134) (Jimenez-Mateos et al., 2015; Jimenez-Mateos et al., 2012; Reschke et al., 2017). When given shortly after SE, this potently inhibits the development of epilepsy. If the miRNAs are biomarkers of epilepsy, then this disease-modifying therapy should alter levels towards baseline. To test this, we again utilized the IAKA model. Blood was taken from BL animals and then 2 weeks after SE in animals given Ant-134 or a scrambled antagomir. Plasma from a third group of mice not subjected to SE were used to obtain background levels of each miRNA (Fig. 5A). qPCR analysis revealed that plasma levels of miR-93-5p and miR-182-5p were no longer elevated in IAKA mice that received Ant-134 (Fig. 5B, F). Furthermore,

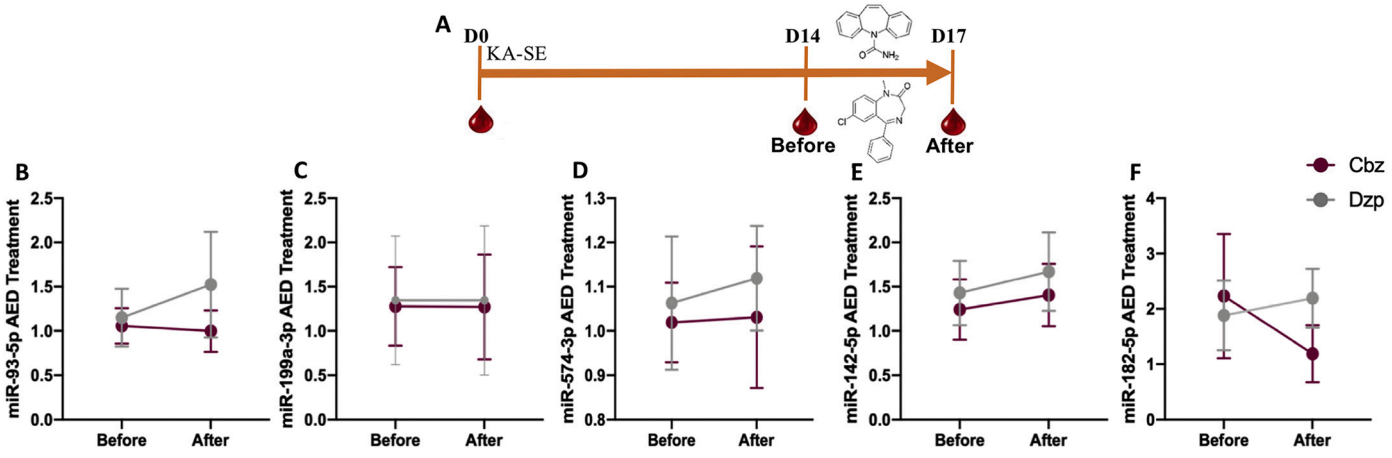

Fig. 4. Anti-epileptic drugs do not affect plasma levels of potential miRNA biomarkers. (A) Schematic of the experimental design to test whether common AED/anti-convulsant drugs affect circulating miRNA levels. (B - F) RT-qPCR analysis of circulating miRNA levels failed to detect any differences in epileptic mice after dosing with either Carbamazepine (Cbz) or Diazepam (Dzp) compared to levels pre-dosing (n = 6 per group).

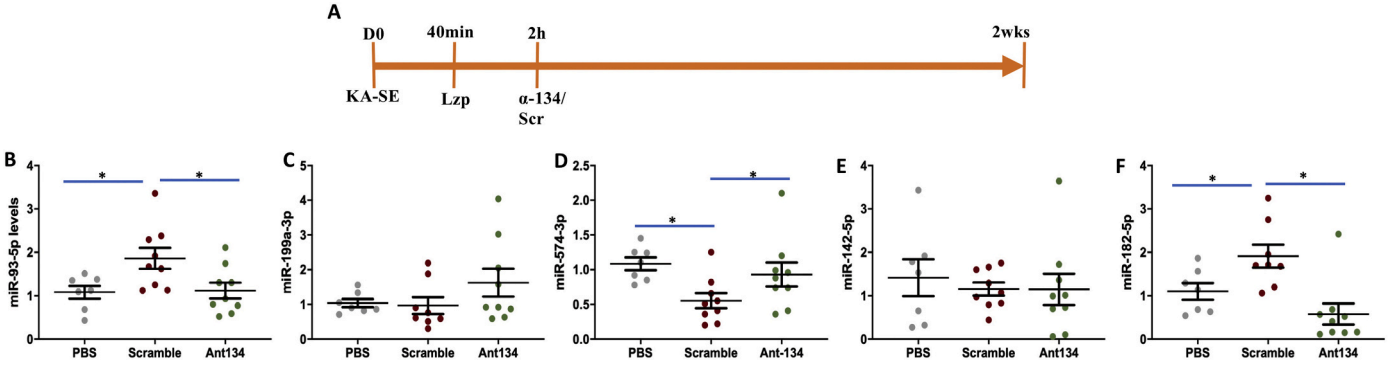

Fig. 5. Blocking epileptogenesis prevents dysregulation of circulating plasma miRNA levels. (A) Schematic of the experimental design to test the effect of blocking epilepsy development in mice destined to become epileptic on miRNA levels. (B – F) RT qPCR analysis of individual miRNA from plasma from control mice (n = 7), epileptic mice (n = 9) and mice which experience an epilepsy-inciting event but then receive an intervention treatment (n = 9). qPCR analysis revealed that suppressing epileptogenesis prevented dysregulation of miR-93-5p (B), miR-574-3p (D) and miR-182-5p (F). (*p < 0.05).

Ant-134 treatment prevented the reduction of miR-574-3p levels normally seen in this model (Fig. 5D). Ant-134 had no observable effects on miR-199a-3p or miR-142-5p (Fig. 5C, E).

3.5. Neuronal and microglial expression of circulating miRNA biomarkers of epilepsy and potential route of entry from brain

Determining the cellular origin of circulating epilepsy-associated biomarkers is important support for mechanistic links to the disease process. To address this, we used transgenic mice which express FLAG-tagged Ago2 in specific brain cell types, allowing us to elute miRNAs from either neurons or microglia. As the mice conditionally express FLAG-Ago2 in neurons or microglia upon tamoxifen treatment (Supplemental Fig. 1A), we compared levels of the five miRNAs in either line with or without recombination. In pilot studies we established the validity of this approach, showing FLAG pulldowns from tamoxifen-treated mice from the Thy1 (neuronal) line were enriched for the neuron-specific miRNA miR-124-3p (Supplemental Fig. 1B) but were lacking this miRNA in FLAG pull-downs from the microglia line (Supplemental Fig. 1C). Conversely, we detected an abundance of miR-146a in the microglia-FLAG-Ago2 line immunoprecipitates (Supplemental Fig. 1D) while in neurons it was almost undetectable (Supplemental Fig. 1E).

Next, we analysed FLAG pull-downs from each mouse line for the presence of the five miRNA biomarkers of epilepsy. Consistent with known tissue expression in brain (Ludwig et al., 2016), FLAG-Ago2 immunoprecipitates from the two mice lines detected miR-93-5p in hippocampal neurons and microglia (Fig. 6A, B). We also detected miR-199a-3p in both neurons and microglia (Fig. 6C, D). Within the hippocampus we detected abundant levels of miR-574-3p in both neurons and microglia (Fig. 6E, F). MiR-182-5p was also detected in mouse brain, both in neurons and microglia (Fig. 6G, H) while miR-142-5p was also detectable in Flag-Ago2 mice hippocampi (Fig. 6I, J).

We detect persistent changes in the levels of circulating miRNAs lasting up to 2 weeks post epilepsy-inciting insult and also in chronic human TLE where patients have been living with epilepsy for many years. Having identified that our miRNAs of interest are expressed in neurons and microglia we next sought to establish how miRNAs may enter peripheral circulation in stable persons with epilepsy. To test whether the BBB is leaky in epilepsy we injected epileptic mice systemically with BDA. With a molecular weight of 70 kDa this molecule cannot cross an intact BBB. However we were able to detect small amounts of this molecule in the brains of epileptic mice suggesting BBB disturbance in epilepsy (Fig. 6K).

3.6. Predicted miRNA targets and potential involvement in the pathogenesis of epilepsy

Finally, to explore the biological functions potentially regulated by the miRNA differentially expressed in human epilepsy patients, we identified the mRNA targets of these miRNA (Fig. 7A). We filtered specifically for human miRNA-mRNA interactions with low-throughput experimental validation. Epilepsy-associated mRNA targets included PTEN, MTOR, CXCL8 and the TGFβ pathway, with all three human-

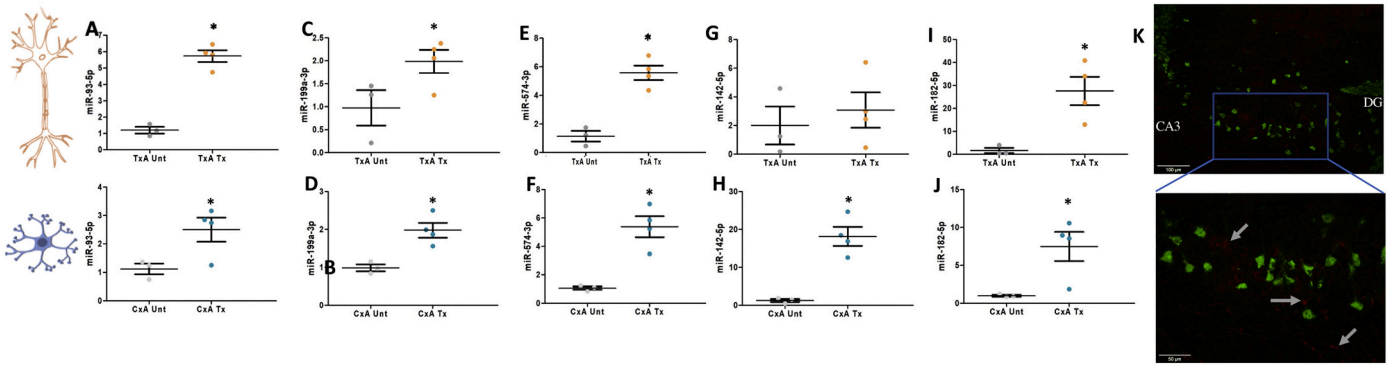

Fig. 6. Neurons and microglia as potential cellular origin of circulating biomarkers of epilepsy (A-J) qRT-PCR analysis of individual miRNAs from transgenic mice either treated or untreated with tamoxifen to determine whether circulating miRNA are expressed in neurons and/or microglia . PCR analysis detected all miRNAs examined (miR-93-5p, miR-199a-3p, miR-574-3p, miR-142-5p and miR-182-5p) in both neurons and microglia in mice treated with tamoxifen whereas we were unable to detect miRNA in untreated mice (n = 3–4 per group). (K) Representative photomicrographs of BDA entry into the brain from the periphery. Entry was confirmed via immunofluorescent detection of BDA (red, indicated with arrows) in the hippocampus of epileptic mice (NeuN in green). The presence of BDA in the brain suggests BBB disruption in epilepsy as the size of the molecule prevents brain entry when BBB is intact (n = 3 mice injected, BDA detectable in all 3).

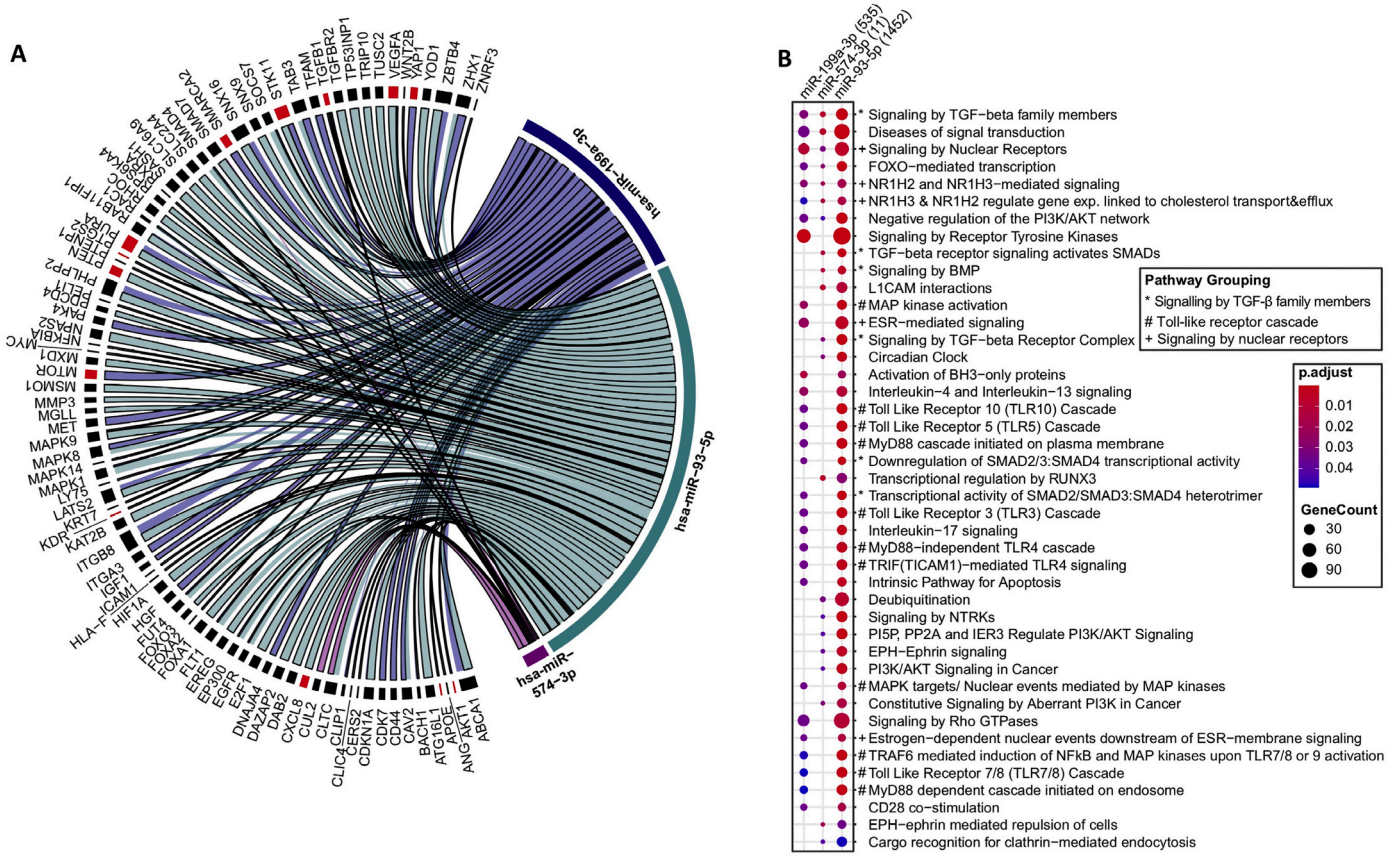

Fig. 7. Gene ontology and miRNA-targeting analysis. (A) Circos plot showing experimentally validated targets (with strong low-throughput evidence) and targets in common among the three most differentially regulated miRNA in human TLE. The three miRNA and their targets are represented on the outside of the circle and connecting lines indicate interactions. Targets previously implicated in epilepsy are coloured red. Experimentally validated interactions are outlined in black. The weight of connecting lines corresponds to the miRDIP predicted interaction score (Tokar et al., 2018), and interactions with experimental evidence are outlined in black. (B) Significantly enriched Reactome pathways for the high confidence mRNA targets of hsa-miR-93-5p, hsa-miR-199a-3p and hsa-miR-574-3p. The total number of mRNA targets included in the analysis is shown in brackets after the miRNA name. Adjusted *p*-values (p.adjust; Benjamini-Hochberg) < 0.05 were considered significant. Pathways significantly enriched in targets of 2 or more of the miRNAs are shown, and are sorted by the number of targeting miRNAs and descending sum of *p*-values (Supplemental file 2). Dot size indicates the number of mRNA in the pathway targeted by each miRNA (GeneCount). Related sub-pathways are grouped into their Reactome parent pathways: TGF-β, Signalling by TGF-β family members; TLR, Toll-like receptor cascade; NR, Signalling by nuclear receptors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

epilepsy associated miRNA targeting the TGFB1 protein. Red targets in the circus plot have been previously implicated in epilepsy. Reactome pathway enrichment analysis also identified multiple pathways targeted by 2 or more of these miRNA (Fig. 7B). Notably, several of these pathways relate to TGFβ and Toll-like Receptor (TLR) signalling. Nuclear receptor signalling is also targeted. These findings indicate potential overlapping mechanisms through which these miRNAs could exert their affects in epilepsy.

4. Discussion

The present study, through an initial discovery phase in rodent models of epilepsy and subsequent validation in humans, identifies a set of circulating miRNA which represent potential biomarkers of temporal lobe epilepsy. Our study performed initial miRNA profiling in three preclinical rodent models of epilepsy to overcome the possibility of identifying model/species-specific miRNA changes which are not relevant to the human condition. The validity of this approach was confirmed, when we found that three of the miRNA dysregulated in rodent models of epilepsy were also dysregulated in plasma from epilepsy patients compared to healthy control plasma. Interestingly, patients with NEAD did not have altered levels of epilepsy-associated peripheral biomarkers. An experimental disease-modifying therapy restored levels of several of the biomarkers to baseline whereas anti-seizure drugs did not, further validating these miRNA as diagnostic of epilepsy rather than acute seizures. Finally, we report the presence of these miRNA in neurons and microglia, while *in-silico* analysis of their targets reveal potential roles in the regulation of molecular and cellular mechanisms associated with epileptogenesis. Together the results support the use of circulating miRNA as a potential class of molecular biomarker to aid in the diagnosis of epilepsy.

Recent investigations have identified potential miRNA biomarkers for several neurological conditions in biofluids such as CSF (Raoof et al., 2017; Wiedrick et al., 2019), peripheral blood plasma (Manna et al., 2018), umbilical cord blood plasma (Brennan et al., 2018) and tears (Kenny et al., 2019). Previously we have analysed miRNA profiles in human CSF (Raoof et al., 2017) and human plasma samples (Raoof et al., 2018) from patients with TLE. Both biofluids contained differentially expressed miRNA in epilepsy patients compared to controls however there was little overlap between the two, suggesting the use of either biofluid may uncover epilepsy-associated miRNA biomarkers. For this study we focussed on plasma miRNAs for several reasons. Firstly they are more abundant per microlitre in plasma than in CSF (Raoof et al., 2017, 2018) enabling more rapid and accurate profiling which might be more useful in the clinical setting. Second, the use of plasma allows for repeated sampling in a non-invasive way in patients compared to CSF sampling. CSF is not routinely sampled from epilepsy patients in many countries. Finally plasma is less prone to some of the

contamination issues which serum is affected by (Moldovan et al., 2014; Wang et al., 2012).

In the present study, we employed an initial discovery phase whereby we profiled peripheral plasma miRNA at key stages of disease (epileptogenesis and chronic epilepsy) from serially taken samples from three well established and widely employed pre-clinical models of epilepsy. This is the first use of multiple models to discover plasma miRNA biomarkers of epilepsy, offering important advantages over previous studies. First, by profiling plasma from three different models in two different species we reduced the risk of identifying model- or species-specific changes in miRNA levels as disease related biomarkers. As each model was performed by a different laboratory we further minimise single or novice user bias. Second, by taking serial samples from the same individual animals it allowed us to follow the expression profile of individual miRNAs as each animal went through the different stages of the disease and reduces animal-animal variability. Constant video EEG was employed in each model to confirm disease stage. Epileptogenic blood samples were collected 48 h post SE from both the IAKA model and the Pilo model. No seizure activity was detected at this time-point which correlates with previous studies from our group and others (Mouri et al., 2008; Smith et al., 2018). We collected serially sampled blood from PPS rats at early, mid and late epileptogenic timepoints (again confirmed with EEG) and a final blood sample was collected upon confirmation of spontaneous seizures from each model. We followed recommendations on miRNA biomarker profiling in rodent biofluids including using repeated sampling from the same animal, appropriate collection tubes, plasma over serum, appropriate hemolysis quality control steps and the use of fragment analyser analysis of plasma RNA quality (van Vliet et al., 2017). Our analysis of baseline plasma miRNA revealed a plasma profile which was similar between mouse strains and between rodent species and strong overlap among the most abundant miRNA with healthy human plasma. This supports the validity of using rodent plasma as a discovery platform for human based biomarkers.

When analysing plasma from experimental animals we noticed a stronger overlap in miRNA plasma composition between models in the chronically epileptic animals than the epileptogenic groups. This is perhaps not surprising when we consider the strong influence of disease aetiology on gene expression. Indeed, a previous study investigating DNA methylation pattern commonalities among different rodent models of epilepsy found vastly divergent methylation patterns in each model (Debski et al., 2016). Even among the IAKA model and pilocarpine models, both performed in mice and both chemoconvulsant models of epilepsy, the miRNA profiles during epileptogenesis were more disparate than the chronically epileptic mice. This leads us to speculate that the precipitating insult is the key influence on the biomarker profile during epileptogenesis. Upon establishment of chronic epilepsy, a more signature miRNA profile emerges, regardless of aetiology. This raises challenges for identifying a biomarker of epileptogenesis in humans. It may be necessary, therefore, to sub-phenotype patients according to presumed aetiology for biomarker profiling. For example, tailoring the search for molecular signatures of epileptogenesis to very specific (sub) types of insult such as TBI or stroke. Anti-epileptogenic treatments may too need to be tailored to the causal insult.

OpenArray profiling identified a set of three miRNAs which were differentially expressed in plasma from chronically epileptic animals in three animal models (miR-93-3p, miR-574-3p and miR-182-5p). Dysregulation of each miRNA was confirmed by independent assays. These are all novel plasma miRNA biomarkers of epilepsy, supporting the benefit of our multi-model discovery strategy. Of particular interest for biomarker identification are those molecules whose expression is limited to plasma in a given cohort of individuals (e.g. found only in controls or only in epilepsy). We chose to further investigate a number of miRNA which were found only in the plasma from rodents with epilepsy and were undetectable in healthy animals as these are likely easier to interpret when developing a molecular diagnostic test (miR-199a-3p, miR-182-5p).

The present study shows high translatability of rodent model-identified miRNA biomarkers to clinical epilepsy. Using plasma from healthy individuals and from patients admitted to the EMU who had a diagnosis of TLE, we validated changes for miR-93-5p, miR-199a-3p and miR-574-3p as dysregulated in human epilepsy. Notably, while we were able to detect miR-199a-3p in control patients, control animals did not have detectable levels. Because we used samples from each epilepsy patient before and after seizures we demonstrate the potential biomarkers to be predictive of the disease itself rather than an acute response to seizure activity and therefore likely to represent underlying pathological mechanisms of epilepsy.

While this is the first study to propose these specific miRNA as circulating biomarkers of epilepsy, prior studies have reported dysregulation of these miRNA in experimental and human epilepsy in brain. Specifically, miR-199a-3p is significantly upregulated in experimental TLE in rat while levels of the opposing arm, miR-199a-5p, are found at elevated levels in blood from patients with TLE with hippocampal sclerosis. Similarly, levels of miR-93-5p and miR-142-5p are upregulated in tissue from experimental rodent models of epilepsy while miR-574-3p is downregulated in synaptoneurosomes from epileptic rats (Gorter et al., 2014; Risbud and Porter, 2013). The current data therefore suggests that differential levels of miRNA in plasma may reflect and mirror transcriptional and pathway-specific changes which occur in the diseased brain or in the pathogenesis of epilepsy. We used high-confidence target and target pathway analysis to identify potential involvement of the identified miRNAs in the pathogenesis of epilepsy. Indeed we found that many validated targets of the dysregulated miRNAs have been implicated in epilepsy and epilepsy-associated conditions like Tuberous Sclerosis (Rensing et al., 2015; Tsai et al., 2014). These included PTEN, MTOR, CXCL8 and components of the TGFβ signalling pathway including TGFβ1. A recent complimentary study by our group analysed dysregulation of Ago2-bound miRNAs in brain using these same models of epilepsy and identified TGFβ signalling as enriched among miRNA targeted pathways in epilepsy (Venø et al., 2020). This suggests that the miRNAs identified in the current study may functionally contribute to the pathogenesis of the disease. We also see strong regulation of TLR mediated signalling pathways and apoptosis pathways, both of which have been implicated in epilepsy (Gross et al., 2017; Korgaonkar et al., 2020; Vezzani et al., 2019).

The composition of peripheral blood plasma is influenced by the complex effects of age, diet, lifestyle and environmental factors. Plasma from diseased patients is also likely influenced by the disease itself but also drug regimens patients may be exposed to. This can hamper the identification of bona fide biomarkers of disease and is difficult to control for. To exclude the possibility that dysregulation of miRNA identified in our study may result from drug influences we dosed mice with common anti-epileptic/convulsant drugs. To include drug refractory effects in our model we dosed epileptic mice with both carbamazepine, which is ineffective in the IAKA model and diazepam, which has potent anti-convulsant effects in our model (Raoof et al., 2018). Neither carbamazepine nor diazepam had any influence on the miRNA we tested, suggesting that these miRNA at least, are more closely associated with epilepsy and disease processes rather than external influences or transient seizure- or anti-seizure effects. This is perhaps an additional advantage of focussing on miRNA whose expression is limited to plasma in the diseased state. While it was encouraging that neither drug affected the levels of these plasma miRNA we cannot discount that other drugs administered to patients as well as certain diets/lifestyle factors may affect the levels of these miRNA which might limit their use as biomarkers of disease.

To further consolidate our hypothesis that dysregulation of these miRNA is associated with the presence of epilepsy we performed an intervention study whereby we blocked epilepsy development in mice following an epilepsy-inciting insult and then measured the levels of

miRNA in plasma and compared to chronically epileptic mice. MiR-134 inhibition using specific antagomirs has been shown to effectively abolish epileptogenesis when delivered soon after an epilepsy-inciting event in multiple models of epilepsy and across multiple species (Jimenez-Mateos et al., 2015; Jimenez-Mateos et al., 2012; Reschke et al., 2017). The anti-epileptogenic mechanism of this intervention is thought to be partially mediated via the rescue of the LimK1 protein which is a critical regulator of dendritic spine structure (21). When we block epileptogenesis in a cohort of mice and then measure plasma miRNA 2 weeks later when epilepsy is usually well established in this model, we were no longer able to detect dysregulation of epilepsy-associated miRNA miR-93-5p, miR-574-3p, miR-199a-3p and miR-182-5p. This suggests that the levels of these miRNA are linked with the development of disease and are unlikely to represent the molecular milieu following an epilepsy-inciting event. Furthermore, this data suggests that miRNA plasma levels could be useful in tracking the efficacy of novel anti-epileptogenic therapies.

Finally, we sought to establish potential mechanisms of persistent miRNA dysregulation in plasma in epilepsy. To determine the potential cellular origin of the identified biomarkers we measured neuronal and microglial specific expression of each miRNA and detected each one at abundant levels in both cell types. The entry mechanisms of brain-derived miRNA into peripheral plasma remains unknown, particularly at disease stages (chronic epilepsy) where it is unclear whether there is still BBB disruption which would facilitate entry of miRNA from the brain into peripheral circulation. We previously found IgG and albumin entry into the brain parenchyma in epileptic mice and a recent study by Ruber et al. presented evidence that a single brief seizure could elicit BBB leakage and allow brain-specific proteins including S100β gain entry to the peripheral circulation (Michalak et al., 2012; Ruber et al., 2018). In the current study we found that BDA, which has a molecular size of 70 kDa can cross the BBB of chronically epileptic mice providing further evidence of BBB disruption in chronic epilepsy which might facilitate the entry of disease-associated biomarkers from the site of disease into peripheral blood plasma even in chronic epilepsy.

While the battery of validation assays we performed provide confidence that miR-93-5p, miR-199a-3p and miR-574-3p plasma levels robustly correlate with epilepsy, there are a number of study limitations which should be considered and which should direct future studies. First, we only selected a number of interesting miRNA targets and it is highly likely that we excluded other potential biomarkers. Second, by concentrating only on miRNA common to multiple rodent models it is possible that we exclude potential human biomarkers which are mirrored only in one animal model. As our patient cohort were mostly TLE patients we cannot say that these miRNA can differentiate between different types of epilepsy, it is possible that analysis of plasma from other types of epilepsy would also contain dysregulated miR-93-5p, 199a-3p and 574-3p. We also cannot discount that these miRNA may signal a neurological disease and not be entirely specific for epilepsy. Inclusion of plasma from patients with other neurological diseases such as multiple sclerosis, Alzheimer's Disease etc. would enhance the utility of these molecules. Future studies might determine whether this is the case or whether miRNA could assist in diagnosis of subclasses of epilepsy. When analysing human plasma samples we have not adjusted for time-of-day sampling, patient diet, fasting etc. which could all influence the levels of plasma miRNA. These effects however are partly mitigated by sourcing all patients from a single test centre. Finally analysis of potential cellular origin of epilepsy biomarkers was characterised using non-epileptic transgenic mice. It is possible that the cellular origin in the epileptic brain may differ due to context-dependent signalling.

In conclusion, using a multi-rodent, multi-model approach, and including an extensive set of validation experiments we have identified a set of miRNA which may be biomarkers of epilepsy and assist in the diagnosis of the disease. We demonstrate that these miRNA are also dysregulated in human TLE patient plasma and can be readily profiled. Additionally, we show that dysregulation of these specific miRNA is related to the presence of disease as intervention or inhibition of epileptogenesis in animals destined to become epileptic prevents disruption of the levels of these miRNA. Although further efforts are required to identify biomarkers of epileptogenesis, altogether these data suggest that a miRNA or panel of miRNA may assist in the diagnosis of epilepsy.

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Declaration of competing interest

None.

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