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RESEARCH ARTICLE

Plasma microRNAs as prognostic biomarkers for development of severe epilepsy after experimental traumatic brain injury—EpiBioS4Rx Project 1 study

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Abstract

Objective: To test a hypothesis that acutely regulated plasma microRNAs (miRNAs) can serve as prognostic biomarkers for the development of post-traumatic epilepsy (PTE).

Methods: Adult male Sprague–Dawley rats ($n = 245$) were randomized to lateral fluid-percussion–induced traumatic brain injury (TBI) or sham operation at three study sites (Finland, Australia, United States). Video-electroencephalography (vEEG) was performed on the seventh post-injury month to detect spontaneous seizures. Tail vein plasma collected 48 h after TBI for miRNA analysis was available from 209 vEEG monitored animals (45 sham, 164 TBI [32 with epilepsy]). Based on small RNA sequencing and previous data, the seven most promising brain enriched miRNAs (miR-183-5p, miR-323-3p, miR-434-3p, miR-9a-3p, miR-124-3p, miR-132-3p, and miR-212-3p) were validated by droplet digital polymerase chain reaction (ddPCR).

Results: All seven plasma miRNAs differentiated between TBI and sham-operated rats. None of the seven miRNAs differentiated TBI rats that did and did not develop epilepsy ($p > .05$), or rats with ≥3 vs <3 seizures in a month ($p > .05$). However, miR-212-3p differentiated rats that developed epilepsy with seizure clusters (i.e., ≥3 seizures within 24 h) from those without seizure clusters ($.34 \pm .14$ vs $.60 \pm .34$, adj. $p < .05$) with an area under the curve (AUC) of .81 (95% confidence interval [CI] .65–.97, $p < .01$, 64% sensitivity, 95% specificity). Lack of

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elevation in miR-212-3p also differentiated rats that developed epilepsy with seizure clusters from all other TBI rats ($n = 146$, $.34 \pm .14$ vs $.55 \pm .31$, $p < .01$) with an AUC of .74 (95% CI .61–.87, $p < .01$, 82% sensitivity, 62% specificity). Glmnet analysis identified a combination of miR-212-3p and miR-132-3p as an optimal set to differentiate TBI rats with vs without seizure clusters (cross-validated AUC .75, 95% CI .47–.92, $p < .05$).

Significance: miR-212-3p alone or in combination with miR-132-3p shows promise as a translational prognostic biomarker for the development of severe PTE with seizure clusters.

KEYWORDS
harmonization, lateral fluid-percussion, post-traumatic epilepsy, preclinical, receiver-operating characteristic analysis, video-EEG monitoring

1 | INTRODUCTION

Traumatic brain injury (TBI) is a major etiology for epilepsy, causing about 15% of structural and 5% of all epilepsies.[1] More than 20 preclinical proof-of-concept studies have shown favorable anti-epileptogenic or disease-modifying effects in rodent models of post-traumatic epilepsy (PTE); however, none has progressed to clinical trials.[2] A major reason for the slow progress from laboratory discoveries to the clinic is the lack of prognostic biomarkers that could be used to select TBI patients at high risk for developing PTE for enrollment into anti-epileptogenesis clinical trials. Such a prognostic biomarker would reduce the overall number of patients required to be recruited, and consequently the duration and cost of the trials.[3,4]

Due to its easy accessibility and repeatability, blood is an appealing biofluid for the discovery of microRNA (miRNA) and other molecular biomarkers for PTE. miRNAs are short (~22-nucleotide) non-coding RNAs that function in the post-transcriptional regulation of gene expression.[5,6] Many miRNAs regulate cellular processes that are involved in epileptogenesis, such as dendritic plasticity, neuroinflammation, and neurodegeneration.[7] Several studies have reported regulated miRNA expression in the brain and blood of patients with epilepsy.[8,9] Altered plasma or serum levels of brain-enriched miRNAs have also been detected after TBI in preclinical models,[10–12] and in patients with TBI.[9] Importantly, some of the regulated circulating miRNAs are common to epilepsy and TBI, such as miR-212, miR-132, and miR-9.[11–15] However, none has yet been shown to be a prognostic miRNA biomarker for the development of PTE.

The Epilepsy Bioinformatics Study for Antiepileptogenic Therapy (EpiBioS4Rx) is a National Institute of Neurological Disorders and Stroke (NINDS)-funded multicenter project that aims to facilitate the development of antiepileptogenic treatments after TBI, including the identification of preclinical biomarkers (https://epibios.loni.usc.edu/). One of its objectives is to overcome the low reproducibility in preclinical studies related to small sample size and variability in the methods between laboratories.[16,17] To improve reproducibility, preclinical EpiBioS4Rx Project 1 is performed in a multicenter design at three different study sites located in Finland (University of Eastern Finland (UEF)), Australia (Monash University), and the United States (University of California, Los Angeles [UCLA]).[18,19] In all centers, PTE was induced in rats by using lateral fluid-percussion–induced TBI, which has been demonstrated to trigger epileptogenesis by several laboratories.[20–22] Model production and blood-sampling procedures were harmonized between the study sites.[19] Consequently, we were able to analyze large sample numbers from PTE-phenotyped animals, which could not have been generated with the resources of a single laboratory.

Key points

- This is the first large-scale, preclinical, multicenter study on microRNA (miRNA) biomarkers in epilepsy.
- miR-212-3p, alone or in combination with miR-132-3p, is a sensitive and specific prognostic biomarker for severe post-traumatic epilepsy with seizure clusters after lateral fluid-percussion–induced traumatic brain injury.
- Intracerebral electrode implantation, craniotomy, and early seizures can affect plasma miRNA levels.
- Preclinical multicenter studies are feasible for the discovery of circulating molecular biomarkers.

To advance the discovery of circulating prognostic biomarkers for PTE that could be translated to clinical use, we hypothesized that some of the circulating miRNAs that show altered expression at acute post-TBI phase will present as prognostic biomarkers for the later development of PTE and/or its severity.

2 | MATERIALS AND METHODS

The study design summarized in Figure 1A was applied and harmonized at all study sites (UEF, Monash, and UCLA).[18,19] Small RNA sequencing and droplet digital polymerase chain reaction (ddPCR) were used to analyze plasma miRNAs on D2 after TBI or sham operation. ddPCR analysis also included baseline (BL) samples. Figure 1B shows a flowchart of the animal numbers for the small RNA sequencing and ddPCR analysis. Methods and additional results are described in Figures S1–S21 and Tables S1–S9.

3 | RESULTS

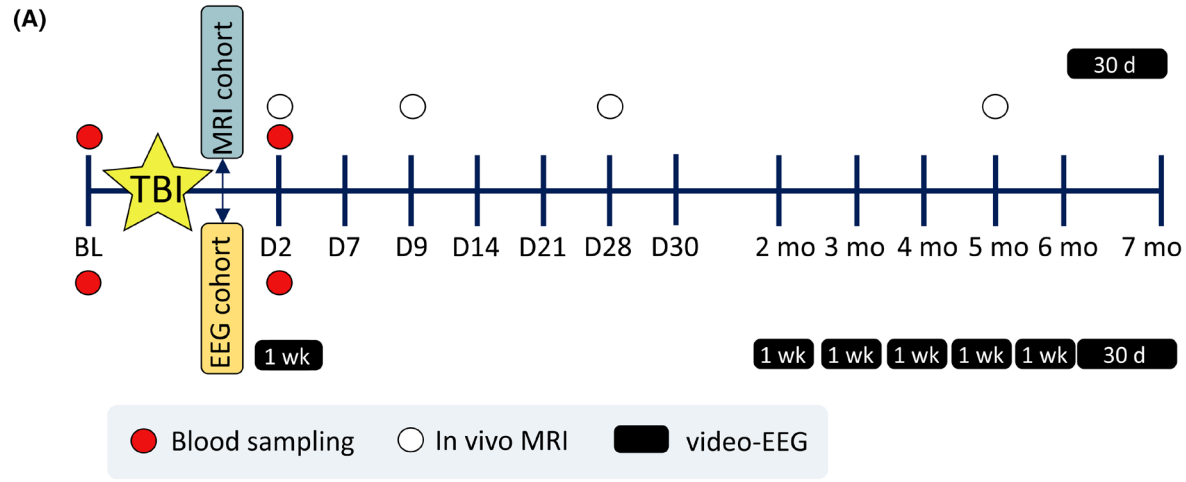
3.1 | Selection of miRNAs for analysis

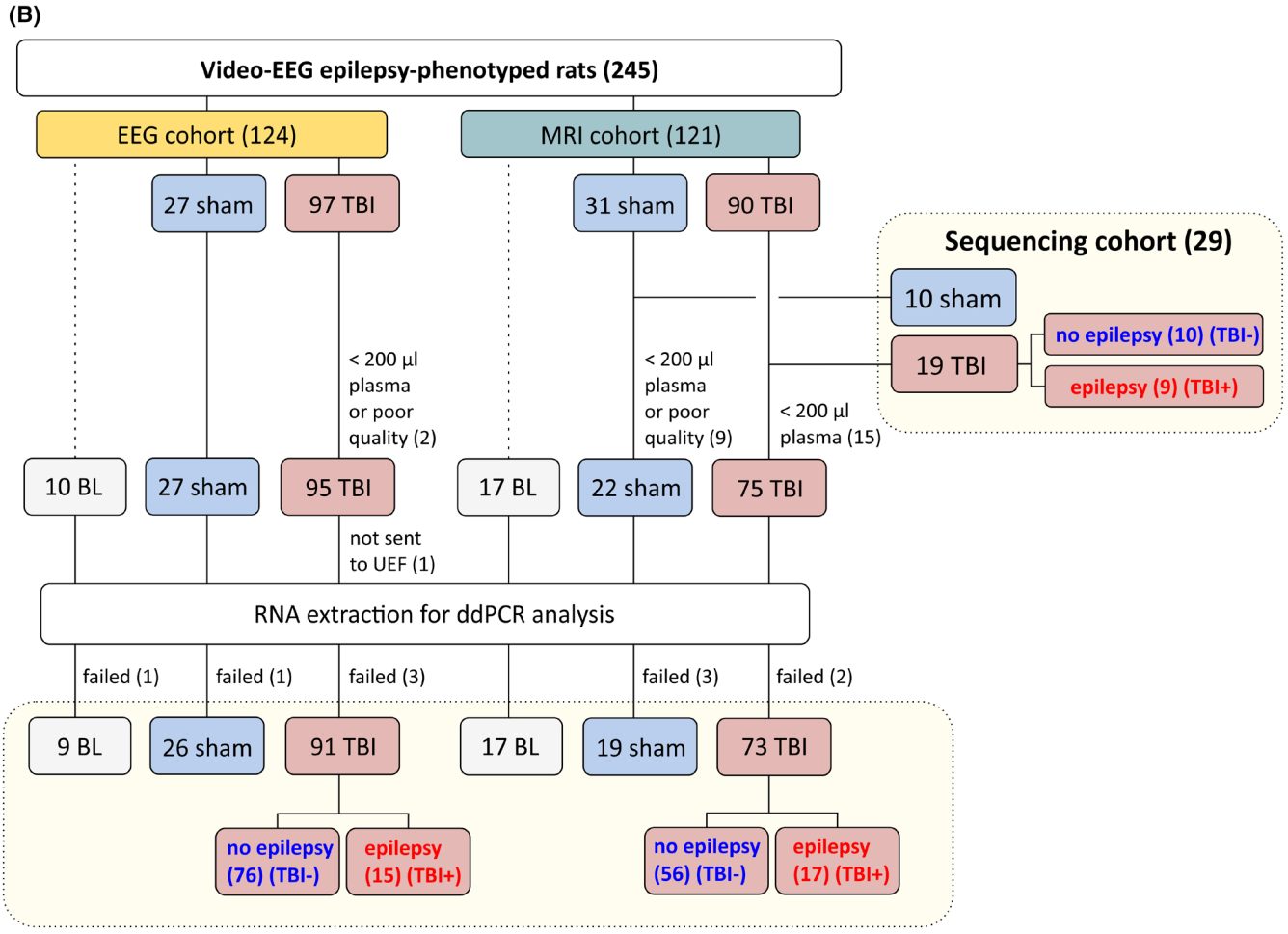
The miRNAs for ddPCR analysis were selected from the list of 23 differentially expressed (DE) miRNAs between the sham and TBI groups based on miRNA sequencing (Tables S1–S3). We selected miRNAs that were brain enriched,[23,24] upregulated in TBI group with log2FC ≥1.0, and had a mean count per million (CPM) mapped reads ≥30, as our preliminary studies revealed that miRNAs with lower CPM are unlikely detected by PCR. Consequently, rno-miR-323-3p (log2FC 1.24, mean CPM 44), rno-miR-434-3p (log2FC 1.20, mean CPM 286), and rno-miR-183-5p (log2FC 1.09, mean CPM 661) were selected. The list of three selected miRNAs was complemented by four additional miRNAs—rno-miR-9a-3p, rno-miR-124-3p, rno-miR-132-3p, and rno-miR-212-3p—which were not differentially expressed in the DESeq2 analysis, but which we have previously found upregulated in rat plasma on D2 after lateral fluid-percussion (FPI)-induced TBI.[10–12]

3.2 | Robust injury effect on expression of plasma miRNAs on D2

First, we analyzed whether the plasma levels of the seven analyzed miRNAs on D2 were regulated by TBI or sham-operation (Figure 2). The number of samples available from each study site is summarized in Table S4. Reproducibility of the injury effect on plasma miRNAs by each study site is summarized in Supplementary

FIGURE 1 Study design and a flowchart of animals included in the plasma micro ribonucleic acid (miRNA) analysis. (A) The EpiBioS4Rx Project 1 is a National Institute of Neurological Disorders and Stroke (NINDS)-funded Centers-Without-Walls international multicenter study, designed to perform statistically powered preclinical biomarker discovery and validation. We expected to find a miRNA biomarker that will differentiate the rats with traumatic brain injury (TBI) and epilepsy (TBI+) from those without epilepsy (TBI–) with area under curve (AUC) .700 ($p < .05$ as compared to .500; χ^2 test, MedCalc software).[18,19] At each of the three study sites located in Finland (University of Eastern Finland), Australia (Monash University, Melbourne University) or United States (David Geffen School of Medicine at University of California, Los Angeles), the rats were divided into electroencephalography (EEG) and magnetic resonance imaging (MRI) cohorts. In each cohort, the rats were randomized into the sham or TBI groups using the random number generator in Excel. In both cohorts, blood was collected at the baseline (BL, naïve samples) and on day (D) 2, D9, and D28, and 5 months after TBI or sham-operation (BL and D2 highlighted in red in panel A). Blood collected at BL and on D2 were used for the miRNA analysis to identify early prognostic biomarkers for post-traumatic epilepsy. Both cohorts also underwent neuroscore testing before TBI and on D2, D7, D14, D21, and D28 after TBI. The rats in the MRI cohort were imaged on D2, D9, and D28–30, and 5 months after TBI or sham-operation. They were implanted with epidural and intracerebral electrodes at 6 months after injury and underwent a 1-month continuous video-EEG (vEEG) monitoring during the seventh post-TBI month to determine the epilepsy phenotype. Ex vivo MRI was performed after the rats were killed in the end of the study. The rats in the EEG cohort were implanted with the electrodes immediately after sham-operation or TBI induction, monitored for 1 week, and thereafter, monthly in 1-week periods. As in the MRI cohort, they also underwent a 1-month continuous monitoring during the seventh post-TBI month. A detailed description of the procedures and epilepsy phenotype of the study cohort has been presented by Ndode-Ekane et al.[18,19] (B) The D2 samples of the MRI cohort from all study sites were used for small RNA sequencing to discover differentially expressed miRNAs. These included 10 sham-operated and 19 TBI rats (9 with epilepsy [TBI+] and 10 without epilepsy [TBI–]) that had completed the vEEG monitoring on the seventh post-injury month. Then the differentially expressed plasma miRNA levels were investigated by ddPCR. The analysis included a total of 235 plasma samples (26 BL, 45 sham, 164 TBI) from all three study sites. Of these, 109 (17 BL, 19 sham, 73 TBI) were from the MRI and 126 (9 BL, 26 sham, 91 TBI) from the EEG cohort. Three samples from sham-operated and four from TBI rats were included both in the sequencing and ddPCR analysis The BL ("naïve") group, consisted of baseline samples from 18 TBI and 8 sham-operated rats. Causes and numbers (in parenthesis) for sample exclusions are indicated in panel B No samples were excluded based on miRNA levels detected.





Materials (paragraph 2.3), Figures S10 and S11, and Table S6.

3.2.1 | Sham vs BL (naïve)

In the entire animal cohort, sham-operated rats (with craniotomy) had 6.5-fold increased miR-323-3p [.13 ± .19 vs .02 ± .01, adjusted (adj.) $p < .05$], 14-fold increased miR-9a-3p (.42 ± .68 vs .03 ± .04, adj. $p < .001$), 8.9-fold increased miR-124-3p (1.51 ± 1.97 vs .17 ± .27, adj. $p < .001$), and 2-fold increased miR-132-3p (.84 ± .74 vs .43 ± .13, adj. $p < .05$) compared with the BL. The levels of miR-183-5p, miR-434-3p, and miR-212-3p in the sham-operated controls were comparable to the BL samples ($p > .05$).

3.2.2 | TBI vs BL (naïve) or sham

miR-183-5p. TBI rats had 2.3-fold higher miR-183-5p levels compared with the BL samples (.68 ± .60 vs .30 ± .38, adj. $p < .001$) and 2-fold higher levels compared with the sham group (.68 ± .60 vs .34 ± .22, adj. $p < .001$).

miR-323-3p. TBI rats had 22.5-fold higher miR-323-3p levels compared with the BL samples (.45 ± .41 vs .02 ± .01, adj. $p < .001$) and 3.5-fold higher levels compared with the sham group (.45 ± .41 vs .13 ± .19, adj. $p < .001$).

miR-434-3p. TBI rats had 5.4-fold higher miR-434-3p levels compared with the BL samples (1.36 ± 1.16 vs .25 ± .17, adj. $p < .001$) and 2.5-fold higher levels compared with the sham group (1.36 ± 1.16 vs .55 ± .56, adj. $p < .001$).

miR-9a-3p. TBI rats had 27.7-fold higher miR-9a-3p levels compared with the BL samples (.83 ± .98 vs .03 ± .04, adj. $p < .001$) and 2-fold higher levels compared with the sham group (.83 ± .98 vs .42 ± .68, adj. $p < .001$).

miR-124-3p. TBI rats had 17.3-fold higher miR-124-3p levels compared with the BL samples (2.94 ± 3.88 vs .17 ± .27, adj. $p < .001$) and 1.9-fold higher levels compared with the sham group (2.94 ± 3.88 vs 1.51 ± 1.97, adj. $p < .001$).

miR-132-3p. TBI rats had 2.9-fold higher miR-132-3p levels compared with the BL samples (1.26 ± .78 vs .43 ± .13, adj. $p < .001$) and 1.5-fold higher levels compared with the sham group (1.26 ± .78 vs .84 ± .74, adj. $p < .001$).

miR-212-3p. TBI rats had 1.8-fold higher miR-212-3p levels compared with the BL samples (.53 ± .31 vs .30 ± .14, adj. $p < .001$) and 1.5-fold higher levels compared with the sham group (.53 ± .31 vs .36 ± .34, adj. $p < .001$).

In summary, all seven plasma miRNAs showed a clear injury effect, being elevated on D2 after TBI as compared to the sham or BL groups. Moreover, four of seven miRNAs were elevated in plasma on D2 after sham surgery as compared to BL.

3.3 | Injury effect on plasma miRNA was found both in the MRI and EEG cohorts

3.3.1 | Injury effect

Next we analyzed whether the injury effect was present in both cohorts and whether it was more pronounced in the electroencephalography (EEG) cohort with immediate post-injury intracerebral electrode operation than in the magnetic resonance imaging (MRI) cohort with intracerebral electrode implantation at 6 months (Figure 3, Table S5).

We found that miRNA levels on D2 were typically higher in the EEG than MRI cohort in both the sham-operated control and TBI groups, but not in samples collected at BL (no electrode implantation) (Supplementary Materials, paragraph 3.4). Reproducibility of injury effect on plasma miRNA levels at each study site when the EEG and MRI cohorts were analyzed separately is summarized in Supplementary Materials (paragraph 3.4, Figures S12–S15, Tables S6 and S7).

3.3.2 | Receiver-operating characteristic (ROC) analysis

Receiver-operating characteristic (ROC) analysis of normalized plasma miRNA levels was performed in the entire animal cohort, and thereafter, separately in the MRI and EEG cohorts (Figures 2B and 3C; Table S5).

Sham vs BL (naïve). In the whole cohort, all miRNAs analyzed except miR-212-3p differentiated sham-operated experimental controls from the BL with area under curve (AUC) ≥.65. The best-performing miRNA was miR-9a-3p (AUC .81, $p < .001$).

In the MRI cohort, none of the plasma miRNAs distinguished the sham-operated controls from the BL in ROC analysis. In the EEG cohort, five of seven miRNAs (all except miR-183-5p and miR-212-3p) separated the sham and BL samples with AUC ≥.75. The best-performing miRNA was miR-124-3p (AUC 1.00, $p < .001$).

TBI vs BL (naïve). In the whole cohort, all seven miRNAs differentiated TBI animals from the BL with AUC ≥.75. The best-performing miRNAs were miR-9a-3p and miR-323-3p (both AUCs .99, $p < .001$).

In the MRI cohort, all seven miRNAs differentiated the TBI and BL samples with AUC ≥.69. The best-performing miRNAs were miR-9a-3p and miR-323-3p (both AUCs .99, $p < .001$). Also in the EEG cohort, all seven miRNAs differentiated the TBI and BL samples with AUC ≥.77. The best-performing miRNAs were miR-9a-3p and miR-124-3p (both AUCs 1.00, $p < .001$).

TBI vs sham. In the whole cohort, all seven miRNAs differentiated TBI and sham with AUC ≥.71. The best performing miRNA was miR-323-3p (AUC .86, $p < .001$).

In the MRI cohort, all seven miRNAs differentiated the TBI and sham animals with AUC ≥.77. The best-performing miRNA was miR-323-3p (AUC .92, $p < .001$). Also in the EEG cohort, all seven miRNAs differentiated the TBI and sham animals with AUC ≥.67. The best-performing miRNA was miR-323-3p (AUC .84, $p < .001$).

In summary, all plasma miRNAs differentiated TBI animals from the sham or BL groups. Data were rather similar whether the entire cohort or the MRI and EEG sub-cohorts were analyzed. The differentiation between the sham and BL groups observed in the whole animal cohort related to elevated miRNA levels in sham-operated rats in the EEG cohort.

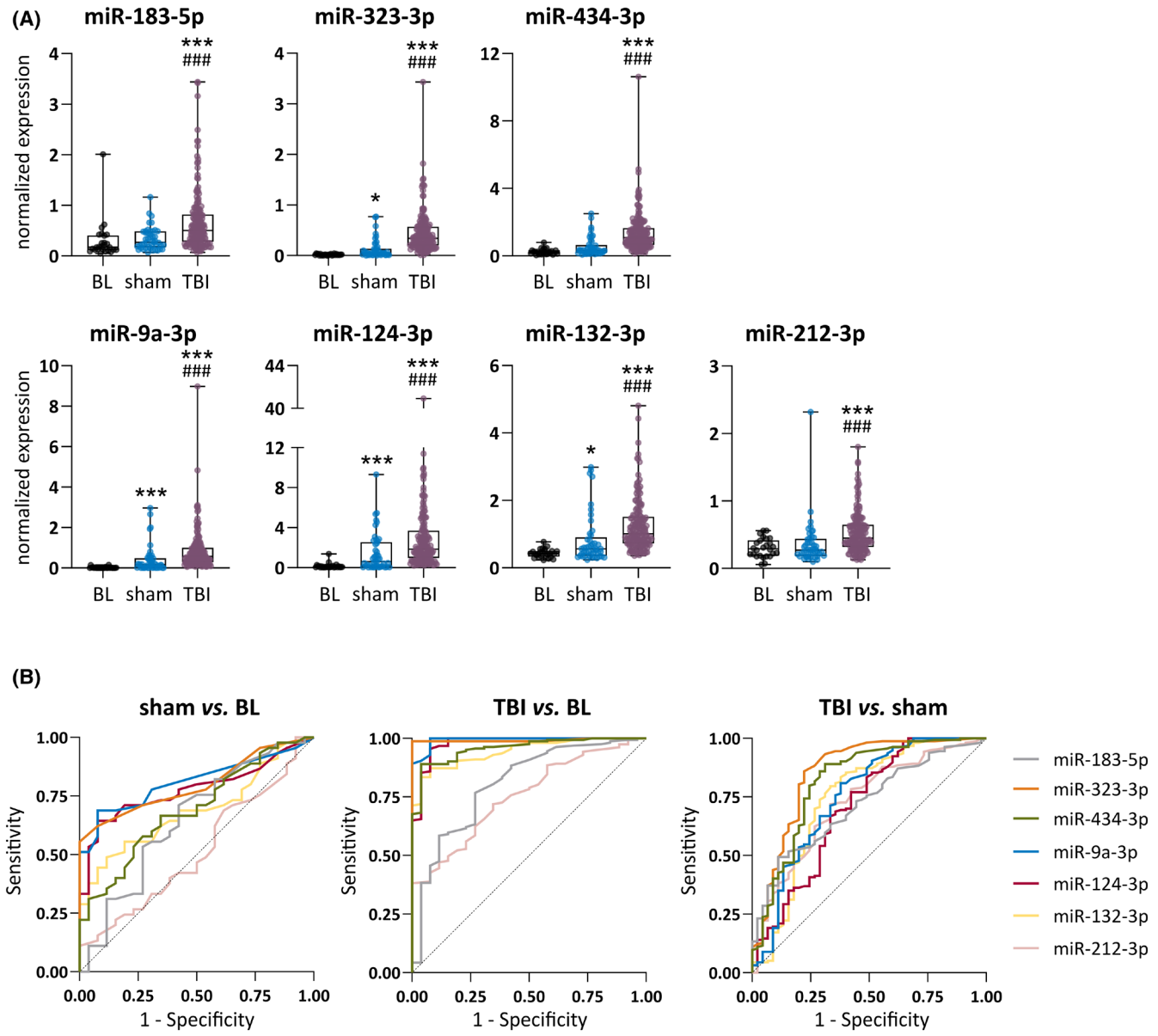


FIGURE 2 Was there an injury effect on expression of plasma miRNAs on D2 after TBI?—Yes. (A) Box and whisker plots (whiskers: minimum and maximum; box: interquartile range; line: median) showing the levels of miR-183-5p, miR-323-3p, miR-434-3p, miR-9a-3p, miR-124-3p, miR-132-3p, and miR-212-3p in rat plasma at baseline (BL) or on D2 after TBI or sham-operation. All seven miRNAs were increased in the TBI rats compared with BL samples or sham-operated controls. Furthermore, miR-323-3p, miR-9a-3p, miR-124-3p, and miR-132-3p were increased in the sham-operated controls compared with BL (naïve samples). Statistical significance: *$p < .05$; ***, $p < .001$ (compared with BL); ###, $p < .001$ (compared with sham). (B) ROC analysis showed that six of seven miRNAs (all except miR-212-3p, AUC .52) separated the sham-operated rats from BL, AUC ranging from .65 (miR-183-5p) to .81 (miR-9a-3p) (Table S5). All seven miRNAs separated the TBI rats from BL samples and from sham-operated controls. In the TBI vs BL analysis, the highest AUC was observed for miR-323-3p and miR-9a-3p, which both had AUCs of .99 (Table S5). In the TBI vs sham analysis, the highest AUCs were observed for miR-323-p (AUC .86) and miR-434-3p (AUC .82) (Table S5).

3.4 | Plasma miRNA as prognostic biomarkers for epileptogenesis and epilepsy severity after TBI

In the whole EpiBioS4Rx cohort, unprovoked late seizures were detected in 22% (41/187) of the TBI rats (TBI+ group) when the animals underwent 1-month vEEG monitoring during the seventh follow-up month.[19] Of the 32 TBI+ animals included in the miRNA analysis, 63% (20/32) had ≥3 seizures per monitoring month and 34% (11/32) had seizure clusters (≥3 seizures/24 h).[25]

Next we investigated whether plasma miRNA expression profile on D2 after TBI would serve as a prognostic biomarker for epileptogenesis, that is, whether the

elevations in plasma miRNA levels differed between the rats (a) that did (TBI+) or did not (TBI–) develop PTE, (b) with different epilepsy severities. Data analyzed in the whole animal cohort are shown in Figure 4 and Table 1. Data analyzed separately in the MRI and EEG cohorts are summarized in Supplementary Materials (paragraph 3.5) and in Figures S16–S18 and Table S8. Finally, data analyzed separately at different study sites are presented in Supplementary Materials (paragraph 3.6) and Figures S19 and S20 and Table S9.

3.4.1 | Plasma miRNA levels on D2 between epilepsy outcome groups

Epilepsy vs no epilepsy. The normalized miRNA expression levels for the TBI+ and TBI– groups are summarized in Figure 4A. There were no differences in D2 miRNA levels between the TBI+ and TBI– groups ($p > .05$ for all).

ROC analysis indicated that none of the seven plasma miRNAs separated the TBI+ rats ($n = 30$) from the TBI– rats ($n = 127$) on D2 after TBI (Figure 4D, Table 1).

Epilepsy severity—seizure frequency <3 vs ≥3 seizures per monitoring month. Next we investigated whether the plasma miRNA levels differed within the TBI+ group, depending on the epilepsy severity. TBI+ rats were categorized into two groups based on the seizure frequency (<3 seizures vs ≥3 seizures during the 1-month vEEG monitoring). However, we did not detect any differences in the miRNA levels between the seizure frequency groups ($p > .05$ for all miRNAs) (Figure 4B).

ROC analysis indicated that none of the seven plasma miRNAs separated the TBI+ rats with ≥3 seizures ($n = 20$) from TBI+ rats with <3 seizures ($n = 10$) on D2 after TBI (Figure 4D, Table 1).

Epilepsy severity – clusters vs no clusters. In addition to seizure frequency, we categorized TBI+ rats in two groups based on the occurrence of seizure clusters (≥3 seizures within 24 h) during the vEEG monitoring. Both ddPCR rounds contained 11 TBI+ rats with seizure clusters (TBI + C), whereas the number of TBI+ rats without clusters (TBI + noC) was 21 (round 1) or 19 (round 2).

Rats in the TBI + C group had lower levels of miR-212-3p ($n = 11$, $.34 \pm .14$) compared with the TBI + noC group ($n = 19$, $.60 \pm .34$, adj. $p < .05$) or TBI– group ($n = 127$, $.54 \pm .31$, adj. $p < .05$) (Figure 4C). The miR-212-3p levels in TBI + C group (n = 11) were also lower ($.34 \pm .14$ vs $.55 \pm .31$, $p < .01$) when compared to all other TBI rats (TBI + noC and TBI- combined, $n = 146$). No differences were observed in the other six miRNAs ($p > .05$).

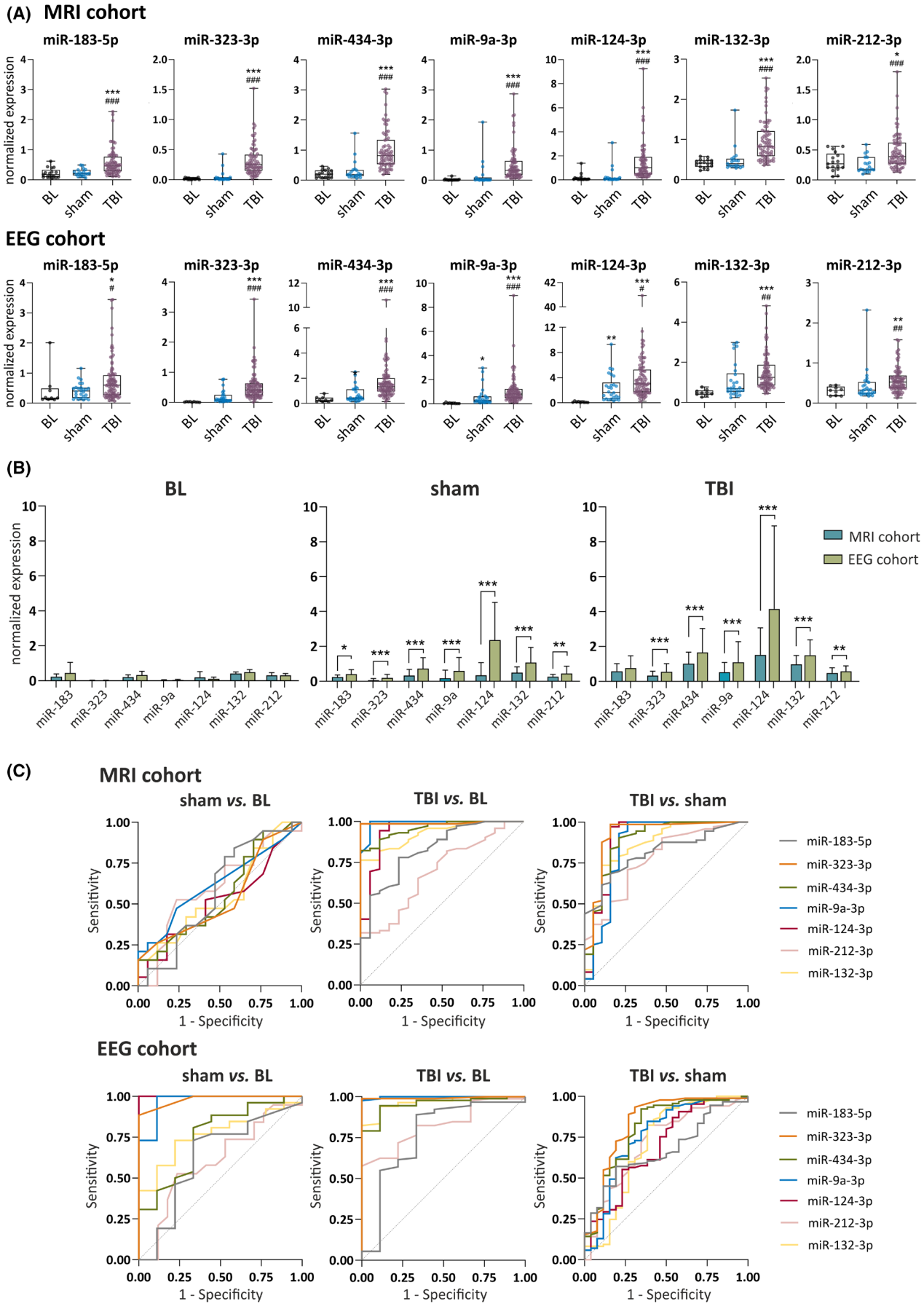
ROC analysis revealed that lower plasma miR-212-3p levels on D2 separated the TBI+ rats with seizure clusters (n = 11) from TBI+ rats without clusters ($n = 19$) with AUC .81 (95% CI .65–.97, $p < .01$) and 64% sensitivity and 95% specificity (cut-point .30). In addition, lower miR-212-3p levels separated the TBI+ rats with seizure clusters from all other TBI rats (TBI– and TBI + noC combined, $n = 146$) with AUC .74 (95% CI .61–.87, $p < .01$) and 82% sensitivity and 62% specificity (cut-point .39).

3.4.2 | Glmnet analysis supports D2 circulating miRNA levels as prognostic biomarkers for epilepsy severity

Finally, we used elastic-net-regularized logistic regression (glmnet) to determine the optimal combination of miRNAs to differentiate between the rats in the TBI+ and TBI– groups and in the epilepsy severity groups (Figure 5).

Epilepsy vs no epilepsy. Glmnet analysis did not identify any set of miRNAs that would differentiate the TBI+ rats ($n = 30$) from the TBI– rats ($n = 127$), as all coefficients were zero in the majority of the cross-validation folds.

FIGURE 3 Was the injury effect on plasma miRNAs found both in the EEG and MRI cohort?—Yes. (A) Box and whisker plots (whiskers: minimum and maximum; box: interquartile range; line: median) show that the levels of all seven investigated miRNAs (miR-183-5p, miR-323-3p, miR-183-5p, miR-9a-3p, miR-124-3p, miR-132-3p, and miR-212-3p) were increased in the plasma of TBI rats compared with the sham or BL groups both in the MRI and EEG cohorts. In addition, miR-9a-3p and miR-124-3p were increased in the sham-operated rats compared with the BL samples in the EEG but not in the MRI cohort. Statistical significance: *$p < .05$; **$p < .01$; ***$p < .001$ (compared with BL); #, $p < .05$; ##, $p < .01$; ###, $p < .001$ (compared with sham). (B) Bar graphs (mean and standard deviation) showing the differences between the MRI and EEG cohorts in individual miRNA levels separately in the BL, sham, and TBI groups. In the TBI group, the levels of six of seven miRNAs were higher in the EEG than MRI cohort (multiple Mann–Whitney tests with Benjamini-Hochberg FDR correction, adj. $p < .01$–$.001$). In the sham group, the levels of all seven miRNAs were higher in the EEG than MRI cohort (adj. $p < .05$–$.001$). At BL (no electrode operation or craniotomy), both cohorts had similar miRNA levels. (C) ROC curves. In the EEG cohort, five miRNAs (miR-323-3p, miR-434-3p, miR-9a-3p, miR-124-3p, and miR-132-3p) separated the sham from the BL samples on D2 with AUC .75–1.00. In the MRI cohort, none of the miRNAs differentiated the shams from the BL samples. In both the EEG and MRI cohorts, all miRNAs separated the TBI group from the BL and sham groups. Details of ROC analysis are summarized in Table S5.


(A) MRI cohort
miR-183-5p
miR-323-3p
miR-434-3p
miR-9a-3p
miR-124-3p
miR-132-3p
miR-212-3p
normalized expression
BL
sham
TBI
EEG cohort
(B)
BL
sham
TBI
MRI cohort
EEG cohort
miR-183
miR-323
miR-434
miR-9a
miR-124
miR-132
miR-212
(C)
MRI cohort
sham vs. BL
TBI vs. BL
TBI vs. sham
Sensitivity
1 - Specificity
miR-183-5p
miR-323-3p
miR-434-3p
miR-9a-3p
miR-124-3p
miR-212-3p
miR-132-3p
EEG cohort

Epilepsy severity—seizure frequency <3 vs ≥3 seizures per monitoring month. Glmnet analysis did not identify any set of miRNAs to differentiate the TBI+ rats categorized by seizure frequency. Furthermore, we investigated whether miRNA levels could differentiate the TBI+ rats with ≥3 seizures ($n = 20$) from all other TBI rats (TBI− and TBI+ <3 seizures combined, $n = 137$), but no miRNA combination was identified.

Epilepsy severity—clusters vs no clusters. Glmnet analysis identified miR-212-3p and miR-132-3p as the optimal set of miRNAs to differentiate TBI+ rats with seizure clusters ($n = 11$) from TBI+ rats without clusters ($n = 19$). The produced logistic regression model had a cross-validated AUC .75 (95% CI .47–.92, $p < .05$) (Figure 5A). Moreover, miR-212-3p and miR-132-3p were identified as the optimal miRNA set to differentiate TBI+ rats with seizure clusters from all other TBI rats (TBI− and TBI+ without clusters combined, $n = 146$) with a cross-validated AUC .67 (95% CI .42–.82, $p < .05$) (Figure 5B).

4 | DISCUSSION

Our objective was to identify prognostic plasma miRNA biomarkers for post-traumatic epileptogenesis in a rat model of PTE that could be translated into clinical trials.

4.1 | TBI induces a robust regulation of plasma miRNA levels

To identify potential miRNA biomarker candidates in rat plasma, we performed a small RNA sequencing of plasma samples collected on D2. This identified about 750 miRNAs, of which 9 were upregulated and 14 downregulated in the TBI group compared with sham-operated controls. Our validation strategy focused on brain-enriched upregulated miRNAs, increasing the likelihood of being detected using ddPCR. After preliminary ddPCR experiments, three upregulated DE miRNAs from the miRNA-seq data set (miR-183-5p, miR-323-3p, and miR-434-3p) and four miRNAs that we had previously demonstrated to be acutely upregulated in TBI rat plasma (miR-9a-3p, miR-124-3p, miR-132-3p, miR-212-3p) were included in validation.[10–12]

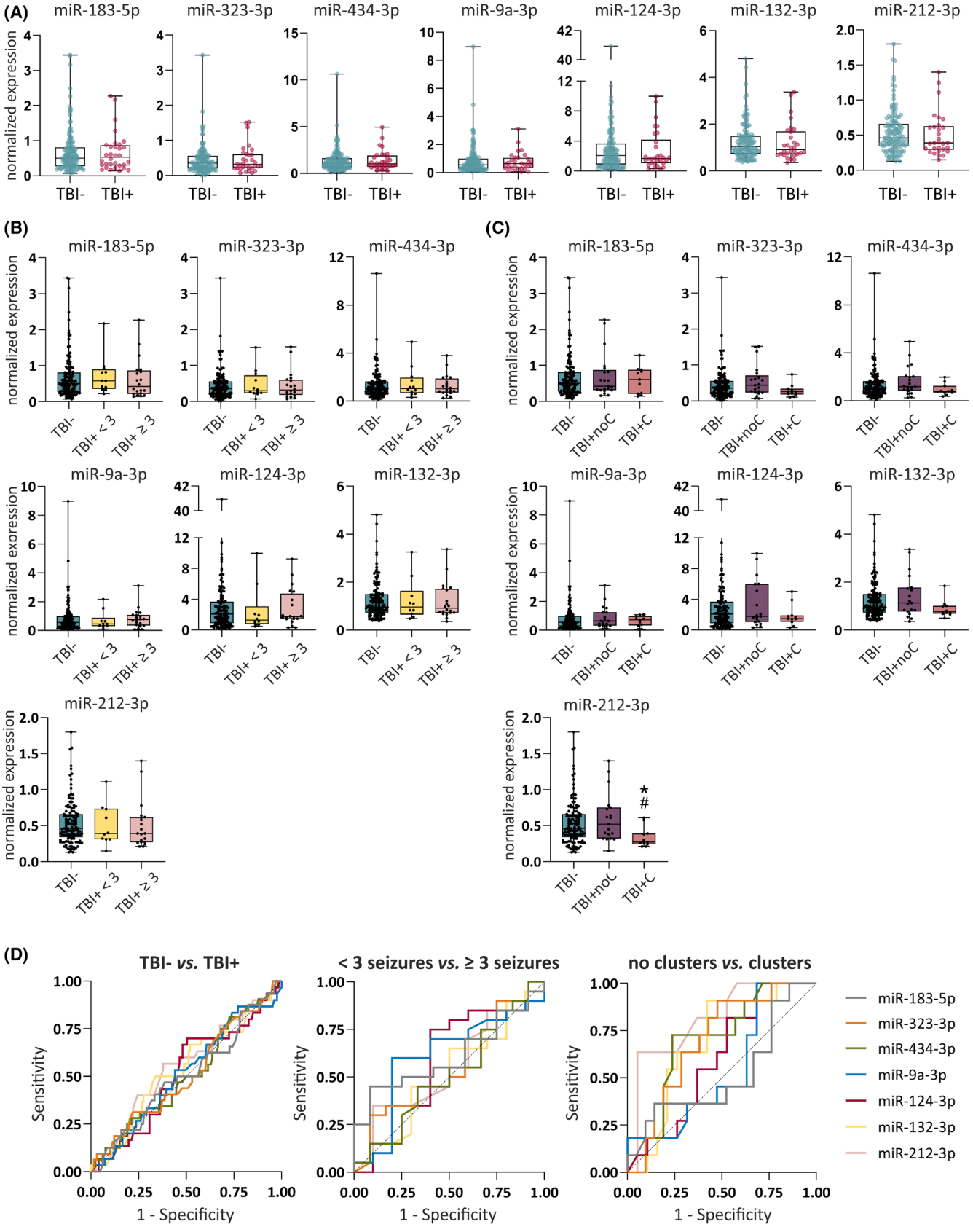
Validation of the selected miRNAs by ddPCR revealed that all seven miRNAs were upregulated 1.5- to 3.5-fold on D2 post-injury as compared to the sham group. The slightly elevated levels of miR-323-3p, miR-9a-3p, miR-124-3p, and miR-132-3p in the craniectomized sham group as compared to BL (naïve) samples suggest that these four miRNAs present potential diagnostic biomarkers for mild brain injury. Increased plasma miRNA levels after craniectomy were also reported in our earlier studies.[11,12] Moreover, we found that the levels of all investigated miRNAs except miR-183-5p were greater both in injured and sham-operated animals in the EEG cohort as compared to the MRI cohort. The difference between the cohorts may relate to acute brain injury from the electrode implantation in the EEG cohort. In this cohort, three bipolar intracerebral and four epidural electrodes were implanted right after the impact or craniectomy, about 2 days before plasma sampling for miRNA analysis. In the MRI cohort, electrode implantation was performed at the end of the fifth follow-up month, thus not affecting the plasma sampling on D2. Finally, data available from the UEF subcohort showed that rats with early seizures had elevated miR-183-5p levels, suggesting that early post-TBI seizures can also affect the plasma levels of some miRNAs.

Taken together, the results showed that severe TBI causes a robust regulation of plasma miRNAs on D2 post-injury. In addition, other procedures like craniectomy and intracerebral electrode implantation as well as early seizures can affect circulating miRNA levels. Craniectomy and early seizures are clinically relevant factors to be considered in the analysis of circulating miRNA levels in humans with severe TBI.

4.2 | Circulating miR-212-3p alone and in combination with miR-132-3p was identified as a potential prognostic biomarker for development of severe PTE

In the whole EpiBioS4Rx cohort, 22% of the TBI rats developed unprovoked late seizures and were defined as

FIGURE 4 Plasma miRNAs as prognostic biomarkers for epileptogenesis and epilepsy severity after TBI. (A) Box and whisker plots (whiskers: minimum and maximum; whiskers; interquartile range; line: median) showing that all investigated plasma miRNAs had similar expression levels on D2 after TBI in the rats with (TBI+) and without epilepsy (TBI−) (Mann–Whitney *U* test, $p > .05$ all). (B) No differences were detected in plasma miRNA levels between the TBI− group, TBI+ animals with <3 seizures, or TBI+ rats with ≥3 seizures (Kruskal–Wallis test, $p > .05$ for all). (C) TBI+ rats that experienced seizure clusters (TBI + C, $n = 11$) had lower plasma miR-212-3p levels on D2 after injury compared with the TBI+ rats without seizure clusters (TBI + noC, $n = 19$, adj. $p < .05$) or TBI− rats ($n = 127$, adj. $p < .05$). Statistical significance: *$p < .05$ (compared with TBI−); #$p < .05$ (compared with TBI + noC) (Kruskal–Wallis test followed by post hoc Dunn's test). (D) ROC analysis. None of the miRNAs differentiated the TBI− and TBI+ groups or the TBI+ rats with <3 seizures or ≥3 seizures. However, miR-212-3p levels differentiated the TBI + C rats from TBI + noC rats with AUC .81 (95% CI .65–.97, $p < .01$). Details of ROC analysis are summarized in Table 1.


(A)
miR-183-5p
miR-323-3p
miR-434-3p
miR-9a-3p
miR-124-3p
miR-132-3p
miR-212-3p
normalized expression
TBI-
TBI+
(B)
(C)
TBI+ < 3
TBI+ ≥ 3
TBI+noC
TBI+C
*
#
(D)
TBI- vs. TBI+
< 3 seizures vs. ≥ 3 seizures
no clusters vs. clusters
Sensitivity
1 - Specificity
0.00
0.25
0.50
0.75
1.00
miR-183-5p
miR-323-3p
miR-434-3p
miR-9a-3p
miR-124-3p
miR-132-3p
miR-212-3p

having PTE.[19] Of the animals with PTE, 34% showed seizure clusters and 63% had at least three seizures during the 1-month monitoring period. Next, we addressed the question: does the level of miRNA upregulation relate the risk of epileptogenesis or epilepsy severity? Even though RNA sequencing did not identify any differentially expressed miRNAs on D2 between the rats that did or did not develop PTE, we found a robust upregulation of some plasma miRNAs and variability in the count numbers between the animals, encouraging us to validate the top candidates with ddPCR in the whole cohort of 164 TBI animals. It is disappointing that ROC analysis revealed that none of the seven investigated miRNAs distinguished the TBI+ rats from the TBI− rats. It is important to note that we found that normalized miR-212-3p levels in TBI+ rats with seizure clusters were 63% of that in TBI+ rats without seizure clusters and 57% of that in TBI− rats. The lower elevation in miR-212-3p levels differentiated the TBI+ rats with seizure clusters from TBI+ rats without seizure clusters with an AUC .81. Moreover, the lower elevation in miR-212-3p levels separated the TBI+ rats with seizure clusters from all other TBI rats with an AUC .71.

Next, we investigated whether a combination of miRNAs would predict post-traumatic epileptogenesis better than a single miRNA by using elastic-net-regularized logistic regression (glmnet).[26] In correlation with our other analyses, algorithm did not identify any miRNA sets to separate the TBI+ and TBI− rats. However, the logistic regression model containing both miR-212-3p and miR-132-3p separated the TBI+ rats with seizure clusters from the TBI+ rats without seizure clusters with a cross-validated AUC .75 and from all other TBI rats with an AUC .67.

Our previous single-center study conducted in the EPITARGET cohort of 114 TBI rats, using a different small RNA sequencing platform, did not reveal plasma miR-212-3p or miR-132-3p as candidate prognostic biomarkers for PTE.[12] Because the current study in the EpiBioS4Rx cohort included six miRNAs (miR-323-3p, miR-434-3p, miR-9a-3p, miR-124-3p, miR-132-3p, and miR-212-3p) that were also analyzed in the EPITARGET cohort, we investigated whether combining the two cohorts, with a total number of 254 TBI animals in the ddPCR analysis, could improve the performance of the models in the glmnet analysis (Figure S21). Of interest, the analysis of the combined cohorts identified miR-212-3p as before and miR-9a-3p as the optimal miRNAs to distinguish the TBI+ rats with or without seizure clusters with an AUC .75. In addition, miR-212-3p, miR-9a-3p, and miR-124-3p were identified as the optimal set to distinguish TBI+ clusters group from all other TBI rats with an AUC .68.

Separate analysis of data generated by each study site revealed that the injury effect on most miRNAs was reproduceable at each site, particularly in the MRI cohort. However, analysis of miRNAs as prognostic biomarkers for PTE and epilepsy severity gave variable results, emphasizing the importance of large sample sizes for the analyses.

Taken together, our data suggest that the lowered trajectory of plasma miR-212-3p levels can predict the development of more severe PTE, reflected by seizure clusters, whereas development of "milder" PTE might remain undetected. Moreover, the performance of the logistic regression models obtained from the glmnet analysis of a large cohort of animals, combining the EpiBioS4Rx and EPITARGET cohorts, strengthened the data observed in the EpiBioS4Rx cohort only, suggesting that miRNA combinations can present prognostic biomarkers for the development of PTE with seizure clusters.

4.3 | miR-212-3p and miR-132-3p in epileptogenic process

The best combinatory predictors of severe PTE, miR-212-3p and miR-132-3p, belong to the same miRNA family and locate in the same cluster in the genome.[27] They are brain enriched both in humans and rodents[28–30] and are expressed in neurons[31,32] and glial cells.[33]

Previous studies have reported regulated expression of miR-212 and miR-132 in the brain tissue in several experimental models and human epilepsy. A meta-analysis of differentially expressed miRNAs across different status epilepticus models identified miR-212 and miR-132 to be consistently upregulated in acute, latent, and chronic stages of epileptogenesis,[34] particularly in the dentate gyrus[13,33,35] and hippocampus proper.[14,36–38] In human epilepsy, two studies have reported downregulation of miR-212 in the hippocampus of patients with temporal lobe epilepsy (TLE).[39,40] Cai et al. also reported decreased miR-212 levels in the serum of patients with TLE.[40] Hippocampal miR-132 was found upregulated in children with mesial TLE[41] and in patients with TLE and hippocampal sclerosis.[33] However, temporal neocortex miR-132 expression was decreased in patients with TLE.[42]

Neuronal miR-212 and miR-132 are involved in the regulation of dendritic maturation[43] and synaptic plasticity.[44,45] Suppression of miR-212 by a long non-coding RNA in astrocytes was found to increase the expression of matrix metalloproteinases, which have been linked to epileptogenesis.[40,46] Silencing of miR-132 had a neuroprotective and seizure-suppressing effect in rodent epilepsy

TABLE 1 ROC analysis—Plasma miRNAs as prognostic biomarkers for epileptogenesis and epilepsy severity after TBI.

Comparisons	ROC AUC (*p*-value)	95%CI	Cutoff	Sensitivity (%)	Specificity (%)
miR-183-5p					
TBI+ vs TBI−	.504 (ns)	.39–.62	≤.42	47	61
≥3 sz vs <3 sz	.608 (ns)	.41–.80	≥.31	45	92
clusters vs no clusters	.517 (ns)	.29–.74	≤.23	36	86
miR-323-3p					
TBI+ vs TBI−	.511 (ns)	.40–.62	≥.58	31	78
≥3 sz vs <3 sz	.540 (ns)	.33–.75	≤.20	30	92
clusters vs no clusters	.701 (ns)	.52–.88	≤.42	91	52
miR-434-3p					
TBI+ vs TBI−	.505 (ns)	.39–.62	≥.68	81	27
≥3 sz vs <3 sz	.515 (ns)	.30–.73	≤.87	45	67
clusters vs no clusters	.697 (ns)	.51–.88	≥.87	73	76
miR-9a-3p					
TBI+ vs TBI−	.515 (ns)	.40–.63	≥.26	87	23
≥3 sz vs <3 sz	.608 (ns)	.38–.83	≥.69	60	80
clusters vs no clusters	.543 (ns)	.33–.76	≤1.08	100	32
miR-124-3p					
TBI+ vs TBI−	.512 (ns)	.40–.62	≤2.08	70	50
≥3 sz vs <3 sz	.580 (ns)	.34–.82	≥1.45	75	60
clusters vs no clusters	.605 (ns)	.40–.81	≤5.00	100	32
miR-132-3p					
TBI+ vs TBI−	.535 (ns)	.42–.65	≤.84	50	66
≥3 sz vs <3 sz	.508 (ns)	.28–.74	≤.95	50	50
clusters vs no clusters	.691 (ns)	.50–.89	≤1.09	91	58
miR-212-3p					
TBI+ vs TBI−	.552 (ns)	.44–.67	≤.40	57	62
≥3 sz vs <3 sz	.538 (ns)	.32–.76	≤.30	35	90
clusters vs no clusters	**.811** (**)	**.65–.97**	**≤.30**	**64**	**95**

Note: Number of animals included in analyses: Round 1 (miR-183-5p, miR-323-3p, and miR-434-3p) 132 TBI− group, 12 TBI+ animals with <3 seizures and 20 with ≥3 seizures. Round 2 (miR-9a-3p, miR-124-3p, miR-132-3p, and miR-212-3p) 127 TBI−, 10 TBI+ animals with <3 seizures and 20 with ≥3 seizures.

Receiver-operating characteristic (ROC) analysis between the epilepsy outcome groups (epileptogenesis: TBI+ vs TBI−; epilepsy severity: ≥3 sz vs <3 sz per month, seizure cluster vs no clusters). The results are shown as AUC (*p*-value, 95%CI, optimal cutoff, sensitivity, and specificity) for all seven circulating miRNAs analyzed (rno-miR-183-5p, rno-miR-323-3p, rno-miR-434-3p, rno-miR-9a-3p, rno-miR-124-3p, rno-miR-132-3p, and rno-miR-212-3p). The labels ≥ and ≤ in the cutoff column indicate whether the values in the positive group (e.g., TBI+) are higher (≥) or lower (≤) than in the negative group (e.g., TBI−).

Abbreviations: AUC, area under the curve; CI, confidence interval; ROC, receiver-operating characteristic; sz, seizure; TBI, traumatic brain injury; TBI+, TBI rats with epilepsy; TBI−, TBI rats without epilepsy.

**$p < .01$ (bolded); ns, not significant.

models.[37,47,48] On the other hand, Korotkov et al. showed that overexpression of miR-132 in cultured human astrocytes decreased the expression of epilepsy-associated pro-inflammatory genes, suggesting that miR-132 can also have a protective role.[33]

Taken together, we identified miR-212 alone and in combination with miR-132 as potential circulating biomarkers to identify the injured rats at risk of developing severe PTE with seizure clusters. These miRNAs have been shown to be regulated in epileptogenic brain tissue and to modulate epileptogenic brain pathologies like neuroinflammation, neurodegeneration, extracellular matrix, dendritic maturation,[43] and synaptic plasticity.[33,44,45] Even though miR-212 and miR-132 are brain enriched, it remains to be explored whether their peripheral regulation plays a role in modulation of epileptogenesis, and in addition, whether the specificity and sensitivity of miR-212/miR-132 as biomarkers for epilepsy severity can be enhanced by combining them with other circulating, EEG, or MRI markers.

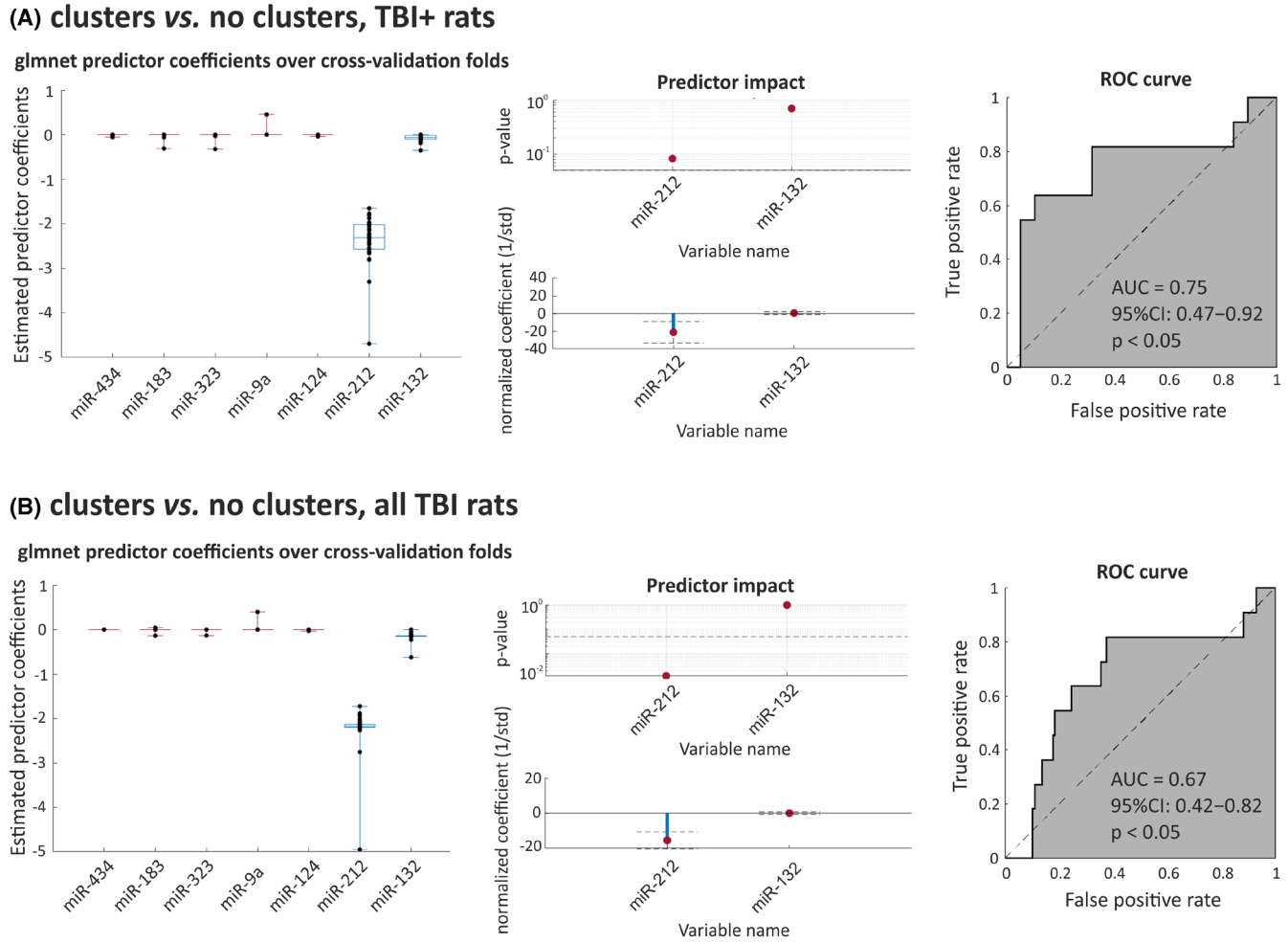


FIGURE 5 Elastic-net-regularized logistic regression (glmnet) analysis of plasma miRNAs in epilepsy severity groups on D2 after TBI. (A) Left panel: Boxplots of predictor coefficients over cross-validation folds. The glmnet analysis identified miR-212-3p and miR-132-3p (boxplots shown in blue) as the optimal miRNA set to distinguish TBI+ rats with seizure clusters (≥3 seizures within 24 h, $n = 11$) from TBI+ rats without clusters ($n = 19$). The other five miRNAs had coefficient of zero in most cross-validation folds, and therefore, were excluded (boxplots shown in red). Center panel: p-value and normalized coefficient for each predictor (miRNA) in the standard logistic regression analysis. Right panel: ROC analysis yielded a cross-validated AUC of .75 with 95% confidence interval (CI) of .47–.92 ($p < .05$). (B) The glmnet analysis of TBI+ rats with seizure clusters ($n = 11$) and all other TBI rats (TBI- and TBI+ no clusters combined, $n = 146$) identified miR-212-3p and miR-132-3p as the optimal miRNA set to distinguish the groups (boxplots shown in blue). ROC analysis yielded a cross-validated AUC of .67 with 95% CI .42–.82 ($p < .05$). D2, day 2; TBI, traumatic brain injury; TBI+, TBI with epilepsy; TBI–, TBI without epilepsy.

5 | CONCLUSIONS

Our study is the first preclinical multicenter, prospective, biomarker discovery study for PTE. We show that plasma miR-212 levels on D2, alone or in combination with miR-132, can differentiate the animals that will or will not develop PTE with seizure clusters. It remains to be explored whether these miRNAs will be useful for stratification of subjects that will develop a greater frequency of seizures within the clinical follow-up, thus being more likely detected even without a long-term EEG. We also show that craniectomy, electrode implantation, and early post-impact seizures can regulate plasma miRNA levels on D2, and this needs to be considered in future preclinical and clinical study designs and data analysis. Even though TBI-induced miRNA profiles across the three sites involved were comparable, we found site differences in epilepsy severity–related analyses that probably related to small cohort sizes. The need for large cohort size in data analysis was further supported when the data from the EpiBioS4Rx and EPITARGET cohorts were combined, showing consistency of the results and revealing additional biomarker candidates.

AUTHOR CONTRIBUTIONS

A.P., T.O.B., and R.S. designed the study. M.H., S.D.G., X.E.N.-E., I.A., C.S.-G., P.C.-E., P.A., N.P., R.I., E.M., G.S., N.G.H., N.C.J., D.K.W., O.G., and S.R.S. set-up the methodologies. M.H., X.E.N.-E., I.A., C.S.-G., P.C.-E., J.S., E.B., G.R.Y., and R.D.B. performed the experiments. M.H., S.D.G., N.P., and E.M. analyzed the microRNA (miRNA) data. P.A., I.A., C.S.-G., R.B.D., M.H., R.S., and A.P. analyzed the EEG data. A.P., M.H., N.P., X.E.N.-E., I.A., C.S.-G., R.S., and T.O.B. compiled the data and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

None of the authors has any conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

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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REFERENCES

1. Herman ST. Epilepsy after brain insult. Neurology. 2002;59(9 suppl 5):S21–P-S26. Available from: http://n.neurology.org/content/59/9_suppl_5/S21.abstract
2. Dulla CG, Pitkänen A. Novel approaches to prevent Epileptogenesis after traumatic brain injury. Neurotherapeutics. 2021;18(3):1582–601. https://doi.org/10.1007/s13311-021-01119-1
3. Engel JJ, Pitkänen A, Loeb JA, Dudek FE, Bertram EH 3rd, Cole AJ, et al. Epilepsy biomarkers. Epilepsia. 2013;54:61–9.
4. Klein P, Tyrlikova I. No prevention or cure of epilepsy as yet. Neuropharmacology. 2020;168:107762.
5. Lee RC, Feinbaum RL, Ambros V. The C. Elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843–54.
6. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. Elegans. Cell. 1993;75(5):855–62.
7. Brennan GP, Henshall DC. microRNAs in the pathophysiology of epilepsy. Neurosci Lett. 2018;667:47–52.
8. Henshall DC, Hamer HM, Pasterkamp RJ, Goldstein DB, Kjems J, Prehn JHM, et al. MicroRNAs in epilepsy: pathophysiology and clinical utility. Lancet Neurol. 2016;15(13):1368–76.
9. Pitkänen A, Paananen T, Kyyriäinen J, Das Gupta S, Heiskanen M, Vuokila N, et al. Biomarkers for posttraumatic epilepsy. Epilepsy Behav. 2021;121:107080. https://doi.org/10.1016/j.yebeh.2020.107080
10. Vuokila N, Das Gupta S, Huusko R, Tohka J, Puhakka N, Pitkänen A. Elevated acute plasma miR-124-3p level relates to evolution of larger cortical lesion area after traumatic brain injury. Neuroscience. 2020;433:21–35.
11. Das Gupta S, Ciszek R, Heiskanen M, Lapinlampi N, Kukkonen J, Leinonen V, et al. Plasma miR-9-3p and miR-136-3p as potential novel diagnostic biomarkers for experimental and human mild traumatic brain injury. Int J Mol Sci. 2021;22(4):1563–90.
12. Heiskanen M, Das Gupta S, Mills JD, van Vliet EA, Manninen E, Ciszek R, et al. Discovery and validation of circulating microRNAs as biomarkers for Epileptogenesis after experimental traumatic brain injury-the EPITARGET cohort. Int J Mol Sci. 2023;24(3):2823–57.
13. Bot AM, Dębski KJ, Lukasiuk K. Alterations in miRNA levels in the dentate gyrus in epileptic rats. PLoS One. 2013;8(10):e76051.
14. Venø MT, Reschke CR, Morris G, Connolly NMC, Su J, Yan Y, et al. A systems approach delivers a functional microRNA catalog and expanded targets for seizure suppression in temporal lobe epilepsy. Proc Natl Acad Sci USA. 2020;117(27):15977–88.
15. Szydlowska K, Bot A, Nizinska K, Olszewski M, Lukasiuk K. Circulating microRNAs from plasma as preclinical biomarkers of epileptogenesis and epilepsy. Sci Rep. 2024;14(1):708.
16. Lapinlampi N, Melin E, Aronica E, Bankstahl JP, Becker A, Bernard C, et al. Common data elements and data management: remedy to cure underpowered preclinical studies. Epilepsy Res. 2017;129:87–90.
17. Simonato M, Brooks-Kayal AR, Engel JJ, Galanopoulou AS, Jensen FE, Moshé SL, et al. The challenge and promise of anti-epileptic therapy development in animal models. Lancet Neurol. 2014;13(9):949–60.

18. Ndode-Ekane XE, Ali I, Santana-Gomez C, Andrade P, Immonen R, Casillas-Espinosa P, et al. Epilepsy phenotype and its reproducibility after lateral fluid percussion-induced traumatic brain injury in rats: multicenter EpiBioS4Rx study project 1. Epilepsia. 2024;65(2):511–26.
19. Ndode-Ekane XE, Ali I, Santana-Gomez CE, Casillas-Espinosa PM, Andrade P, Smith G, et al. Successful harmonization in EpiBioS4Rx biomarker study on post-traumatic epilepsy paves the way towards powered preclinical multicenter studies. Epilepsy Res. 2024;199:107263.
20. Kharatishvili I, Nissinen JP, McIntosh TK, Pitkänen A. A model of posttraumatic epilepsy induced by lateral fluid-percussion brain injury in rats. Neuroscience. 2006;140(2):685–97.
21. Bragin A, Li L, Almajano J, Alvarado-Rojas C, Reid AY, Staba RJ, et al. Pathologic electrographic changes after experimental traumatic brain injury. Epilepsia. 2016;57(5):735–45.
22. Shultz SR, Cardamone L, Liu YR, Edward Hogan R, MacCotta L, Wright DK, et al. Can structural or functional changes following traumatic brain injury in the rat predict epileptic outcome? Epilepsia. 2013;54(7):1240–50.
23. Bushel PR, Caiment F, Wu H, O'Lone R, Day F, Calley J, et al. RATEmiRs: the rat atlas of tissue-specific and enriched miRNAs for discerning baseline expression exclusivity of candidate biomarkers. RNA Biol. 2020;17(5):630–6.
24. Keller A, Gröger L, Tschernig T, Solomon J, Laham O, Schaum N, et al. MiRNATissueAtlas2: an update to the human miRNA tissue atlas. Nucleic Acids Res. 2022;50(D1):D211–D221.
25. Casillas-Espinosa PM, Andrade P, Santana-Gomez C, Paananen T, Smith G, Ali I, et al. Harmonization of the pipeline for seizure detection to phenotype post-traumatic epilepsy in a preclinical multicenter study on post-traumatic epileptogenesis. Epilepsy Res. 2019;156:106131.
26. Zou H, Hastie T. Regularization and variable selection via the elastic net. J R Stat Soc Series B Stat Methodol. 2005;67(2):301–20. https://doi.org/10.1111/j.1467-9868.2005.00503.x
27. Wanet A, Tacheny A, Arnould T, Renard P. miR-212/132 expression and functions: within and beyond the neuronal compartment. Nucleic Acids Res. 2012;40(11):4742–53. https://doi.org/10.1093/nar/gks151
28. Ludwig N, Leidinger P, Becker K, Backes C, Fehlmann T, Pallasch C, et al. Distribution of miRNA expression across human tissues. Nucleic Acids Res. 2016;44(8):3865–77. https://doi.org/10.1093/nar/gkw116
29. Isakova A, Fehlmann T, Keller A, Quake SR. A mouse tissue atlas of small noncoding RNA. Proc Natl Acad Sci USA. 2020;117(41):25634–45.
30. Bushel PR, Caiment F, Wu H, O'Lone R, Day F, Calley J, et al. RATEmiRs: the rat atlas of tissue-specific and enriched miRNAs database. BMC Genomics. 2018;19(1):825. https://doi.org/10.1186/s12864-018-5220-x
31. Jovičić A, Roshan R, Moisoi N, Pradervand S, Moser R, Pillai B, et al. Comprehensive expression analyses of neural cell-type-specific miRNAs identify new determinants of the specification and maintenance of neuronal phenotypes. J Neurosci. 2013;33(12):5127–37.
32. Wong H-KA, Veremeyko T, Patel N, Lemere CA, Walsh DM, Esau C, et al. De-repression of FOXO3a death axis by microRNA-132 and -212 causes neuronal apoptosis in Alzheimer's disease. Hum Mol Genet. 2013;22(15):3077–92.
33. Korotkov A, Broekaart DWM, Banchaewa L, Pustjens B, van Scheppingen J, Anink JJ, et al. microRNA-132 is overexpressed in glia in temporal lobe epilepsy and reduces the expression of pro-epileptogenic factors in human cultured astrocytes. Glia. 2020;68(1):60–75.
34. Korotkov A, Mills JD, Gorter JA, van Vliet EA, Aronica E. Systematic review and meta-analysis of differentially expressed miRNAs in experimental and human temporal lobe epilepsy. Sci Rep. 2017;7(1):11592.
35. Gorter JA, Iyer A, White I, Colzi A, van Vliet EA, Sisodiya S, et al. Hippocampal subregion-specific microRNA expression during epileptogenesis in experimental temporal lobe epilepsy. Neurobiol Dis. 2014;62:508–20.
36. Nudelman AS, DiRocco DP, Lambert TJ, Garelick MG, Le J, Nathanson NM, et al. Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, in vivo. Hippocampus. 2010;20(4):492–8.
37. Jimenez-Mateos EM, Bray I, Sanz-Rodriguez A, Engel T, McKiernan RC, Mouri G, et al. miRNA expression profile after status epilepticus and hippocampal neuroprotection by targeting miR-132. Am J Pathol. 2011;179(5):2519–32.
38. Bencurova P, Baloun J, Hynst J, Oppelt J, Kubova H, Pospisilova S, et al. Dynamic miRNA changes during the process of epileptogenesis in an infantile and adult-onset model. Sci Rep. 2021;11(1):9649.
39. Haenisch S, Zhao Y, Chhibber A, Kaiboriboon K, Do LV, Vogelgesang S, et al. SOX11 identified by target gene evaluation of miRNAs differentially expressed in focal and non-focal brain tissue of therapy-resistant epilepsy patients. Neurobiol Dis. 2015;77:127–40.
40. Cai X, Long L, Zeng C, Ni G, Meng Y, Guo Q, et al. LncRNA ILF3-AS1 mediated the occurrence of epilepsy through suppressing hippocampal miR-212 expression. Aging. 2020;12(9):8413–22.
41. Peng J, Omran A, Ashhab MU, Kong H, Gan N, He F, et al. Expression patterns of miR-124, miR-134, miR-132, and miR-21 in an immature rat model and children with mesial temporal lobe epilepsy. J Mol Neurosci. 2013;50(2):291–7.
42. Guo J, Wang H, Wang Q, Chen Y, Chen S. Expression of p-CREB and activity-dependent miR-132 in temporal lobe epilepsy. Int J Clin Exp Med. 2014;7(5):1297–306.
43. Magill ST, Cambronne XA, Luikart BW, Lioy DT, Leighton BH, Westbrook GL, et al. microRNA-132 regulates dendritic growth and arborization of newborn neurons in the adult hippocampus. Proc Natl Acad Sci USA. 2010;107(47):20382–87.
44. Impey S, Davare M, Lesiak A, Fortin D, Ando H, Varlamova O, et al. An activity-induced microRNA controls dendritic spine formation by regulating Rac1-PAK signaling. Mol Cell Neurosci. 2010;43(1):146–56.
45. Remenyi J, van den Bosch MWM, Palygin O, Mistry RB, McKenzie C, Macdonald A, et al. miR-132/212 knockout mice reveal roles for these miRNAs in regulating cortical synaptic transmission and plasticity. PLoS One. 2013;8(4):e62509.
46. Gorter JA, Van Vliet EA, Rauwerda H, Breit T, Stad R, van Schaik L, et al. Dynamic changes of proteases and protease inhibitors revealed by microarray analysis in CA3 and entorhinal cortex during epileptogenesis in the rat. Epilepsia. 2007;48(Suppl 5):53–64.
47. Huang Y, Guo J, Wang Q, Chen Y. MicroRNA-132 silencing decreases the spontaneous recurrent seizures. Int J Clin Exp Med. 2014;7(7):1639–49.

48. Zhang W, Ye F, Xiong J, He F, Yang L, Yin F, et al. Silencing of miR-132-3p protects against neuronal injury following status epilepticus by inhibiting IL-1β-induced reactive astrocyte (A1) polarization. FASEB J. 2022;36(10):e22554.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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