Set-up and comparison of the Droplet Digital PCR method with quantitative RT-PCR in assessing the levels of microRNA-124 from plasma and brain tissue of rats with traumatic brain injury

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Abstract

Traumatic brain injury (TBI) is the predominant cause of acquired epilepsy. The 30-y cumulative incidence of post-traumatic epilepsy (PTE) is 2.1% for mild, 4.2% for moderate, and 16.7% for severe injuries, which makes the pursuit of biomarkers that determine which injuries culminate in PTE paramount.

The hypothesis that plasma levels of brain-specific miR-124 may serve as a diagnostic marker in lateral FPI model of TBI, was tested in this work. Expression level of miR-124 was studied in plasma at both acute and chronic time points. The study of ipsilateral cortex was made only in the chronic time point. The ddPCR method was set up for miR-124 quantification from multiplexed and miR-124 specific cDNAs, and its sensitivity was compared with qRT-PCR. The relation between brain lesion size and corresponding miR-124 levels in plasma was also investigated.

The study underscored the potential of miR-124 as a biomarker for TBI at acute timepoint. In comparison to baseline, miR-124 elevations in 2 days post-TBI were 260% in qRT-PCR ($p<0.01$), 223% ($p<0.05$) in multiplexed ddPCR and 147% ($p<0.05$) in miR-124 specific ddPCR datasets. In comparison to 2 days controls, miR-124 elevations were 180% in qRT-PCR ($p<0.05$), 209% ($p<0.05$) in multiplexed and 137% ($p<0.05$) in miR-124 specific ddPCR datasets.

In perilesional cortex, miR-124 expression in TBI group was comparable to controls. Higher plasma miR-124 level was observed in animals with larger brain lesions.

Keywords:Biomarker, brain lesion, ddPCR, plasma, post-traumatic epilepsy, traumatic brain injury

I. Introduction

Epilepsy is a family of brain disorders, characterized by recurrent and unpredictable interruptions of the normal brain function, leading to seizures, periods of unusual behaviour and momentary loss of consciousness [1], [2]. It affects both sexes and people from all age group and geographical distribution [3]. About 50 million people worldwide are affected, with a significant majority of patients being drug-refractory [4], [5]. Epilepsy is strongly associated with psychological imbalances and social stigmatization, and most patients experience a discriminatory behaviour in various aspects of daily life [6]. Symptomatic epilepsies are the most common forms of epilepsy syndrome encountered in adults, with traumatic brain injury (TBI) being the predominant cause of post-traumatic epilepsy (PTE) in both civilian and military population [7], [8]. The brain insults trigger epileptogenic cascades, involving abnormal neurodegeneration and neuronal circuitry reorganization that predisposes the brain towards ictogenesis [9]. Current treatment methods for epilepsy focus on prevention/suppression of epileptic seizures with antiepileptic drugs (AEDs). However, reviews suggest that a large section of the treated population suffer from adverse side-effects of the medication [10], and 20-30% of the patients become unresponsive to any AED treatment [4], [11]. Thus, rather than suppressing seizure occurrence, prevention of the development of epilepsy (antiepileptogenesis) is gaining attention now.

Molecular biomarkers may play a significant role in determining susceptibility towards epilepsy after brain insults, characterization of epileptogenic progression, and for predicting response to implemented therapeutic approaches [12]. Recent studies particularly stress on understanding changes in microRNA (miRNA) profiles in the epileptogenic brain, since they are powerful players regulating and fine tuning a multitude of gene expression patterns [5], [13], [14], [15], [16]. The human brain exhibits an exceptionally high miRNA expression level, suggesting significant association between neurological disorders and miRNA dysfunction [5]. It is also predicted that miRNA alterations occur much earlier in response to a disease state than conventional protein biomarkers, making them crucial for detection in early stages of disease development [17]. In fact, up-regulation of brain enriched miRNAs has been observed in blood samples of rats subjected to the lateral fluid percussion injury (FPI) model of TBI, at 2 days post-trauma [18].

Thus, the aim of this thesis was to assess the expression levels of a brain enriched miRNA, miR-124, from plasma and ipsilateral cortex of rats subjected to lateral FPI. The hypothesis that plasma levels of brain enriched miRNAs can serve as potential diagnostic markers for development of brain injury, was tested in this work. Quantification of miR-124 was performed using quantitative reverse-transcription PCR (qRT-PCR) and droplet digital PCR (ddPCR), at both acute and chronic timepoints. Sensitivity of the two techniques in miR-124 profiling from plasma was compared. In addition, brain lesions developed as an impact of injury was studied with cresyl violet staining, and relation between lesion size developed at a chronic timepoint and corresponding miR-124 levels in plasma at an acute timepoint was investigated. Finally, miR-124 expression in the ipsilateral cortex at a chronic timepoint was studied using ddPCR.

II. Materials and Methods

Adult male Sprague-Dawley rats (Harlan Netherlands B.V., Horst, The Netherlands) were used for this study. All rats were individually housed in a controlled environment (temperature 22 ± 10^0C, humidity 50-60%, lights on from 07:00 to 19:00 h). Pellet food and water were provided ad libitum. All animal procedures were conducted in accordance with the guidelines of the European Community Council Directives 86/609/EEC and Committee for the Welfare of Laboratory animals.

2.1 Procedure for induction of lateral FPI

Induction of TBI was performed according to the lateral FPI protocol [9], [19]. Animals were placed in a Kopf stereotactic frame (David Kopf Instruments, Tujunga, CA,

USA). First, the skull was exposed with a midline incision followed by extraction of the periosteum. The left temporal muscle was detached from the lateral ridge and then a circular craniectomy ($\oslash$ 5 mm) was performed over the left parietal lobe midway between lambda and bregma keeping the dura mater intact. The edges of the craniectomy were sealed with a modified Luer-Lock cap that was filled with saline while the calvaria was covered with dental acrylate (Selectaplus CN, Dentsply DeTrey GmbH, Dreieich, Germany). FPI was induced 90 minutes after the administration of anesthesia by connecting the rat to the fluid-percussion device (AmScien Instruments, Richmond, VA, USA), via a female Luer-Lock fitting. For control animals, anesthesia and all surgical procedures were performed, except for the induction of injury.

2.2 Sample preparation for microRNA profiling from plasma

For miRNA profiling from plasma, the study was performed on a cohort of 16 rats (cohort A:6 control and 10 TBI), with an average body weight of 375g (360-385g). MiRNA profiling was performed at four timepoints: baseline (1 week prior to injury), 2 days, 1 week and 3 months post-injury. For inducing lateral FPI, rats were anesthetized with an intraperitoneal (i.p) injection, containing a solution of sodium pentobarbital (58mg/kg), magnesium sulphate (127.2 mg/kg), propylene glycol (42.8%) and absolute ethanol (11.6%). A severe impact of about 3.15 $\pm$ 0.02 atm (3.05 - 3.25 atm) pressure was induced. For blood sampling, rats were anesthetized with 4% isoflurane and 500μL of tail vein blood was collected in K2-EDTA tubes (Product code: 368841, BD Vacutainer, Belliver Industrial estate, Plymouth UK). The samples were immediately processed by centrifuging at 1500xg for 10 minutes at 4^0C. Plasma was extracted and stored in aliquots of 50μL at -70^0C until further use. At 3 months post-injury, the rats were deeply anesthetized with 4% isoflurane and decapitated with guillotine. Brains were quickly extracted and flash frozen in -70^0C isopentane, followed by subsequent storage at -70^0C until further use.

2.3 Sample preparation for microRNA profiling from brain tissue

In case of brain tissue samples (ipsilateral cortex), the study was performed only at a 3 months post-injury time point. A separate cohort of 11 rats were used for this purpose (cohort B:5 control and 6 TBI). The initial set consisted of 30 healthy adult rats with an average body weight of 326g (300-354g), out of which 18 were subjected to lateral FPI. For injury induction, rats were anesthetized with an intraperitoneal (i.p) injection, containing a solution of sodium pentobarbital (58mg/kg), chloral hydrate (60mg/kg), magnesium sulphate (127.2 mg/kg), propylene glycol (42.8%) and absolute ethanol (11.6%). A severe impact of about 3.23 $\pm$ 0.01 atm (3.13 - 3.35 atm) pressure was induced to cause the brain injury. At 3 months post-injury, the animals were deeply anesthetized with CO_2 and decapitated with guillotine. Brains were quickly extracted and cortex, thalamus and hippocampus were separated and flash frozen in liquid nitrogen, and subsequently stored at -70^0C until further use.

2.4 RNA extraction from plasma and brain tissue samples

Total RNA extraction from plasma samples was performed using the Exiqon miRCURY^{TM} RNA isolation kit - biofluids (Product code: 300112, Exiqon, Denmark) [20]. According to the protocol, 50μL of rodent tail vein plasma was used as the starting sample, topped up with nuclease free water (Product code: AM9937, Ambion, Life Technologies, Carlsbad, CA, USA) to a final volume of 200μL. Also to reduce the effect of inhibitors/nucleases, the 50μL plasma volume was centrifuged at 3000xg for 5 minutes, to pellet any debris or insoluble cellular components. To minimize technical variation among replicates and to enhance isolation of low abundance miRNAs, 1μg of MS2 carrier RNA (Product code: 10165948001, Roche Diagnostics GmbH, Mannheim, Germany) was added to each sample during the extraction procedure. Also, to monitor the efficiency of RNA extraction step, 1μL of Exiqon RNA Spike-in template mixture (Product code: 203203, Exiqon, Denmark) per sample was added. Total RNA extraction was perfomed according to the guidelines [20]. Extracted RNA samples were eluted in 25μL of nuclease free water (provided with the Exiqon RNA isolation kit). For long-term storage, the purified RNA samples were kept at -70^0C. All centrifugations were performed at room temperature and the optional Proteinase K treatment was omitted.

Total RNA extraction for ipsilateral cortex samples was performed using the Qiagen miRNeasy Mini kit (Product code: 217004, Qiagen GmbH, Hilden Germany), following the protocol guidelines [21]. Total RNA was eluted from the spin column in two elution steps of 50μL of nuclease free water (provided with the Qiagen miRNeasy kit). Finally, total RNA eluate was obtained in 100μL of nuclease free water. For the tissue samples, total RNA concentration was measured in both NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and in Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) with the Agilent RNA 6000 Nano kit (Product code: 5067–1511, Agilent Technologies, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania). All RNA elutions were diluted to 1:20, prior to estimation of small RNA concentration using the Agilent Small RNA kit (Product code: 5067–1548, Agilent Technologies, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania).

2.5 cDNA synthesis with Exiqon miRCURY LNA^{TM} Kit

The Exiqon miRCURY LNA^{TM} Universal RT microRNA PCR kit (Universal cDNA synthesis kit II, 8-64 rxns, Product code: 203301, Exiqon, Denmark) was implemented for monitoring RNA extraction and RT reaction efficiencies [22]. This quality control was performed only for elutions from plasma samples, since the extracted RNA volumes were too low to be detected reliably by NanoDrop or Agilent Bioanalyzer. Since RNA volumes from tissue samples could be reliably measured, quality control was omitted for those samples. For the plasma RNA elutions, a 10μL universal cDNA synthesis reaction mixture was set up as follows: 2μL of 5x reaction buffer, 4μL of nuclease free water, 1μL of the enzyme mix, 1μL of UniSp6 spike–in template and 2μL of undiluted template RNA.

The exogenous RNA spike-in template UniSp6 (provided with the cDNA synthesis kit) was added during cDNA synthesis to monitor RT reaction efficiency. Since it is presumed that the abundance of miR-124 in cell-free plasma samples would be considerably low, undiluted template RNA samples were used. The reaction mixture was then vortexed well and RT reactions were performed in a 96-well thermal cycler (BIO-RAD T100^{TM} thermal cycler, CA, USA). The thermal cycling conditions were as follows: Incubation for 60 minutes at 42^0C, heat-inactivation of the reverse transcriptase for 5 minutes at 95^0C, followed by immediate cooling to 4^0C and storage at -20^0C for later usage.

2.6 qRT-PCR amplification with Exiqon kit for quality control

qRT-PCR amplifications of UniSp4 and UniSp6 templates were performed using the miRCURY LNA^{TM} Universal RT microRNA PCR system designed for biofluids (ExiLENT SYBR Green master mix, Product code: 203403, Exiqon, Denmark) [22]. The UniSp4 template was chosen for amplification from the template spike-in mix (UniSp2, UniSp4 and UniSp5 mix), as its amplification corresponded with consistent threshold cycles (C_t) across all samples from all tested time-points, indicating successful RNA extraction. In this step, the cDNA samples were diluted to 40x in nuclease free water according to the protocol (195μL nuclease free water to each 5μL cDNA sample). ROX is a passive reference dye that allows normalization of signals from individual PCR wells, enabling comparison of amplification signals across an entire PCR plate. Since the Exiqon PCR Master Mix (ExiLENT SYBR Green master mix) does not contain this dye, 0.2μL of ROX (Product code: 12223-012, Invitrogen, Life Technologies, Carlsbad, CA, USA) per PCR replicate was added to each of the diluted cDNA samples prior to PCR amplification. For spike-ins, reactions were performed in triplicates. The 10μL qRT-PCR reaction mixture was designed as stated in the protocol (Protocol A: Table 3) [22]. The qRT-PCR amplification followed by melt curve analysis was then performed in a StepOnePlus^{TM} system (Applied Biosystems), as indicated in the guidelines (protocol A: Table 4) [22]. Results were analysed with StepOne Software v2.1 (Applied Biosystems, Foster City, CA, USA).

Amplification efficiencies of possible endogenous controls miR-103 (hsa-miR-103a-3p, Product code: 204063, Exiqon, Denmark) and miR-127 (hsa-miR-127-3p, Product code: 204048, Exiqon, Denmark), and the target miRNA miR-124 (hsa-miR-124-3p, Product code: 206026, Exiqon, Denmark) were also checked in pilot studies with the Exiqon kit, in order to compare with the TaqMan system. Amplification in plasma for these miRNAs were tested at 40x, as well as for 20x and undiluted template cDNA conditions. No template controls (NTCs) were included in every PCR plate.

2.7 MicroRNA specific cDNA synthesis with TaqMan assay

Next, the TaqMan small RNA assay (TaqMan MicroRNA Reverse Transcription Kit, Product code: 4366596, Applied Biosystems, Foster City, CA, USA) was used to perform miRNA specific reverse transcription (RT) of the RNA samples extracted from brain tissue and plasma, followed by quantitative PCR amplification of the target miRNAs with probe based TaqMan qRT-PCR chemistry [23]. The cDNA synthesis reaction master mix was designed as indicated in the protocol [23]. The 15μL RT reaction was then obtained by adding 7μL of the master mix, with 3μL of 5x miR-124 RT primer (Product code: RT 001182, Applied Biosystems, Foster City, CA, USA) and 5μL of the undiluted template RNA. In order to test if miRNAs miR-103 and miR-127 can act as possible endogenous controls, a second multiplexed cDNA synthesis reaction was also set up as follows: 7μL of the master mix, with 1μL each of 5x miR-124 (Product code: RT 001182, Applied Biosystems, Foster City, CA, USA), miR-103 (Product code: RT 000439, Applied Biosystems, Foster City, CA, USA) and miR-127 RT primers (Product code: RT 000452, Applied Biosystems, Foster City, CA, USA), and 5μL of the undiluted template RNA.

For tissue samples, the small RNA concentration for each diluted RNA elution (1:20 dilution) was estimated in the Agilent Bioanalyzer, and from these 1:20 dilutions, 5μL of RNA template with 10ng of small RNA concentration was prepared. Since small nuclear RNA U6 is a known endogenous control, multiplexed cDNA synthesis reaction for the cortex samples was set up as follows: 7μL of the master mix, with 1.5μL each of 5x snRNA U6 (Product code: RT 001973, Applied Biosystems, Foster City, CA, USA) and miR-124 RT primers, and 5μL of the diluted template RNA (small RNA concentration 10ng). The reaction components were mixed well and loaded on the thermal cycler (BIO-RAD T100^{TM} thermal cycler, CA, USA). Reverse transcription was performed as instructed in the protocol [23].

2.8 MicroRNA specific qRT-PCR reaction with TaqMan chemistry

MicroRNA specific quantitative PCR (qRT-PCR) was setup to estimate the concentrations of miR-103 and miR-124 in purified plasma RNA samples. For cortex samples, qRT-PCR amplification step was omitted and quantification was only performed using droplet digital PCR. In qRT-PCR, each sample was run in duplicate with suitable NTCs. The reaction setup was designed according to the protocol [23]. The thermal cycling conditions for qRT-PCR were also designed in the StepOnePlus^{TM} system (Applied Biosystems), as indicated in the guidelines. Results were analysed with StepOne Software v2.1 (Applied Biosystems, Foster City, CA, USA) [23].

Due to the absence of established endogenous controls for quantifying miRNA expression in circulating biofluids, the expression levels for individual miRNAs were obtained by normalising the C_t values (from TaqMan qRT-PCR) with the exogenous spike-in UniSp4 expression data obtained from the Exiqon qRT-PCR amplification system. For both miR-103 and miR-124, normalization of qRT-PCR expression to UniSp4 was performed using the $\Delta\Delta C_t$ method.

2.9 Absolute quantification of target miRNA with Droplet Digital PCR

The reaction mixtures for ddPCR based quantification of miR-124, in brain tissue and plasma samples, were prepared according to the Droplet Digital^{TM} PCR protocol. Reverse transcription of the target miRNA template was performed with the TaqMan reverse transcription kit (TaqMan MicroRNA Reverse Transcription Kit, Product code: 4366596, Applied Biosystems, Foster City, CA, USA). Amplification was then carried out by adding 1.33μL of the cDNA template (undiluted cDNA template for plasma samples, and diluted cDNA template for cortex samples) to a 20μL reaction mixture, containing

10μL BioRad 2x ddPCR supermix for probes (Product code: 186-3010, BIO-RAD, CA USA), 1μL 20x miR-124 PCR primer (Product code: TM 001182, Applied Biosystems, Foster City, CA, USA) and 7.67μL nuclease-free water [24]. The 20μL reaction mixtures were loaded in disposable droplet generator cartridges (Product code: 186-4008, DG8^{TM} Cartridges for QX100^{TM}/QX200^{TM} Droplet Generator, BIO-RAD, Germany) along with 70μL of droplet generation oil for probes (Product code: 186-3005, Droplet generation oil for probes, BIO-RAD, CA, USA). The cartridges were then covered with gaskets (Product code: 186-3009, Droplet Generator DG8^{TM} Gaskets, BIO-RAD, CA, USA) and placed in the droplet generator (Product code: 186-3001, BIO-RAD QX100^{TM} Droplet Generator, Solna, Sweden) for creation of oil-emulsion droplets. Once droplets were generated, they were transferred to 96-well PCR plates (Twin. tec PCR plate 96, semi-skirted, colorless, Product code: E951020303, Hamburg, Germany) sealed (Product code: 181-4040, Pierceable foil heat seal, BIO-RAD, UK), thermal cycled (96-well PTC-200 thermal cycler, MJ Research) and quantified at end-point in the droplet reader (Product code: 186-3001, BIO-RAD QX100^{TM} Droplet Reader, Solna, Sweden) using the QuantaSoft software (v1.7, Product code: 186-4011, BIO-RAD, CA, USA). In this study, for each sample, reaction was performed in duplicates, and miR-124 expression was calculated as the sum of target copies / 20μL well for the two replicates.

To identify positive droplets, the threshold amplitude was set at 6000 for all ddPCR runs in the QuantaSoft software. This was performed to ensure maximum possibility of considering only true positive amplifications and neglecting false positive droplets with lower amplitudes.

For cortex samples, ddPCR based amplification of snRNA U6 (Product code: TM 001973, Applied Biosystems, Foster City, CA, USA) as an endogenous control was also performed. To obtain detectable limits of target concentration in ddPCR, the cortex cDNA samples were diluted to 1:6 for U6 and 1:4 for miR-124, and 1.33μL of these dilutions were taken for ddPCR amplification. Expression of miR-124 was studied with and without normalisation to U6 expression.

The thermal cycling conditions for TaqMan based ddPCR were as follows [24]: 95^{0}C for 10 minutes, 40 cycles of 95^{0}C for 15 seconds and 60^{0}C for 1 minute, and a final inactivation step at 98^{0}C for 10 minutes. NTCs were included in every PCR plate.

2.10 Cresyl violet staining for characterization of lesion size

At 3 months post-TBI, 10-μm-thick coronal sections were cut from brains of the cohort A animals, with a cryostat (Leica CM3050 S, Leica Microsystems Nussloch GmbH, Germany). The sections were then transferred to Superfrost glass slides (Menzel-Glser Superfrost Plus Microscope slides, Thermo Scientific, Gerhard Menzel GmbH, Braunschweig, Germany) and allowed to dry at room temperature for 5 minutes, followed by storage at -70^{0}C until further use. The sections were stained with cresyl violet (Merck, Darmstadt, Germany). First, sections were placed in 96% ethanol for 30 seconds, then 70% ethanol for 30 seconds, 50% ethanol for 30 seconds, followed by staining with 1% cresyl violet in 100% ethanol for 40 seconds. Next, the sections were placed in 50% ethanol for 30 seconds, 70% ethanol for 30 seconds, 96% ethanol for 30 seconds and 100% ethanol for 30 seconds. Finally, the sections were washed twice in xylene for 5 minutes each. The slides were immediately coverslipped using DePeX (Product code: 361254D, BDH Chemical, Poole, UK) as the mounting medium, and left to dry for overnight. For each rat, digital RGB colour images were captured using Leica DMRB microscope equipped with a Nikon DXM1200F camera operated by a Nikon ACT-1 2.7 software. A scale bar taken with the same magnification as the digital images was used.

2.11 Statistical analysis

Independent-samples Mann-Whitney U test was performed to compare miRNA expression levels between control and TBI samples at a particular time-point. The related-samples Friedman′s two-way ANOVA test was then performed to check for differences in expression across all TBI samples over the four timepoints. If the Friedman′s test was found to be significant, variation in expression between pairwise TBI samples was tested using the related-samples Wilcoxon signed rank test. Similarly, the Friedman′s two-way ANOVA tests and Wilcoxon signed rank tests were also performed for control samples. Receiver Operator Characteristic (ROC) curve analyses were also performed to determine the potential of miR-124 to act as a reliable biomarker for head injury. All analyses were performed in IBM SPSS Statistics 21 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 5.03, GraphPad software). All data were represented as mean $\pm$ standard error of the mean. Statistical significance was considered at $p<0.05$.

III. Results

3.1 Optimisation of the droplet digital PCR protocol

Optimisation of the ddPCR protocol in conjunction with TaqMan chemistry, for quantification of miR-124 expression, was first perfomed by running 1:5 dilution series from synthetic miR-124 (Integrated DNA Technologies, Coralville, IA, USA) and one brain tissue sample available from animal cohort B. A linear curve was obtained for the tested dilution points, indicating specificity of the primers and high accuracy of ddPCR in estimating target concentrations (figure 1). Dilution series could not be made for plasma samples because of low target concentration.

Also, a thermal gradient was set up to determine the suitable annealing temperature for ddPCR amplifications. Reaction specificities in tissue samples were first tested. A temperature gradient of 52-63^{0}C was set and run for a single tissue sample (the first dilution from the 1:5 serial dilution prepared earlier). In this test, proper separation between clusters was observed for T_m=58.7^{0}C and T_m= 56.2^{0}C, with T_m= 56.2^{0}C being the best. At higher temperatures (60.8 - 62.2^{0}C), as well as for lower T_m (54.1 – 52^{0}C), poor separation between clusters was observed (figure 2(A)). Also, from this test, fluorescence amplitude of 6000 was considered suitable to avoid inclusion of non-specific amplifications. Thus, the amplitude threshold for all ddPCR runs was manually set to 6000 prior to estimation of target concentration.

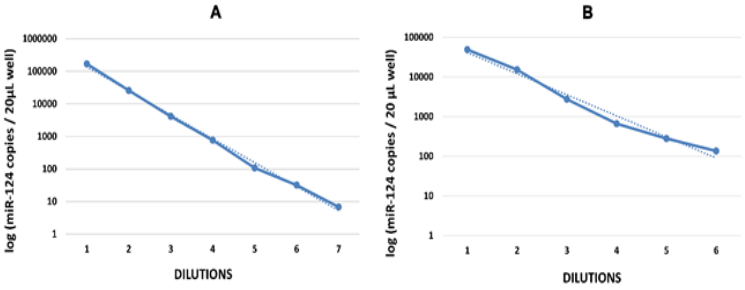


Fig. 1: Target concentration / 20μL ddPCR well for the 1:5 dilution series of (A) synthetic miR-124, (B) ipsilateral cortex (exponential ddPCR based amplifications of miR-124 plotted to the logarithmic scale)

Similar annealing temperature gradients were then run for few plasma samples (with undiluted RT products). However, in this case, no proper separation between positive and negative droplets was observed at any temperature. Too high temperatures led to very few positive droplets, whereas at too low temperatures, increase in positive droplets at a lower threshold level was observed, indicating non-specific amplifications. For T_m=58.7^{0}C and T_m= 56.2^{0}C, no difference in positive droplet number was observed above the amplitude threshold of 6000, whereas increased positive droplets were observed at lower threshold level (at $\sim$1500-2000) for T_m= 56.2^{0}C (figure 2(B)). Thus, as the most optimal annealing temperature could not be established with certainty, it was decided that ddPCR amplifications will be performed with the already specified T_m=60^{0}C only.

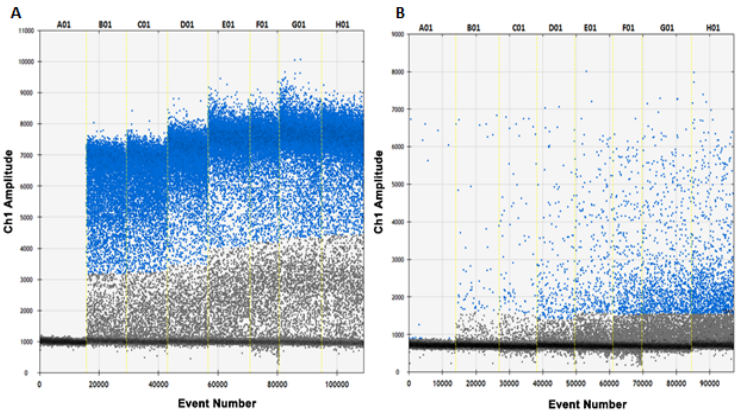


Fig. 2: Annealing temperature gradient for ddPCR based miR-124 expression in (A) Ipsilateral cortex, (B) Plasma (blue-positive droplets, black-negative droplets)

3.2 Quality control for plasma samples with exogenous RNA spike-ins using Exiqon qRT-PCR

Amplification of the exogenous RNA spike-in UniSp4 was tested for all RNA elutions from plasma samples, to monitor RNA extraction efficiency. For each sample, three PCR replicates were run and the mean threshold cycle (C_t) was considered for analysis. Mean C_t values for UniSp4 ranged from 26.9–29.8 across all

samples at the four different time-points, with good agreement among technical replicates (standard deviation between C_t values of technical replicates $<=$ 0.1). UniSp6 amplification was also tested for monitoring efficiency of the RT reactions. Three PCR replicates were run and mean threshold cycle (C_t) ranged from 17.08-18.08 across all samples at the four different time-points, with good agreement among the technical replicates for each sample (standard deviation between C_t values of technical replicates $<=$ 0.3) (figure 3). qRT-PCR amplifications with the Exiqon system were then tested for miR-103, miR-127 and miR-124 at 40x, 20x and undiluted template cDNA conditions, for pilot study samples. Successful amplification of miR-103 was observed in all cases, whereas miR-127 and miR-124 amplifications failed for all.

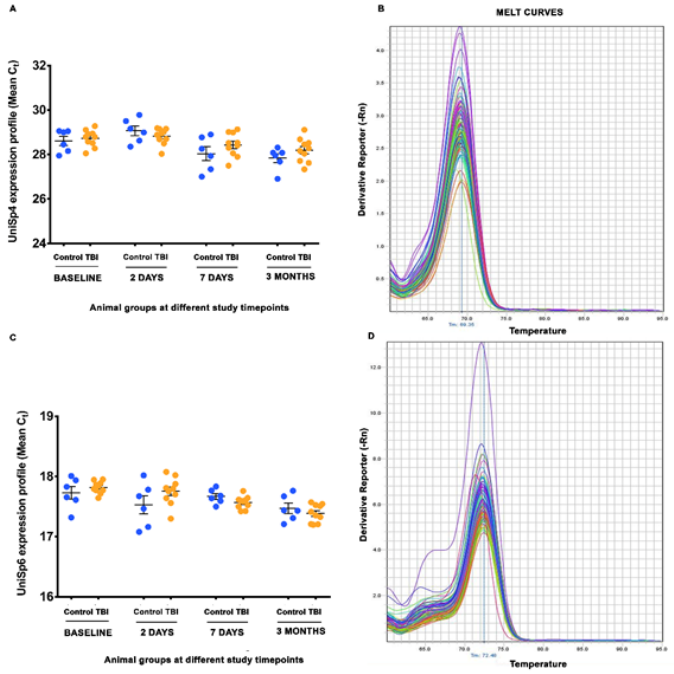


Fig. 3: (A) UniSp4 amplification represented as the mean C_t for the three qRT-PCR replicates, (B) UniSp4 melt curve analysis, (C) UniSp6 amplification represented as the mean C_t for the three qRT-PCR replicates, (D) UniSp6 melt curve analysis

3.3 Amplification of miR-103 in plasma as a possible endogenous control using TaqMan qRT-PCR

To check if miR-103 can act as a possible endogenous control, its amplification efficiency was tested in qRT-PCR from the multiplexed cDNA templates, using the TaqMan chemistry. No variation in miR-103 expression was observed at a particular time-point, between control and injured animals. However, differential expression was observed for the controls at the four studied timepoints ($p<0.01$), as well as for the TBI samples across the four timepoints ($p<0.01$). From this, it could be deduced that expression of miR-103 remains similar across biological conditions (control vs. TBI) at a particular timepoint, whereas, within a single animal, difference in miR-103 expression can be observed at different time-points, irrespective of the biological condition. This variability in expression over time for both uninjured and injured cases indicated that the plasma enriched miR-103 is not a suitable endogenous control candidate for qRT-PCR data normalization (figure 4). Amplification efficiencies of miR-127 as a possible endogenous control was not tested with TaqMan systems, since pilot studies already indicated no amplification of this miRNA in plasma samples (with the Exiqon kit).

3.4 Expression analysis of miR-124 in plasma across different time-points with TaqMan qRT-PCR

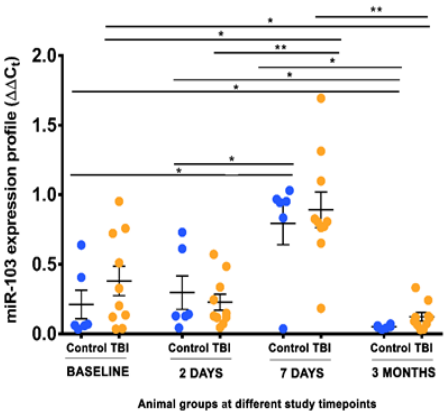
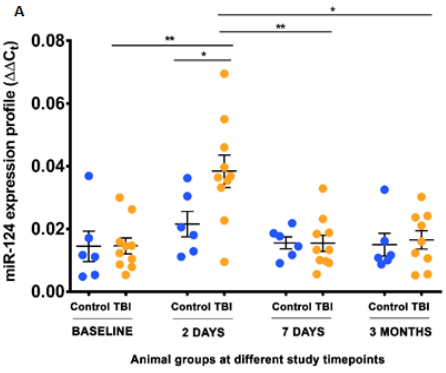
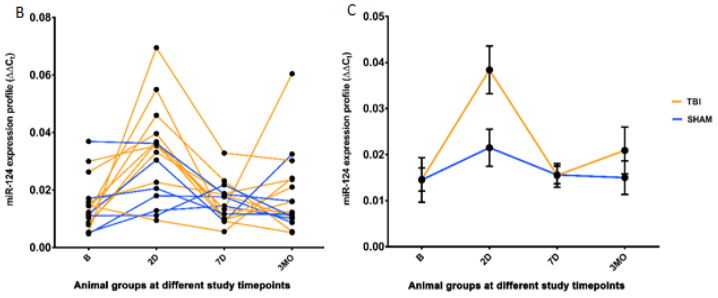


Fig. 4: Analysis of miR-103 expression in plasma samples using TaqMan qRT-PCR chemistry

Expression of miR-124 remained similar across control samples at all timepoints. Also, at baseline, 1 week and 3 months post trauma, no difference in miR-124 expression was observed between the control and injured cases. However, difference in expression was observed between control and TBI samples at 2 days post-injury timepoint ($p<0.05$). Also, up-regulation was observed at 2 days post-injury, compared to the same samples at baseline ($p<0.01$), 1 week post-injury ($p<0.01$), and 3 months post-injury ($p<0.05$, excluding one outlier from the 3 months post-injury group). For the tests that showed difference in expression, Receiver Operator Characteristic (ROC) analyses were good since all tests demonstrated an AUC$>$0.81 with $p<0.05$ (figure 5).





3.5 Pattern of miR-124 expression in relation with lesion size obtained from qRT-PCR analysis

Statistical analysis revealed up-regulation of miR-124 expression in plasma at 2 days post-injury, for the rats characterized with big sized cavities, compared to controls ($p<0.05$). Also, expression of miR-124 was higher in these animals compared to the ones with mid-size

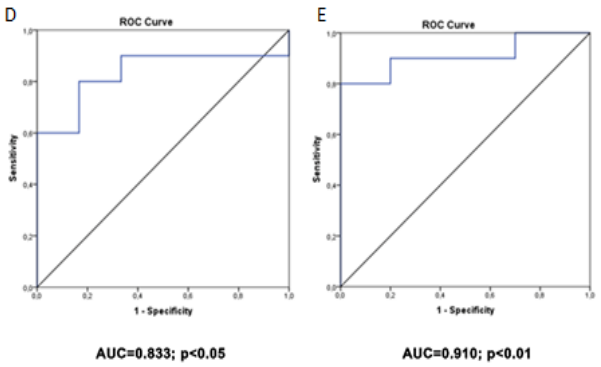


Fig. 5: (A) Analysis of miR-124 expression in plasma samples using TaqMan qRT-PCR chemistry, (B) Individual animal variability in miR-124 expression across the four timepoints of study, (C) Average miR-124 expressions for control and TBI animals across the four timepoints, ROC analysis for (D) 2 days control vs. 2 days post-TBI and (E) Baseline TBI vs. 2 days post-TBI

and smaller cavities ($p<0.05$). However, no difference was observed between controls and smaller lesion groups. ROC analysis for miR-124 levels in rats with larger cavities, compared to controls, was perfect with an AUC of 1.0 and $p<0.05$. In comparison to the rats with middle and small sized cavities, an AUC of 0.917 with $p<0.05$ was obtained (figure 6). Thus, larger lesion size correlated with higher expression of miR-124 at an acute timepoint after injury induction.

To confirm the lesion size and extent of cell loss at the site of impact, cresyl violet staining was performed for 10-μm-thick coronal brain sections, for both controls and TBI rats at 3 months post-injury. Rats with larger cavities demonstrated huge lesion sizes, with a greater extent of cell loss at the site of injury, as compared to the ones with smaller and mid-size cavities. Controls demonstrated no cell loss or lesion formation in any brain area (figure 7).

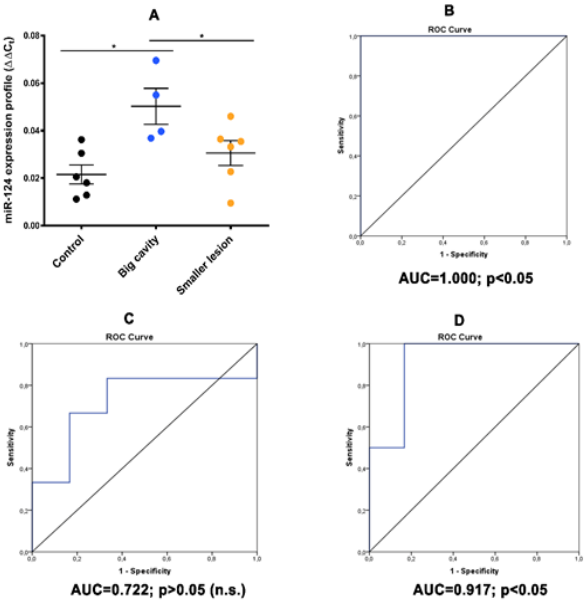


Fig. 6: (A) Up-regulation in miR-124 expression observed in plasma of rats characterized with larger brain lesions at 2 days post-injury, ROC analysis for (B) control vs. big lesion group (C) control vs. smaller lesion group, (D) big lesion vs. smaller lesion group (qRT-PCR data)

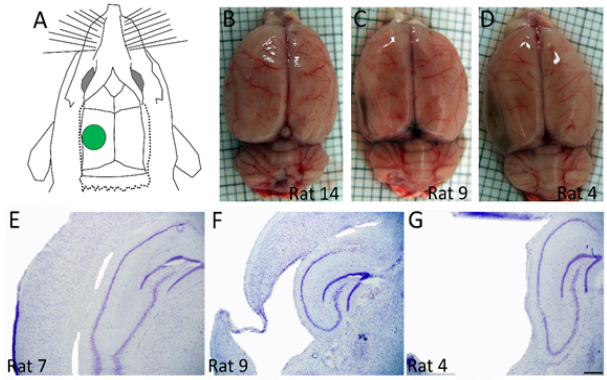
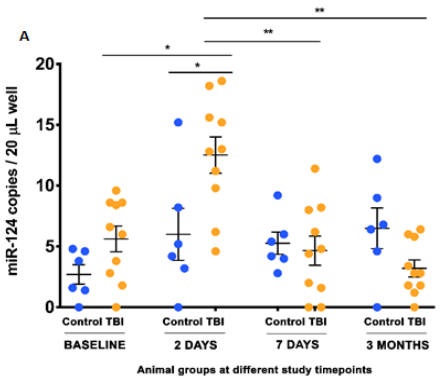


Fig. 7: (A) Site of impact for the lateral FPI model of traumatic brain injury, (B) Whole brain image for control rat 14 with no lesion due to absence of injury, (C) Whole brain image for TBI rat 9 with mid-size cavity, (D) Whole brain image for TBI rat 4 with big cavity, (E) Cresyl violet staining for control rat 7 reveals absence of lesion at the ipsilateral cortex, (F) Middle sized cavity observed at the ipsilateral cortex for TBI rat 9, with cell loss at the area of impact, (G) Large cavity with a higher extent of cell loss observed at the lesion area for TBI rat 4

3.6 Quantification of miR-124 with Droplet Digital PCR from multiplexed TaqMan RT products

Droplet PCR reaction with TaqMan multiplexed cDNA samples could successfully replicate the expression pattern observed in qRT-PCR. Levels of miR-124 remained similar for the controls at all timepoints. Also, at baseline, 1 week and 3 months post trauma, no difference in miR-124 expression was observed between the control and injured cases. Significance was only observed for the 2 days post-injury samples, when compared with the same ones at baseline ($p<0.05$), 1 week post-injury ($p<0.01$), and 3 months post-injury ($p<0.01$). Also, difference in expression was observed between control and TBI samples at 2 days post-injury ($p<0.05$). ROC analyses revealed an AUC>0.84 for all cases ($p<0.05$) (figure 8).



3.7 Droplet PCR based estimation of miR-124 expression in relation with lesion size from multiplexed cDNA

Similar to the previous qRT-PCR analysis, comparison of miR-124 expression in plasma at 2 days post-TBI in relation with the lesion size observed at 3 months, was studied using the multiplexed cDNA. However, for this test, the results obtained from qRT-PCR could not be replicated. No difference in expression was observed in any of the cases (figure 9). It was observed that only one control animal exhibited very high miR-124 copy number (15,2 copies in total in the two replicates). However, since this datapoint was not marked

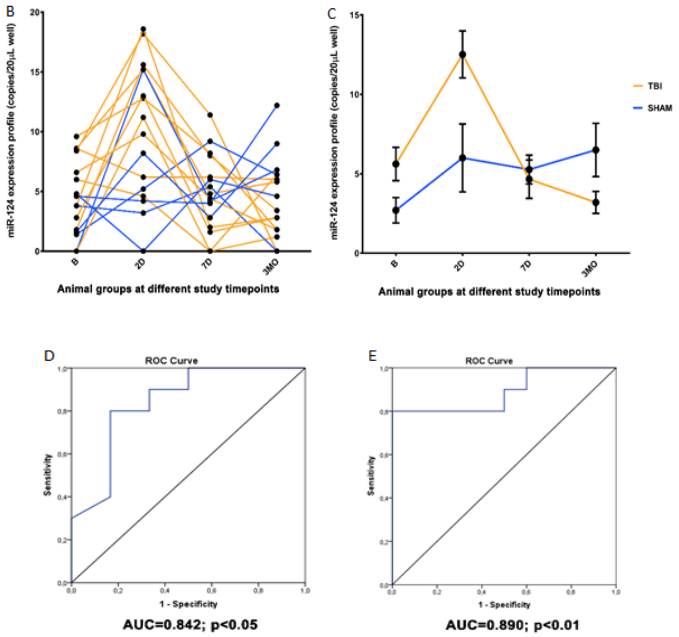


Fig. 8: (A) Expression profile for miR-124 across all timepoints in plasma (multiplexed ddPCR), (B) Individual animal variability in miR-124 expression across the four timepoints of study, (C) Average miR-124 expressions for control and TBI animals across the four timepoints, ROC analysis for (D) 2 days control vs. 2 days post-TBI, (E) Baseline TBI vs. 2 days post-TBI

as an outlier in SPSS analysis, it was not excluded from analysis.

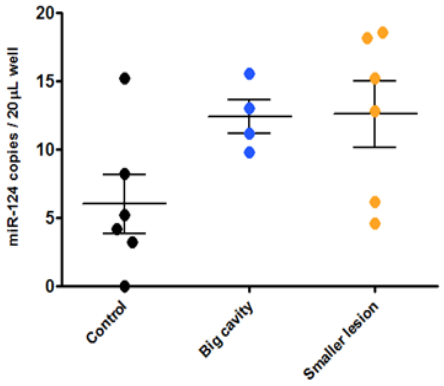
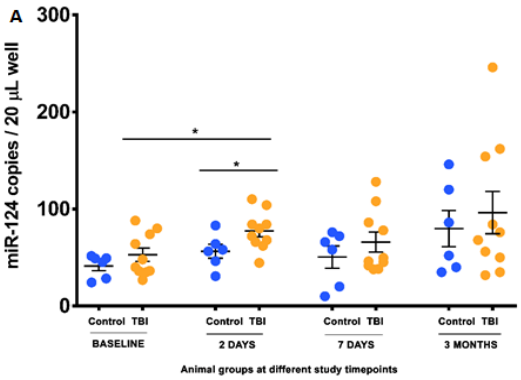


Fig. 9: No up-regulation in miR-124 expression observed in relation with lesion size in the ddPCR multiplex data

3.8 Quantification of miR-124 with Droplet Digital PCR from miR-124 specific RT products

Droplet PCR run with multiplexed cDNA templates could successfully demonstrate up-regulation in miR-124 expression in trauma samples at 2 days post-injury. However, the main drawback of this run was that very few target copies could be obtained for each sample, and with considerably low flourescence amplitudes. Particularly, for few samples, application of the manual threshold of 6000 resulted in no positive amplifications at all. However, when tested without the manual threshold setup (with automatically assigned thresholds from QuantaSoft), the up-regulation pattern of miR-124 at 2 days post-TBI could no more be observed. To check if the multiplex setup of cDNA synthesis reaction with multiple PCR primers was responsible for this low target amplification, ddPCR runs were repeated with only miR-124 specific RT reaction products. For this case, ddPCR runs for samples from different timepoints were run on different days to account for the impact of day-to-day variability on miR-124 expression levels.

Higher copy numbers were in fact observed with these RT products, compared to multiplex samples. The expression pattern observed in qRT-PCR for the acute timepoint (2 days post-injury) could be successfully replicated. However, unlike the previous results, decrease in miR-124 concentration in the trauma samples at chronic timepoints (1 week and 3 months post-injury) could not be observed. For these samples, up-regulation of miR-124 occurred in response to trauma at 2 days post-TBI, and the levels remained consistent till 1 week and 3 months post-TBI timepoints, possibly accounting for the day-to-day variation in reaction preparation. ROC analyses revealed only fair accuracy with AUC in the range of 0.7–0.8 (figure 10).



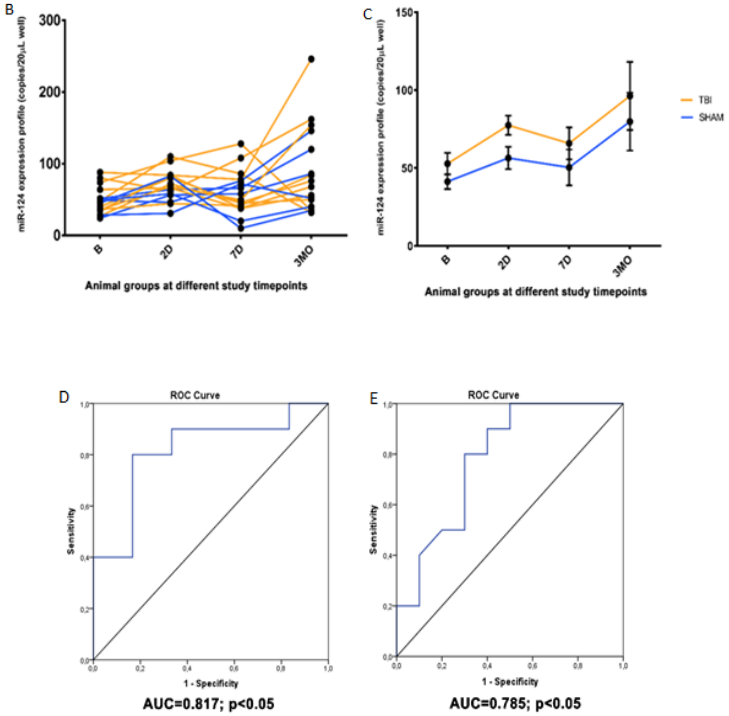


Fig. 10: (A) Expression profile for miR-124 across all timepoints in plasma samples with ddPCR (miR-124 specific ddPCR), (B) Individual animal variability in miR-124 expression across the four timepoints of study, (C) Average miR-124 expressions for control and TBI animals across the four timepoints, ROC analysis for (D) 2 days control vs. 2 days post-TBI and (E) Baseline TBI vs. 2 days post-TBI

3.9 Droplet PCR based estimation of miR-124 expression in relation with lesion size from miR-124 specific RT products

Comparison of miR-124 expression in plasma at 2 days post-TBI in relation with the lesion size observed at 3 months, was also studied using the droplet digital PCR for miR-124 specific RT products. The expression pattern obtained in this case was different from both qRT-PCR and multiplexed ddPCR results. Up-regulation in miR-124 expression was observed in rats characterized with big sized cavities, compared to controls ($p<0.05$). Corresponding ROC analysis revealed high accuracy of miR-124 in differentiating rats with larger lesions from the controls (AUC=0.917 and $p<0.05$). This resembled the qRT-PCR pattern observed earlier. However, no difference in miR-124 expression was observed between the big cavity and smaller lesion groups. Also, no difference was observed between controls and rats with smaller lesions (figure 11).

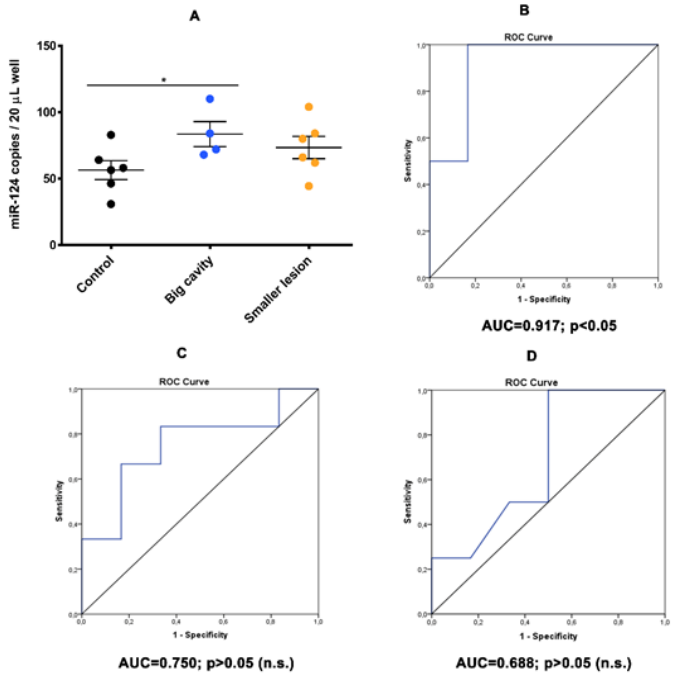


Fig. 11: (A) Up-regulation in miR-124 expression was observed in the ddPCR method only for rats with larger brain lesions compared to controls, ROC analysis for (B) control vs. big lesion group (C) control vs. smaller lesion group, (D) big lesion vs. smaller lesion group (miR-124 specific dataset)

3.10 Correlation between qRT-PCR and ddPCR expression patterns

Bivariate correlation analysis between the qRT-PCR and ddPCR datasets was first performed at 2 days post-TBI timepoint. For the multiplexed dataset, there was a correlation trend, but did not achieve significance (Spearman Correlation coefficient (ρ) = 0.447 and $p>0.05$). For the miR-124 specific dataset, correlation was observed (Spearman Correlation coefficient (ρ) = 0.74 and $p<0.01$) (figure 12 (A) and (B)). However, when analysis between the whole qRT-PCR and ddPCR datasets was performed, correlation was observed for both qRT-PCR vs. multiplexed ddPCR and qRT-PCR vs. miR-124 specific datasets. This was possibly as a result of inclusion of more data points in the analysis. For the multiplexed dataset, Spearman Correlation coefficient (ρ) of 0.387 and $p<0.01$ was obtained. For the miR-124 specific dataset, Spearman Correlation coefficient (ρ) was 0.343 and $p<0.01$(figure 12 (C) and (D)).

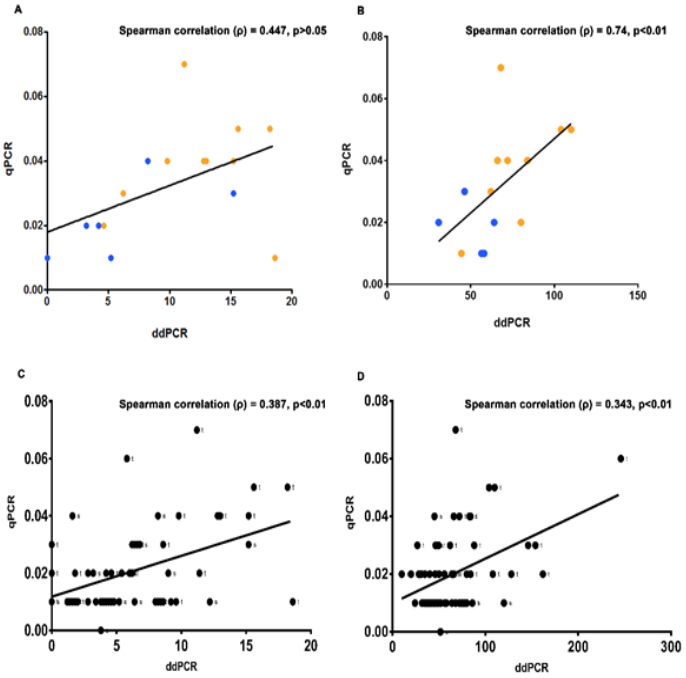


Fig. 12: Correlation between qRT-PCR and ddPCR amplifications at 2 days post-TBI for (A) multiplexed cDNA, (B) miR-124 specific cDNA. Correlation between qRT-PCR and ddPCR amplifications across all timepoints for (C) multiplexed cDNA, (D) miR-124 specific cDNA

3.11 Expression analysis of miR-124 in brain tissue samples (ipsilateral cortex)

To assess changes in miR-124 expression in the perilesional cortex, multiplexed TaqMan RT products (U6 and miR-124 specific) were used. Expression of miR-124 was studied with and without normalisation to U6 expression. Slight upregulation was observed in the trauma samples whereas all controls represented uniform expression (without normalisation). However, this up-regulation was not statistically significant. When normalised to U6, both controls and TBI samples demonstrated similar expression pattern, and no up-regulation was observed in this case (figure 13). Thus, for both normalised and non-normalised conditions, ddPCR demonstrated no difference in miR-124 expression between controls and TBI, indicating the robustness of this technique in miRNA quantification from tissue samples in absence of endogenous controls. Also, this result was in concordance with our previous miRNA sequencing results that demonstrated no change in miR-124 expression in the ipsilateral cortex at 3 month post-TBI (unpublished data).

IV. Discussion

MicroRNA biomarkers are believed to play a significant role in predicting susceptibility towards post-traumatic epilepsy, since alterations in miRNA expression levels have already been identified in ischemia, Alzheimers disease, and several other neurological disorders [5], [14], [25], [26], [27], [28]. Several studies aiming at prevention of epileptogenesis thus involve investigation of biomarkers at acute and/or chronic timepoints, which can reveal susceptibility of an individual towards developing epilepsy in future. Blood based biomarkers are gaining attention, since cytosolic components released from the damaged brain tissue (neurons and glial cells) are believed to reach peripheral blood through leakage in the blood-brain barrier, and also in part by the recently discovered glymphatic system of the

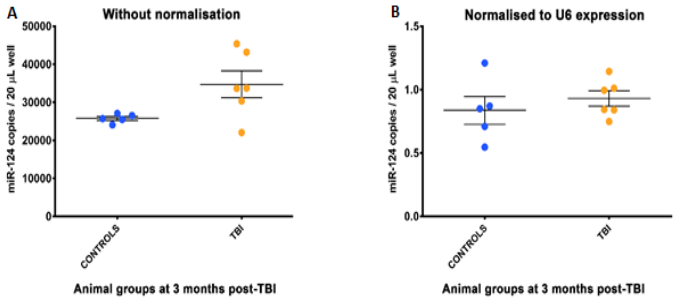


Fig. 13: Comparison of miR-124 expression between controls and TBI samples from ipsilateral cortex at 3 months post-injury, (A) without normalisation to U6, (B) data normalised to U6 expression

CNS [29], [30], [31]. Plasma concentrations of these brain specific macromolecules thus form markers of injury severity and extent of brain damage. Also, blood samples can be easily obtained from patients with minimal surgical intervention, thus making clinical diagnosis easier.

In concordance with this hypothesis, recent studies with blood samples from Sprague-Dawley rats subjected to lateral FPI have indicated up-regulation of brain-enriched miRNAs at an acute time-point (2 days post-TBI) [18]. Thus, the aim of this thesis study was to profile the expression of a brain-enriched miRNA, miR-124 from plasma samples of a similar animal cohort subjected to lateral FPI, over both acute and chronic timepoints. Furthermore, expression levels of miR-124 in the ipsilateral cortex at chronic time post-injury, and relation between plasma miR-124 levels and size of brain lesion developed as a result of the inflicted trauma were investigated.

The significance of this study lies in the increasing demand for identification of non-invasive biomarkers to predict susceptibility towards post-traumatic epilepsy. Obtaining brain tissue samples from individuals who have immediately suffered from a head trauma, but without signs of epilepsy or any cognitive impairment as of yet, can be challenging, as well as unethical in some situations. Also, imaging techniques have limited accuracy in predicting susceptibility towards epileptogenesis post-trauma. In such cases, there is a need to profile blood-based biomarkers, to predict susceptibility towards such conditions. Also, animal model based studies provide significant clues in the pre-clinical stage. For instance, this study not only revealed changes in expression patterns of miR-124 in plasma, but also indicated a probable association between the miR-124 levels and the size of the brain lesion developed. Such associations, if proved consistently with multiple datasets, would allow prediction of lesion size in humans as well based on the miR-124 expression level, to a certain extent, without surgical interventions.

4.1 Methodological considerations

4.1.1 RNA isolation from plasma

Cell-free biofluids such as plasma possess significantly low RNA concentrations, with miRNAs forming only a small fraction of the total RNA volume. Thus, successful extraction of these miRNA targets pose a challenge. Two methods are principally used: chemical extraction and immobilization on glass or silica. The mirVana^{TM} PARIS^{TM} kit is a high performance RNA isolation kit that uses the acid phenol-chloroform method for RNA extraction, followed by immobilization to a glass-fiber filter for elution. The Exiqon miRCURY^{TM} RNA isolation kit - biofluids, on the other hand, is able to purify RNAs smaller than 1000 nucleotides (including mRNAs, tRNAs, miRNAs and siRNAs) based on spin column chromatography, using a proprietary resin as the separation matrix. This protocol is able to extract small RNAs without the use of phenol-chloroform based extraction methods. Both kits were tested in pilot studies and comparable performance was observed. However, based on ease of use, absence of phenol carryover and lower costs, the Exiqon method was finally chosen for total RNA extraction.

4.1.2 Trade-off between Exiqon and TaqMan chemistries

The TaqMan small RNA assays are predesigned primer-probe based assays that aim at targeted quantification, screening and validation of mature microRNA sequences [23]. Due to target specificity of both primers and probes, only miRNA specific reverse transcription and qRT-PCR reactions can be performed with TaqMan chemistry. However, a combination of multiple primers can be used to develop a multiplexed reverse transcription reaction that allows simultaneous amplification of multiple targets from the same cDNA products in downstream qRT-PCR.

On the other hand, the Exiqon miRCURY LNA^{TM} Universal RT microRNA PCR system is a microRNA specific, locked nucleic acid (LNA^{TM}) system that enables specific and accurate quantification of miRNAs using intercalating dye based qRT-PCR amplification [22]. Unlike the miRNA specific TaqMan system, this method involves universal reverse transcription (RT) of all miRNAs followed by PCR amplification with LNA^{TM} enhanced primers. The universal RT reaction saves required sample volume and other reagents, along with lower technical variation and reaction time. However, due to the inherent tendency of intercalating dyes to bind with non-specific amplicons, the reaction specificity of this system is lower as compared to TaqMan. This is also verified in this study, since the Exiqon system consistently failed for miR-124 amplification from plasma samples, whereas successful detection was observed with TaqMan chemistry.

However, the Exiqon kit provided exogenous spike-ins to monitor both RNA isolation and RT reaction efficiencies, and this feature was not available with TaqMan system. Since extracted RNA volumes from plasma samples were below the detection limit of NanoDrop and Agilent Bioanalyzer, monitoring the spike-in RNA expressions was the only available option.

4.1.3 Absence of suitable endogenous control for plasma miRNA quantification

Expression analysis in qRT-PCR requires data normalization to a suitable endogenous control. However, for cell-free plasma samples, suitable endogenous control miRNAs are not known yet. Thus, the plasma enriched miR-103 was tested as a potential candidate. However, significant differences in expression was observed at different timepoints, in both control and injured animals. The exogenous spike-in expression data from Exiqon kit were then utilized. Normalization of miRNA expression from TaqMan was performed to these exogenous spike-ins.

4.1.4 Limitations in optimizing the ddPCR protocol

Annealing temperature (T_m) is the most critical parameter for reaction specificity in PCR, since setting it too low may lead to non-specific amplifications and setting it too high might reduce the yield of the desired PCR product [32]. Thus, a thermal gradient was set up to check for the suitable annealing temperature for miR-124 quantification with ddPCR. For tissue samples, proper separation of positive and negative clusters were obtained at $T_m \sim 60^0$C. However, for plasma no proper separation of clusters was observed at any temperature. Lowering the T_m only resulted in increase of positive droplets at lower fluorescence amplitudes, a possible indication of non-specific amplification. Thus, $T_m = 60^0$C could not be identified to be suitable for the ddPCR method with certainty.

4.2 Potential of plasma miR-124 to act as a TBI biomarker

Since miR-103 failed as an endogenous control candidate, miR-124 expression was also normalized to the exogenous spike-in expression. However, no negative impact in results was observed due to this normalization, since identical expression patterns were also obtained in ddPCR, where absolute miR-124 quantification was performed without requiring normalization controls.

Previous studies in animal models of stroke have demonstrated up-regulation of miR-124 at acute-timepoints post-occlusion, with levels beginning to increase at $\sim$6 hours after surgery, and peaking

at ∼24-48 hours [28], [33]. In this case, levels of miR-124 were assessed both at acute and chronic timepoints post injury. Similar to the stroke models, in all the three tested datasets (qRT-PCR with multiplexed cDNA templates, ddPCR with multiplexed cDNA templates and ddPCR with miR-124 specific templates), up-regulation of miR-124 in injured animals at an acute timepoint (2 days post-TBI) was validated. Also, this was in agreement with the previous study of miR-124 expression profiling post-TBI, in whole blood [18].

A major consideration in profiling plasma levels of miRNA expression for predicting TBI, is that the trauma affects not only the brain, but also peripheral organs. Thus the possibility of the target miRNA expression level to reflect such bodily injuries as well, cannot be ruled out [34]. However, several studies indicate high specificity of miR-124 in brain specific areas (CNS, cerebral cortex, spinal cord) [35], [36], thereby prompting the assumption that most of the altered miR-124 expression patterns observed in plasma reflect only severity of brain injury due to TBI.

The other consideration is the rate of clearance of brain-specific components post injury. Recent studies have indicated impairment of several clearance mechanisms of the brain post-TBI, leading to reduction in brain clearance [29], [30], [37]. Thus, even if brain-specific components profiled from body fluids reveal significant alterations in expression pattern in response to injury, they may not reflect the actual extent of injury severity, because of the impaired clearance. In future studies aiming at profiling brain-specific components from body fluids, this aspect has to be considered.

Another interesting observation is that in in-vitro models of Alzheimer′s disease, miR-124 levels are observed to be inversely proportional with Aβ accumulation, thus indicating that alterations of this miRNA in response to TBI may also pose some relation with Alzheimer′s-like pathology [38].

4.3 Sensitivity of ddPCR in assessing plasma levels of miR-124

Droplet PCR could successfully replicate the up-regulation pattern of miR-124 in injured animals at 2 days post-TBI. However, variability was observed between the multiplexed and miR-124 specific datasets. For the multiplexed products, very few target copies could be obtained, with significantly low fluorescence amplitudes. For few samples, application of the manual threshold of 6000 resulted in no positive amplifications at all, and when tested without the manual threshold setup, the predicted pattern of miR-124 up-regulation at 2 days post-TBI could no more be observed. This indicated the importance of manual adjustment of the threshold, especially for low target abundance samples, to exclude non-specific amplifications and to detect significant changes in expression patterns.

On the other hand, for miR-124 specific cDNA samples, higher copy numbers were obtained, with higher fluorescence amplitudes as well. This indicated that variability in ddPCR results can also be observed when upstream reaction methods are different, possibly in this case due to different mechanisms of cDNA synthesis reaction (multiplex vs. miR-124 specific).

Differences were also observed between the two ddPCR datasets at chronic timepoints post-injury. The multiplexed dataset replicated the qRT-PCR observation pattern, whereas for the miR-124 specific case, up-regulated levels of miR-124 were observed at 1 week and 3 months post-injury timepoints as well. This was possibly because of the day-to-day variability in reaction preparation, since ddPCR runs for the miR-124 specific samples from different timepoints were performed on different days. Importance of performing all ddPCR runs for a single analysis at once, to minimize technical variations, was thus elucidated.

For ddPCR, few no-template controls (NTCs) also exhibited positive amplifications, possibly due to contamination while reaction preparation and the high sensitivity of this technique. However, similar results have been reported in other studies as well [39], [40], where chances of cross-contamination were minimized as far as possible. The extent to which positive amplification of NTCs affect the ddPCR results is not known yet, and needs to be investigated further. In qRT-PCR, none of the NTCs showed any amplification.

4.4 Relation between plasma levels of miR-124 at acute timepoints post-injury with histological changes observable at chronic timepoints

Cresyl violet staining for the coronal brain sections revealed association of elevated miR-124 plasma levels at 2 days post-TBI, with development of larger brain lesions and greater extent of cell loss at the impact site. This association was validated with qRT-PCR. Rats characterized with larger cavities, in fact, demonstrated higher levels of miR-124 in plasma at 2 days post-TBI. One possible explanation for this could be that larger brain lesions are indicative of higher extent of blood-brain barrier damage post-injury, allowing increased infiltration of miR-124 from the injured brain to blood.

However, the ddPCR results failed to identify this association. The ddPCR patterns from multiplexed and miR-124 datasets differed between themselves as well. From the multiplexed dataset, no significant difference in expression pattern was observed among controls, rats with bigger cavities, and rats with smaller lesions. The miR-124 specific dataset, on the other hand, could reveal significant up-regulation of miR-124 in rats with bigger cavities compared to controls, but no difference between these and the ones with smaller lesion was observed. Thus, relation between lesion type and miR-124 levels in plasma at an acute timepoint post-injury was not totally consistent in this study.

V. Conclusion

Thus, the main findings from the study can be summarized as follows:

1. Up-regulation in plasma miR-124 expression was observed in case of animals receiving the head injury, at 2 days post-TBI, with both qRT-PCR and ddPCR methods. This underscored the potential of miR-124 as a biomarker for TBI at acute timepoints. The findings were consistent with previous studies performed with whole blood, wherein up-regulation in miR-124 at 2 days post-TBI was also evident in case of trauma samples. Additionally, in concordance with our previous miRNA sequencing data, similar miR-124 levels were observed for controls and injured animals, in the ipsilateral cortex, at 3 months post-TBI.
2. Both real-time qRT-PCR and ddPCR demonstrated comparable sensitivity in profiling miR-124 levels from plasma. However, absence of a known endogenous reference for qRT-PCR based plasma miRNA quantification was a major limitation. Expression levels were thus normalized to the exogenous spike-ins added to monitor reaction efficiency. No negative impact was observed as a result of this normalization, since ddPCR based absolute quantifications reflected similar expression patterns. On the other hand, ddPCR demonstrated very low rate of target amplification when multiplexed cDNA products were used, although the up-regulation pattern was still evident. Also, when reactions were performed in different days, significant day-to-day variability in expression patterns was observed.
3. Relation between miR-124 levels at acute timepoints, in relation with lesion size developed at chronic periods, was established with qRT-PCR. Rats with small and middle sized cavities at 3 months post-injury demonstrated almost similar miR-124 levels in plasma as controls, whereas rats with larger cavities demonstrated significantly elevated levels of plasma miR-124 at 2 days post-TBI. However, results from ddPCR were not totally consistent with this observation.

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