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MicroRNA Profile Differences in Neonates at Risk for Cerebral Palsy

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Abstract

Background: MicroRNAs; miRs are used as biomarkers in the diagnosis of several diseases. Cerebral palsy; CP, resulting from perinatal brain injury, cannot be diagnosed until 18-24 months old. Biomarkers to predict CP and assess response to investigational therapies are needed. We hypothesized that miRs expressed in neonates with the CP risk factors of abnormal tone and/or intraventricular hemorrhage; IVH differ from those without risk factors.

Methods: This was a cohort study of neonates at risk for CP. Subjects <32 weeks gestation and <1500 grams were recruited from neonatal intensive care units at a large urban delivery hospital and an adjacent children's hospital. Thirty-one plasma samples were evaluated. An unbiased examination was performed by locked nucleic acid quantitative real time – polymerase chain reaction; qRT-PCR. Results were evaluated in the context of IVH and abnormal tone.

Results: Plasma miR profiles in neonates at risk for CP differ when comparing those with and without IVH, and with and without abnormal tone. Restricted profiles were found in each condition with greater differences in the tone comparison than the IVH comparison.

Conclusion: Plasma miR profiles show potential in predicting CP. This study also suggests biologically plausible candidates for future studies.

Keywords: MicroRNAs; Biomarkers; Cerebral palsy

Introduction

Cerebral palsy; CP, the most common motor disability in childhood occurring in 1 in 323 children in the U.S. [1], is characterized by motor and postural control dysfunction resulting from perinatal white matter injury [2]. CP is highly associated with prematurity and very low birth weight; VLBW; 42-47% of individuals with CP were born preterm [3] and 5-10% of VLBW infants develop CP [4]. CP is diagnosed following serial examination findings of non-progressive, persistent delay in motor development [5]. Neonates are considered at-risk based on obstetric and perinatal factors, but these do not reliably predict CP [6]. Abnormal head ultrasounds; HUS are also important predictors with a specificity of 86%, but a sensitivity of only 29% [7]. Early diagnosis would provide a therapeutic window when the central nervous system is most plastic.

MicroRNAs; miRs are small, noncoding RNAs that are important post-transcriptional regulators of gene expression affecting developmental processes [8]. MiRs circulate in a stable extracellular form, and peripheral profiles of miRs are altered in disease states [9]. Rodent adult stroke models and human adult stroke have shown changes in miR expression, demonstrating the value of miRs as potential biomarkers of neuronal injury [10,11]. Premature and VLBW infants are particularly susceptible to oligodendroglial injury, the pathologic substrate for CP [12]. Several miRs are known to regulate oligodendrocyte differentiation [13,14], and we found that miR-138 and miR-338 expression were increased in a mouse model of neonatal brain injury [15]. The aim of the present study was to determine whether miRs are differentially expressed in prematurely born humans at risk for CP.

Methods

Study design

This was a case-control study with subjects assessed for the primary outcome of abnormal motor function by 18 months corrected gestational age.

Subject enrollment

Newborns admitted to the Prentice Women's Hospital Neonatal Intensive Care Unit; NICU or the Ann & Robert H. Lurie Children's Hospital of Chicago NICU who were <32 weeks gestational age at birth and <1500 grams were eligible. Exclusion criteria included newborns who were *in extremis*, had known brain dysgenesis or genetic syndromes associated with brain dysgenesis, were unable to return to the NICU Neurodevelopmental Follow-Up Clinic, or whose parents were members of the Jehovah's Witness faith. The study was approved by institutional review boards at Lurie Children's Hospital and Northwestern University.

Sample collection

One-half milliliter of blood was collected in K_2EDTA containing tubes. Most samples were collected at 1 month of age although some were collected as early as 3 days and as late as 2 months of age. Samples were centrifuged within 1 hour of collection at 1900G for 10 minutes at 4°C, then the supernatant was centrifuged at 16,000G for 10 minutes at 4°C, and finally plasma was collected and stored at -80°C.

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Table 1: Clinical Characteristics.

		IVH			Tone		
Characteristic	Overall Group	Cases	Controls	*p*-value	Cases	Controls	*p*-value
Gestational age, mean (SD), wk	29.0 (1.3)	28.0 (1.3)	29.4 (1.2)	0.04*[a]	28.8 (1.5)	29.2 (1.1)	0.38[a]
Birth weight, mean (SD), g	1144 (149)	1058 (152)	1175 (138)	0.10[a]	1136 (163)	1152 (139)	0.79[a]
Male sex, No. (%)	10 (38.5)	2 (28.6)	8 (42.1)	>0.99[b]	5 (38.5)	5 (38.5)	>0.99[b]
SNAP score, man (SD)	15.1 (12.0)	12.6 (10.5)	16.1 (12.9)	0.50[a]	17.8 (14.5)	12.4 (9.3)	0.27[a]
SNAPPE-II score, mean (SD)	23.3 (16.1)	18.0 (15.0)	25.2 (16.8)	0.31[a]	27.1 (21.1)	19.5 (9.3)	0.25[a]
PPROM, No. (%)	10 (38.5)	3 (42.8)	7 (36.8)	>0.99[b]	5 (38.5)	5 (38.5)	>0.99[b]
≥7d antibiotics, No. (%)	7 (26.9)	2 (28.6)	5 (26.3)	>0.99[b]	3 (23.1)	4 (30.8)	>0.99[b]

[a]*p* values determined using a two-tailed T-test.
[b]*p* values determined using the Kolmogorov-Smirnov test.

Table 2: Comparison of Motor Scores between Groups.

A						
6 months	**No IVH**		**IVH**			
	Mean (SD)	95% CI	Mean (SD)	95% CI	Difference between means (95% CI)	*p*-value
Motor Composite	94.9 (3.0)	87.7 to 102	98.7 (3.8)	88.9 to 108	3.8 (-6.6 to 14.2)	0.444
Gross Motor	9.1 (2.1)	7.4 to 10.9	10.5 (2.7)	7.7 to 13.3	1.4 (-1.4 to 4.1)	0.300
Fine Motor	9.1 (1.2)	8.1 to 10.2	8.8 (1.7)	7.0 to 10.6	-0.3 (-2.0 to 1.4)	0.719
12 months	**No IVH**		**IVH**			
	Mean (SD)	95% CI	Mean (SD)	95% CI	Difference between means (95% CI)	*p*-value
Motor Composite	95.1 (13.6)	86.5 to 104	93.6 (9.5)	81.9 to 105	-1.5 (-15.8 to 12.8)	0.828
Gross Motor	9 (3.7)	6.7 to 11.3	8 (2.5)	5.0 to 11.0	-1 (-4.8 to 2.8)	0.587
Fine Motor	9.3 (1.2)	8.6 to 10.1	9.8 (1.3)	8.6 to 10.1	0.5 (-0.89 to 1.8)	0.475
18 months	**No IVH**		**IVH**			
	Mean (SD)	95% CI	Mean (SD)	95% CI	Difference between means (95% CI)	*p*-value
Motor Composite	100 (3)	92.6 to 108	88 (8.5)	11.8 to 164	-12 (27.9 to 3.9)	0.096
Gross Motor	9 (1)	6.5 to 11.5	8.5 (0.7)	2.1 to 14.9	-0.5 (-3.1 to 2.2)	0.591
Fine Motor	11.4 (0.9)	10.3 to 12.5	8.3 (2.1)	3.2 to 13.5	-3.1 (-5.6 to -0.55)	0.024*

B						
6 months	**Normal Tone**		**Abnormal Tone**			
	Mean (SD)	95% CI	Mean (SD)	95% CI	Difference between means (95% CI)	*p*-value
Motor Composite	105 (5.8)	95.8 to 114	93.1 (7.4)	87.8 to 98.4	-11.9 (-20.9 to -2.9)	0.014*
Gross Motor	11.3 (0.5)	10.4 to 12.1	9.1 (2.6)	7.3 to 10.9	-2.2 (-5.0 to 0.72)	0.129
Fine Motor	10.0 (1.2)	8.2 to 11.8	8.6 (1.4)	7.6 to 9.6	-1.4 (-3.1 to 0.28)	0.095
12 months	**Normal Tone**		**Abnormal Tone**			
	Mean (SD)	95% CI	Mean (SD)	95% CI	Difference between means (95% CI)	*p*-value
Motor Composite	102 (11.1)	93 to 111	88 (92)	80.8 to 95.1	-14 (-24.6 to -3.6)	0.012*
Gross Motor	10.5 (2.8)	8.1 to 12.9	7.1 (3.0)	4.8 to 9.4	-3.4 (-6.4 to -0.38)	0.030*
Fine Motor	10.1 (1.2)	9.1 to 11.1	8.9 (0.8)	8.3 to 9.5	-1.3 (-2.3 to -0.17)	0.025*
18 months	**Normal Tone**		**Abnormal Tone**			
	Mean (SD)	95% CI	Mean (SD)	95% CI	Difference between means (95% CI)	*p*-value
Motor Composite	101.5 (2.1)	82.4 to 121	84.3 (15.0)	60.4 to 108	-17.2 (-48.5 to 14.0)	0.201
Gross Motor	9 (1.4)	-3.7 to 21.7	7 (3.4)	1.6 to 12.4	-2 (-9.2 to 5.2)	0.484
Fine Motor	11.7 (0.58)	10.2 to 13.1	8.8 (2.4)	6.3 to 11.4	-2.9 (-6.3 to 0.60)	0.092

Head imaging

HUS was obtained per usual medical practice: at 7-10 days of life, 1 month of life and 36 weeks corrected gestational age. If IVH was present, then additional imaging studies were obtained per the attending neonatologist's discretion.

Chart review

The inpatient electronic medical record was reviewed to collect clinical data including but not limited to gestational age, birth weight, sex, duration of ruptured membranes, Apgar scores, antibiotic use and HUS findings. Scores of Neonatal Acute Physiology, Version II (SNAP-II) and SNAP, Perinatal Extension (SNAPPE-II) were calculated based on physiological parameters from the first 24 hours of life [16,17]. Outpatient clinical data was collected by review of the electronic medical record from NICU Neurodevelopmental Follow-Up Clinic visits at 3, 6, 12 and 18 months corrected age. Follow-up data included muscle tone on neurologic exam and the Bayley Scales of Infant Development III – Motor Composite Score (BSID III-MCS), and presence or absence of a diagnosis of CP at 18 months corrected age.

Group assignment

Subjects were assigned to the IVH group if IVH was detected on HUS and remained in the IVH group even if subsequent HUS findings normalized. Subjects were assigned to the abnormal tone group based on exam performed by an attending neonatologist. A subject was assigned to the abnormal tone group if other than normal tone was detected on any exam, regardless if tone normalized at later visits.

Quantitative real time-polymerase chain reaction

Plasma was sent to Exiqon Services, Denmark for miR quantification using the miRCURY LNA™ Universal RT microRNA PCR hsa panel I+II to assay for 752 miRs [18]. The LNA or locked nucleic acid method, utilizes synthetic RNA/DNA analogs that possess increased thermo stability when duplexed with oligonucleotides [19]. It allows for bypassing pre-amplification when using small amounts of starting material, thus avoiding bias that could be introduced in that step. In addition, LNA allows for discrimination of miRs that may differ by only one nucleotide [20]. Briefly, all miRs were polyadenylated and reverse transcribed into cDNA using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). The cDNA was transferred to qPCR panels and amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analyzed using the Roche LC software for determination of quantification cycle (Cq) (by the 2nd derivative method) and for melting curve analysis.

Analysis and statistics

Exiqon Services performed comprehensive data analysis to compare differential expression of miRs between the case and control groups. Assay efficiencies were determined by analysis of the amplification curves using algorithms similar to the LinReg software. The assays were inspected for distinct melting curves and the melting temperature (Tm) was confirmed to be within known specifications for the assay. Only samples that exhibited a 3 Cqs lower value than the negative control Cq<37 were included in the data analysis. Norm finder was used to find the best normalizer; this was found to be the average of assays detected on all samples. Data was normalized based on the average of the assays detected in all samples. The equation used to calculate the normalized Cq values was: Normalized Cq (dCq) = average Cq (n=31) – assay Cq (sample). Statistical analysis was performed on the normalized data to compare the average expression levels and to identify miRs that were differentially expressed between the IVH and no IVH groups, between the abnormal tone and normal tone groups and between the IVH/abnormal tone and no IVH/normal tone groups. Differences in expression levels were identified using a two-tailed t-test for 2 group comparisons with a p-value of <0.05 for both. Additional analysis was performed using Excel (Microsoft, Seattle, WA) and Prism 6 (GraphPad, La Jolla, CA).

Results

We obtained 31 plasma samples and followed subjects until 18 months corrected age from August 2013 to December 2016. The majority of samples were collected at 1 month of age but some were collected as early as 3 days and as last as 2 months. Ninety-six percent of subjects were evaluated at 3 months corrected age, 65% were evaluated at 6 and 12 months corrected age, and 58% were evaluated at 18 months corrected age. Two subjects or 7.7% were diagnosed with CP. We compared the characteristics of subjects with IVH, which included severity ranging from grade 1 to 4, to those without IVH. Subjects did not differ with regards to birth weight, sex, illness severity as determined by SNAP and SNAPPE-II scores, PPROM, or exposure to >= 7days of antibiotics. They did, however, differ with respect to gestational age at birth; those with IVH were younger by 1.4 weeks. We also compared subjects who had ever been diagnosed with abnormal tone to those with normal tone. The majority of infants with abnormal tone were identified by 3-6 months corrected age; of these, 54% normalized by 18 months corrected age. These subjects did not differ with regards to gestational age at birth, birth weight, sex, SNAP and SNAPPE-II scores, PPROM, or >= 7days of antibiotics. All subjects received antenatal steroids except for one (Table 1).

Because in practice neonatologists often use the presence of IVH to counsel parents on neurodevelopmental prognoses, we compared Bayley III motor scores of cohort subjects with and without IVH. We found no differences between IVH and no IVH groups when comparing the composite, gross motor and fine motor scores at 6 and 12 months corrected age. We also found no difference between IVH and no IVH groups when comparing composite and gross motor scores at 18 months, but we did observe significantly lower fine motor scores (p=0.024) (Table 2A). By contrast, subjects with abnormal tone scored significantly lower on the Bayley III compared to the normal tone group. At 6 months corrected age, the composite score for infants in the abnormal tone group was lower than for the normal tone group (p=0.014), but gross and fine motor scores were not different between groups. At 12 months corrected age, all three Bayley III scores were different between abnormal tone and normal tone groups (motor composite p=0.012, gross motor p=0.030, fine motor p=0.025) (Table 2B). These differences disappeared at 18 months corrected age.

We performed an unbiased examination for 752 different miRs on all 31 samples. No obvious separation was noted between IVH versus no IVH groups by heat map or principal component analysis. Similarly, heat map analysis did not suggest an obvious

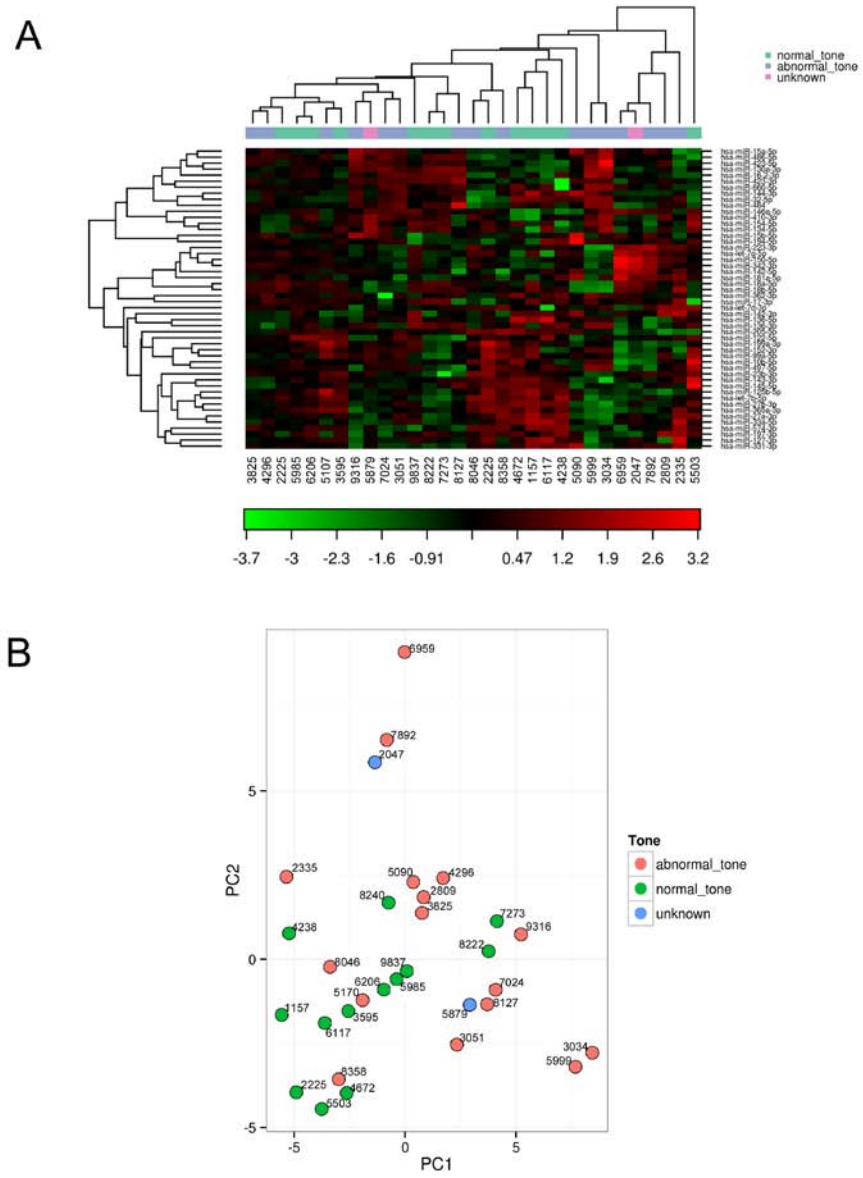

Figure 1: Exploratory data analysis is suggestive of grouping by subjects with abnormal tone. A) Heat map of miR expression in samples from subjects with normal, abnormal and unknown tone. B) Principal component analysis graph shows mild separation of samples from subjects with abnormal and normal tone.

relationship between abnormal tone and miR expression (Figure 1A), but principal component analysis suggested weak separation of abnormal tone versus normal tone samples (Figure 1B). We did find that plasma miR profiles differed between groups. When comparing IVH versus no IVH, we found changes in the peripheral expression of 23 different miRs (Figure 2 and supplementary data A). By contrast, when comparing abnormal tone versus normal tone, we found changes in expression of 70 miRs (Figure 2 and supplementary data B). Finally, when comparing subjects with IVH and abnormal tone versus normal controls (no IVH and normal tone), we found changes in expression of 30 miRs (Figure 2 and supplementary data C).

Only four miRs were differentially expressed in all three of these comparisons (Figure 2). More importantly, none of the same miRs were present at the intersection of the abnormal tone versus normal tone comparison and the IVH versus no IVH comparison. Not surprisingly, many miRs [21] were present at the intersection of the abnormal tone versus normal tone comparison and the IVH/abnormal tone versus normal control comparison. Eight miRs were present at the intersection of the IVH versus no IVH comparison and the IVH/abnormal tone versus normal control comparison.

With regards to miRs known to regulate oligodendroglial differentiation, only miR-9 was differentially expressed (p=0.04) and was downregulated 2-fold in the IVH versus no IVH comparison. The greatest fold change in expression in the IVH versus no IVH comparison was observed for miR-548d-5p (upregulated 4.2-fold, p=0.04) (supplementary data A). The most significant change in expression in the IVH versus no IVH comparison was observed for miR-210-3p (p=0.0001, downregulated 1.5-fold) (supplementary data

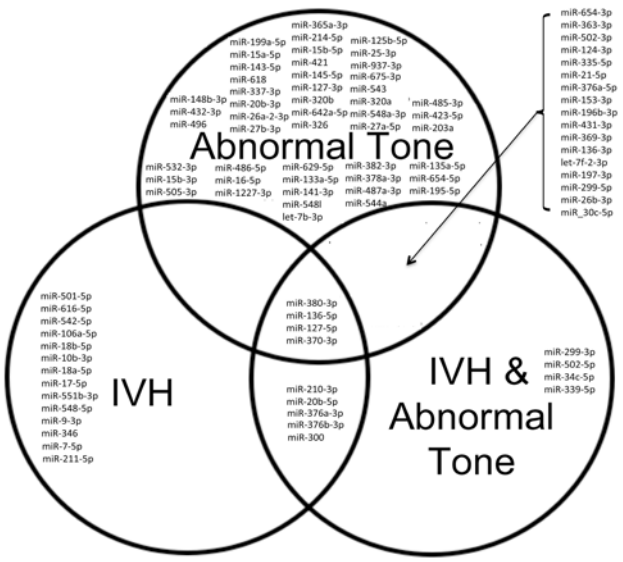

Figure 2: Venn diagrams of A) samples from all time points pooled, and B) samples from discrete time points.

A). The most significant change in expression in the abnormal tone to normal tone comparison was observed for miR-654-3p (p=0.0007, upregulated 2.5-fold) (supplementary data B). The greatest fold change in expression in the abnormal tone to normal tone comparison was observed for miR-26a-2-3p (upregulated 15.6-fold, p=0.01) (supplementary data B). The most significant change in expression in the IVH/abnormal tone to no IVH/normal tone comparison was observed for miR-210-3p, the same as for the IVH to no IVH comparison (p=0.0007, downregulated 1.7-fold) (supplementary data C). The greatest fold change in expression in the IVH/abnormal tone to no IVH/normal tone comparison was observed for miR-300 (p=0.03, downregulated 5.8-fold) (supplementary data C).

Discussion

Although medical history identifies preterm neonates at risk for CP and abnormal brain imaging confers added risk, these indicators are imperfect. Our study provides further evidence that IVH diagnosis by HUS is a poor predictor for CP, as none of our subjects who developed CP by 18 months corrected age had IVH. Pre-discharge MRI has improved sensitivity and specificity over HUS for predicting CP [7], but families are still left with uncertainty at discharge. The burden of diagnosis rests with the NICU follow-up clinic based on serial physical exams, especially evolution of tone abnormalities. However in a systematic review of physical exam findings predictive for CP, tone assessment did not even meet inclusion criteria due to lack of data to assess positive predictive value; PPV and negative predictive value; NPV. Only the asymmetric tonic neck reflex, Moro reflex, parachute reaction, plantar grasp reflex and pull-to-sit maneuver had acceptable PPV and NPV in the first year of life [21]. In our study, of the subjects who displayed abnormal tone at any time, only 15% developed CP by 18 months corrected age. This suggests that abnormal tone between 3-12 months is a better, although still poor, predictor of CP compared to a diagnosis of IVH.

Most importantly, our study is the first to examine serum miR expression in a preterm patient population at risk for CP, and the first to relate miR expression profiles to IVH and tone abnormalities. We demonstrate the feasibility of consistently amplifying miRs from limited volumes of peripheral blood from VLBW infants. We find greater differences in miR expression profiles in subjects with tone abnormalities compared to subjects with IVH, implicating miRs in mechanisms ultimately leading to CP; e.g., post-translational regulation of gene expression by miRs may be important in the pathogenesis of motor dysfunction following preterm brain injury. Finally, our study provides a framework for using serum miR expression as an early disease predictor to enable earlier interventions such as physical therapy, occupational therapy or even experimental therapies.

Our Venn diagram (Figure 2) highlights restricted panels of miRs that are exclusively differentially expressed in the abnormal tone versus normal tone comparison. These could be explored as potential early biomarkers for the later development of CP. A reliable biomarker panel would allow us to trial experimental therapies in patients at highest risk during that window of time before CP becomes manifest. With regards to the use of any individual miR as a biomarker, this study enabled power analyses for the study of individual miRs. We determined that for individual biomarker validation for example, 33 plasma samples would be required to evaluate miR-654, and 13 plasma samples would be required to evaluate miR-26a for significant differences between abnormal tone and normal tone groups with a *p*-value set at <0.01 (supplementary data B).

We showed that several miRs known to play a role in oligodendroglial differentiation, miR-9, miR-138, miR-219 and miR-338, are detectable in peripheral blood samples within the plasma compartment. Of these, only miR-9 was differentially expressed and this was in subjects with IVH. Conceivably, a miR that is exclusively expressed in the CNS might be find its way to the peripheral circulation through disruption of the blood brain barrier that might occur after injury. Many miRs however are expressed both centrally and peripherally. For example, miR-138 and miR-338 are expressed in the peripheral nervous system. Many of the 752 miRs in our unbiased examination are likely expressed in non-brain organ systems. Insults that result in CP might impact the central nervous system alone, the peripheral nervous system alone, the musculoskeletal system alone, any other organ system alone or any of these in combination. Thus, signal from peripheral blood does not specify the organ of origin of any miR. MiRs might be found in the peripheral circulation as a direct result of brain injury or through an indirect process that, for whatever reason, correlates with motor dysfunction. Stated otherwise, if differential expression of a particular miR or set of miRs correlates strongly with an outcome of motor dysfunction, even if the mechanism of involvement is uncertain, it has value as a biomarker.

Finally, our study suggests hypotheses that can be tested in animal or *in vitro* models to understand new mechanisms leading to impaired myelination following perinatal insults. For example, miR-654, which was upregulated in subjects with abnormal tone, may be an important candidate to explore. This miR has been identified as a cancer suppressor in various cancers by decreasing cell proliferation and cell migration [22], and by inducing apoptosis [23]. One could test whether miR-654 mimetics decrease oligodendroglial cell counts

and myelin and whether miR-654 antagonists can block these effects in an animal model of perinatal brain injury. MiR-26a, which was also upregulated in subjects with abnormal tone, similarly induced apoptosis in the setting of cancer [24]. One could similarly test the effects of miR-26a mimetics and antagonists on oligodendroglial cell counts and myelin production. Thus, our study suggests exciting candidates for further investigation.

This study was limited by sample size, attrition and few subjects diagnosed with CP. Nonetheless, it represents an important first attempt in identifying specific plasma miRs important in preterm brain injury leading to motor dysfunction. It also provides evidence of feasibility for a future multicenter study that would be required to study the infrequent outcome of CP.

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