Cord blood microRNA-376c and microRNA-1268a as biomarkers for neonatal hypoxic-ischaemic encephalopathy: a diagnostic accuracy study

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ABSTRACT

Background Hypoxic-ischaemic encephalopathy (HIE) is one of the most common causes of morbidity and mortality among neonates. There is a critical need for non-invasive novel biomarkers to detect HIE early, predict its outcomes and monitor its progression. We conducted this observational study to assess the relative expression of miRNA-376c and miRNA-1268a in cord blood as potential diagnostic and prognostic biomarkers for HIE.

Methods A total of 100 neonates divided into two independent groups were included. The case group included 50 neonates with HIE, while the control group included 50 matched healthy neonates. Relative expressions of miRNA-376c and miRNA-1268a were measured in whole cord blood at birth using real-time PCR.

Results Compared with the control group, patients with HIE had a significantly lower median level of miRNA-376c (0.168, IQR=0.011–0.411 vs 1, IQR=0.80–1.20) and a higher median level of miRNA-1268a (13.46, IQR=2.7–22.8 vs 1, IQR=0.4–1.6). Comparing neonates with HIE who survived versus those who did not survive, no statistically significant difference between the groups in terms of miRNA-376c and miRNA-1268a ($p=0.124$ and $p=0.279$) was elicited. Our diagnostic analysis showed that, at 0.90 points, miRNA-376c has a sensitivity and a specificity of 88% and 68.40%, with an area under the curve of 84%. At 2.70 points, miRNA-1268a has a sensitivity and a specificity of 76% and 100%, with an area under the curve of 96%.

Conclusion The relative expression of miRNA-376c and miRNA-1268a was altered in the cord blood of neonates with HIE. In addition, they have moderate diagnostic accuracy in detecting HIE.

INTRODUCTION

Hypoxic-ischaemic brain injury is a considerable common cause of neonatal encephalopathy.¹ Hypoxic-ischaemic encephalopathy (HIE) is estimated to affect 1.5 out of every 1000 live births.² Up to 60% of neonates will die by 2 years or will have critical disorders

What is known about the subject?

- ▶ Hypoxic-ischaemic brain injury is a considerable common cause of neonatal encephalopathy.
- ▶ Hypoxic-ischaemic encephalopathy is estimated to affect 1.5 out of every 1000 live births, with a considerable mortality rate.
- ▶ miRNA-376c plays a vital role in the regulation of cell growth and proliferation, while miRNA-1268a is involved in embryogenesis and cell differentiation.

What this study adds?

- ▶ The expression of miRNA-376c and miRNA-1268a was altered in the cord blood of neonates with HIE.
- ▶ Patients with HIE had a significantly lower level of miRNA-376c than healthy controls and a higher level of miRNA-1268a.
- ▶ The expression of miRNA-1268a and miRNA-376c was comparable among HIE cases who survived and those who did not survive.

such as mental retardation or epilepsy.³ Finding diagnostic and prognostic biomarkers for HIE helps clinicians identify disease progression, predict the clinical outcomes and provide neuroprotective strategies.³

Previous studies have extensively identified new potential biomarkers for HIE in different types of samples, including cord blood, cerebrospinal fluid, serum and urine.^{4 5} These potential biomarkers include interleukin 6, glial fibrillar acidic protein, myelin basic protein and ubiquitin carboxyl-terminal hydrolase L1 in cord blood.⁴ Interestingly, microRNA (miRNA) expression is altered in neonates with HIE and has been suggested as potential biomarkers for HIE in umbilical cord blood.^{5 6}

miRNAs are small, non-coding RNA molecules with a high degree of conservation that post-transcriptionally regulate the expression of about 30% of human genes.⁷ miRNAs bind to its complementary sequence of 3-untranslated regions of the messenger RNA of the target gene and stimulate its degradation. Further, miRNAs play essential roles in regulating pathways in cell proliferation, migration, apoptosis and drug resistance.^{7,8} miRNA-376c belongs to an evolutionarily conserved miRNA family that includes genes located on human chromosome 14 in a syntenic cluster.⁹ miRNA-376c plays a vital role in the regulation of cell growth and proliferation and was found to have low expression in the umbilical cord whole blood of neonates with HIE. Further, its upregulation reduced cell injury induced by oxygen and glucose deprivation in cell lines.⁶ Furthermore, miRNA-1268a is a critical abundant miRNA functionally involved in embryogenesis and cell differentiation.¹⁰ High expression of miRNA-1268a is considered a risk factor for stroke.¹¹ The RNA-induced silencing complex allows miRNAs to perform their regulatory functions. Production, replication, differentiation and apoptosis are some of the biological processes that miRNAs regulate.¹² Furthermore, exosomal miRNAs are emitted into the fluids of the body and play a role in intercellular communication, suggesting that they may be used as diagnostic biomarkers.¹³ We conducted this study to evaluate the role of miRNA-376c and miRNA-1268a in the diagnosis and prediction of neonatal HIE.

METHODS

We conducted this diagnostic accuracy study at the Neonatal Intensive Care Unit of Menoufia University Hospital, in cooperation with the Biochemistry Department, Faculty of Science, and the Medical Biochemistry and Molecular Biology Department, between January 2020 and January 2021. Written consent was obtained from the guardian of each participant included in the study.

Patient and public involvement statement

Patients and/or the public were involved in the design, conduct, reporting and dissemination plans of this research. Public healthcare clinics, but not families, were involved in the content and design.

Eligibility criteria

Inclusion criteria

For the case group, we targeted all term neonates exposed to perinatal asphyxia and only included neonates with clinical manifestations suggestive of neonatal HIE according to the clinical Sarnat staging system.¹⁴ The control group included a group of age-matched healthy neonates. Controls were full-term neonates with no history of perinatal risk factors for asphyxia and with normal Apgar scores at the first and fifth minute after birth, without the need for resuscitation. Weight was not

used for matching as we aimed to study weight as one of the risk factors.

Exclusion criteria

We excluded the following:

- ▶ Neonates with congenital infections or congenital anomalies.
- ▶ Neonates with chromosomal abnormalities.
- ▶ Neonates with suspected inborn errors of metabolism.
- ▶ Preterm neonates (less than 37 weeks).
- ▶ Infants of mothers with diabetes.
- ▶ Neonates with sepsis.

All neonates were subjected to a detailed history taking, clinical examination as well as laboratory investigations. Routine investigations included complete blood count, quantitative C reactive protein (CRP), blood gases, electrolytes, liver function test, kidney function test, serum ammonia and lactate. Specific investigations assessed miRNA-376c and miRNA-1268a levels using the real-time PCR technique.

Clinical Sarnat staging system

First, all included cases were subjected to a detailed neurological examination for HIE performed within 24 hours of birth. Specifically, physical examination focused on assessing the consciousness level, muscle tone, both tendon and complex reflexes, and autonomic function, as described in the Neonatal Research Network study (NICHD).¹⁵ The results of the neurological examination were augmented with encephalographic findings suggesting the affection. We classified neonates with a diagnosis of HIE based on the Sarnat staging system.¹⁴ According to this staging, neonatal HIE can be categorised into mild cases (stage I), moderate cases (stage II) and severe cases (stage III).

Radiological assessment

Magnetic resonance imaging (MRI) was performed on the eighth day of admission. T1 and T2 images, as well as diffusion-weighted imaging, were used. We assessed the MRI studies for occurrence and magnitude of pathological shortening of T1 as well as prolongation of T2, cerebral haemorrhage, limited diffusion and periventricular leucomalacia. We further graded the MRI findings using the following criteria^{16 17}:

- ▶ Grade 0: normal MRI findings.
- ▶ Grade 1a: MRI findings show minor cerebral impairment that does not affect the basal ganglia or the internal capsule's posterior limb.
- ▶ Grade 1b: MRI findings show more substantial cerebral involvement without involving the basal ganglia or the internal capsule's posterior limb.
- ▶ Grade 2a: MRI findings show abnormalities involving the basal ganglia or the internal capsule's posterior limb with no cerebral abnormalities.
- ▶ Grade 2b: MRI findings show abnormalities involving the basal ganglia and the internal capsule's posterior limb.

- ▶ Grade 3: MRI findings show devastation on all sides of the hemisphere.

Blood sample collection and real-time PCR

Samples were obtained from the umbilical cord blood from all included neonates at birth. We placed 3 mL of cord blood samples from the included neonates into Tempus Blood RNA Tube and were biobanked at -80°C until RNA extraction. miRNA extraction was performed using 100 μL of fresh whole blood samples. Total RNA, including miRNA, was extracted using miRNeasy Isolation Kit (QIAGEN, USA). We assessed the concentration as well as the quality of RNA using a NanoDrop instrument (Thermo Scientific, USA) and stored the RNA extract at -80°C . In order to produce complementary DNA (cDNA), we performed reverse transcription using the miScript II RT Kit (QIAGEN, USA). Preparation of the constituents of the reverse transcription was performed on ice in a total volume of 20 μL . RT Master Mix was prepared with 4 μL miScript HiSpec RT buffer, 2 μL of miScript Nucleics Mix, 2 μL miScript Reverse Transcriptase and 1 μg of miRNA template. The volume was then completed to 20 μL by nuclease-free water.

Incubation was performed using the 2720 Thermal Cycler (Singapore Applied Biosystems) for one cycle: 37°C for 1 hour to activate the reverse transcriptase enzyme, followed by 95°C for 5 min for inactivation. The cDNA obtained was stored at -20°C until real-time PCR. Real-time PCR was carried out using miScript SYBR Green PCR Kit (QIAGEN, USA). Prior to amplification, we diluted the cDNA samples obtained using nuclease-free water, with a 1:5 ratio (16 μL of nuclease-free water and 4 μL cDNA). We applied a cumulative volume of 25 μL , which included SYBR Green Master Mix (12.5 μL), nuclease-free water (3.5 μL), diluted cDNA (4 μL), miScript universal primer (2.5 μL) and miScript primer assay (2.5 μL). For normalisation, we used the minimal nuclear U6. The following specific primers were used: mature miR-376c GGUGGAUAUCCUUCUAUGUU and miR-1268a CGGGCGUGGUGGUGGGGG, as well as mature U6 used as a reference gene (miScript Primer Assay Kit, QIAGEN, USA). A real-time PCR instrument (V.2.0.1, ABI7500) analysed the data. The following cycling conditions were considered: the initial activation process was performed at 95°C for 15 min, then a three-step cycling for 40 cycles: denaturation (at 94°C for 15 s), then primer annealing (at 55°C for 30 s) and primer extension (at 70°C for 30 s).

We normalised the miRNA-376c and miRNA-1268a expression levels to U6, and the relative expression values were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Melting curve analysis was used to validate amplification specificity and the absence of primer dimers in each run.

Statistical analysis

Data normality was assessed, and continuous data were presented as mean $\pm$ SD or median and IQR for symmetric and non-symmetric distributed data, respectively. We

presented qualitative data in frequencies and percentages. χ^2 test was used to assess the association between two or more qualitative variables. We used Fisher's exact test if the expected cell count of more than a quarter of cases was less than five. To compare the quantitative variables between two independent groups, Student's t-test and Mann-Whitney U test were used for parametric and non-parametric data. For non-parametric data, Kruskal-Wallis test was used to compare the quantitative variables between more than two independent groups. Spearman's correlation was used to show the correlation between two continuous non-parametric data. Receiver operating characteristics (ROC) analysis was performed to assess biomarker performance,¹⁸ with respective maximum accuracy points for both sensitivity and specificity. Positive predictive value (PPV) and negative predictive value (NPV) were also calculated. Significant difference was considered when the p value was less than 0.05. All analyses were performed using Statistical Package of Social Science (SPSS) V.22.

RESULTS

Characteristics of the included population

The study included 100 neonates divided into two independent groups. The case group included 50 full-term neonates with HIE, with a mean gestational age of 37.6 weeks (± 1.1), while the control group included 50 matched clinically healthy full-term neonates with no evidence of neonatal HIE, with a mean gestational age of 37.65 weeks (± 0.95). Sixty per cent of the case group and 46% of the control group were male. Participants in the control group had a significantly higher mean weight than neonates with HIE (3.14 kg vs 2.85 kg, $p=0.022$).

With regard to maternal age, we detected no significant difference between the groups ($p=0.248$). Maternal diseases (including gestational diabetes mellitus, hypertension, pre-eclampsia/eclampsia, oligohydramnios/polyhydramnios, placenta previa, cardiac disease, hypothyroidism and premature rupture of membranes PROM), as well as complications during delivery, were prevalent in mothers of neonates with HIE ($p<0.001$). Of the case group, 56% required mechanical ventilation, while none of the participants in the control group required it ($p<0.001$).

Neonates with HIE had significantly lower mean Apgar scores at 1 min and 5 min (3.32 vs 9.36, $p<0.001$, and 5.52 vs 9.90, $p<0.001$). The mean duration of incubation among the case group was 12.7 days (SD=10.3, range 2–47 days). Thirty-four neonates (60%) in the case group and all (100%) in the control group survived. With regard to laboratory findings, compared with the healthy controls, neonates with HIE had statistically significant lower levels of haemoglobin and platelet count ($p=0.026$ and $p<0.001$) and higher urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and CRP levels (all $p<0.05$; table 1).

Table 1 Demographic, clinical characteristics and laboratory findings of the studied groups (N=100)

Variables		Case group (n=50)	Control group (n=50)	P value
Gestational age (weeks)§	Mean±SD	37.6±1.10	37.65±0.95	0.149
Weight (kg)§	Mean±SD	2.85±0.48	3.14±0.34	0.022*
Gender†	Male, n (%)	30 (60)	23 (46)	0.161
	Female, n (%)	20 (40)	27 (54)	
Mode of delivery†	Vaginal, n (%)	26 (52)	15 (30)	0.025*
	Caesarean, n (%)	24 (48)	35 (70)	
Maternal age (years)§	Mean±SD	31.08±6.95	32.52±5.34	0.248
Maternal diseases†	Yes, n (%)	18 (36)	0 (0)	<0.001*
	No, n (%)	32 (64)	50 (100)	
Complications during delivery†	Yes, n (%)	16 (32)	0 (0)	<0.001*
	No, n (%)	34 (68)	50 (100)	
Mechanical ventilation†	Yes, n (%)	28 (56)	0 (0)	<0.001*
	No, n (%)	22 (44)	50 (100)	
Apgar score at 1 min§	Mean±SD	3.32±0.55	9.36±0.631	<0.001*
Apgar score at 5 min§	Mean±SD	5.52±0.60	9.90±0.631	<0.001*
Outcome†	Survived, n (%)	34 (60)	50 (100)	0.001*
	Did not survive, n (%)	16 (40)	0 (0)	
Haemoglobin (g/L)§	Mean±SD	129.8±25.3	142.2±11.7	0.026
White Cell Count ‡(×10 ⁹ /L)	Median (IQR)	16 (12–77)	13 (12–13)	0.065
Platelets (×10 ⁹ /L)‡	Median (IQR)	120 (68–228)	285 (240–350)	<0.001*
pH§	Mean±SD	7.1±0.05	7.37±0.03	<0.001*
PCO ₂ §	Mean±SD	27.3±4.57	37.4±3.80	<0.001*
PO ₂ §	Mean±SD	60.5±7.38	66.2±2.09	0.008*
HCO ₃ §(mEq/L)	Mean±SD	12.01±2.60	21.23±2.18	<0.001*
Urea (mg/dL)‡	Median (IQR)	51 (34–81)	25 (17.5–27.7)	<0.001*
Creatinine (mg/dL)‡	Median (IQR)	1.7 (1.2–1.7)	1.15 (0.9–1.30)	<0.001*
Sodium§(mEq/L)	Mean±SD	132.04±6.96	138.4±4.12	<0.001*
Potassium§(mEq/L)	Mean±SD	4.44±1.16	4±1.50	0.09
ALT (U/L)‡	Median (IQR)	50 (35–100)	29 (27–30)	<0.001*
AST (U/L)‡	Median (IQR)	40 (28–66.5)	21 (19.7–24)	<0.001*
CRP (mg/dL)§	Mean±SD	6.25±1.91	4.80±1.50	0.007*

*P<0.05 is significant.

†χ² test/Fisher's exact test was used.

‡Mann-Whitney test was used.

§t-test was used.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C reactive protein; HCO₃, bicarbonate; PCO₂, partial pressure of carbon dioxide; PO₂, partial pressure of oxygen.

miRNA-376c and miRNA-1268a

Compared with the control group, neonates with HIE had a significantly lower median level of miRNA-376c (0.168, IQR=0.011–0.411 vs 1, IQR=0.80–1.20, $p<0.001$) and a significantly higher median level of miRNA-1268a (13.46, IQR=2.7–22.8 vs 1, IQR=0.4–1.6, $p<0.001$). Comparing neonates with HIE who survived versus those who did not survive, no statistically significant difference between the groups in terms of miRNA-376c and miRNA-1268a ($p=0.124$ and $p=0.279$) was found. There were no significant differences in miRNA-376c and miRNA-1268a among neonates with HIE with regard to clinical staging and MRI findings, as shown in [table 2](#) (all $p>0.05$).

Moreover, assessment of the relationship between miRNA-376c, miRNA-1268a and the demographics of the studied neonates showed no significant differences in terms of sex, mode of delivery, prior maternal diseases and incidence of complications during delivery, except for miRNA-1268a among neonates of mothers with prior diseases ($p=0.037$; [table 3](#)). The results of the correlation analysis are shown in [table 4](#). In terms of miRNA-376c, we detected statistically significant positive correlations with weight, maternal age, Apgar score at 1 min, platelets, pH, partial pressure of carbon dioxide (PCO₂), partial pressure of oxygen (PO₂), bicarbonate (HCO₃) and calcium, as well as negative correlations with white cell count

Table 2 miRNA-376c and miRNA-1268a among the studied groups and among neonates with hypoxic-ischaemic encephalopathy as regards Sarnat staging and MRI finding

		miRNA-376c	miRNA-1268a‡
Case group‡	n=50	0.168 (0.011–0.411)	13.46 (2.7–22.8)
Control group‡	n=50	1 (0.80–1.20)	1 (0.4–1.6)
	P value	<0.001*	<0.001*
Survived‡	n=34	0.263 (0.011–0.472)	4.56 (3.1–24.2)
Did not survive‡	n=16	0.113 (0.012–0.223)	13.9 (2.6–21.9)
	P value	0.124	0.279
Clinical Sarnat staging†	Mild cases	0.259 (0.011–0.397)	13.953 (0.83–21.75)
	Moderate cases	0.095 (0.007–0.401)	11.022 (2.73–26.7)
	Severe cases	0.253 (0.016–0.721)	2.644 (1.8–20.1)
	P value	0.436	0.143
MRI findings†	Normal	0.37 (0.012–0.411)	13.9 (5.1–23.4)
	Grade 1a	0.14 (0.007–0.397)	13.7 (6.1–21.5)
	Grade 1b	0.06 (0.007–0.401)	16.7 (2.7–26.7)
	Grade 2a	0.24 (0.106–0.721)	2.6 (1.9–20.1)
	Grade 2b	0.26 (0.50–1.69)	2.6 (1.61–280.1)
	P value	0.759	0.46

Data are presented as median (IQR).

*P<0.05 is significant.

†Kruskal-Wallis test was used.

‡Mann-Whitney test was used.

(WCC), urea, creatinine, potassium, ALT and AST (all $p<0.05$). With regard to miRNA-1268a, there were statistically significant negative correlations with gestational age, weight, maternal age, Apgar score at 1 min, platelets, pH, PCO_2 , PO_2 , HCO_3 and sodium, as well as positive correlations with WBCs, urea, creatinine, CRP, ALT and AST (all $p<0.05$).

Accuracy of the studied parameters as diagnostic biomarkers for HIE

The usefulness of miRNA-376c and miRNA-1268a as diagnostic markers, compared with Apgar score and pH, was tested through ROC curve analysis (figure 1). At 0.90 points, miRNA-376c showed a sensitivity and a specificity of 88% and 68.40%, with an area under the curve of 84% (PPV=76% and NPV=81%). At 2.70 points, miRNA-1268a

showed a sensitivity and a specificity of 76% and 100%, with an area under the curve of 96% (PPV=77% and NPV=100%). At less than 6 points, Apgar score showed a sensitivity and a specificity of 100% (PPV=100% and NPV=100%), while at less than 7.1 pH showed a sensitivity and a specificity of 90% and 100% (PPV=86% and NPV=100%). The combined test showed an overall sensitivity and specificity of 89% and 85% (PPV=82% and NPV=92%).

DISCUSSION

In the current study, we found that patients with HIE had a significantly lower level of miRNA-376c and a higher level of miRNA-1268a than the control group. We

Table 3 Relationship between miRNA-376c, miRNA-1268a and the demographics of the studied neonates

Variables		miRNA-376c	P value	miRNA-1268a	P value
Gender	Male	0.24 (0.03–0.439)	0.383	8.8 (2.2–26.7)	0.526
	Female	0.66 (0.008–0.353)		15.3 (3.3–21.2)	
Mode of delivery	Vaginal	0.24 (0.007–0.426)	0.756	8.8 (3.3–21.2)	0.938
	Caesarean	0.13 (0.024–0.426)		15.4 (2.3–23.2)	
Maternal diseases	Yes	0.20 (0.021–0.381)	0.733	15.3 (4.5–25.6)	0.037*
	No	0.068 (0.006–2.65)		2.7 (1.43–144.1)	
Complications during delivery	Yes	0.12 (0.007–0.291)	0.067	13.9 (4.9–2396)	0.056
	No	0.39 (0.026–1.81)		2.6 (1.64–22.8)	

Data are presented as median (IQR).

*P<0.05 is significant.

Table 4 Correlation between miRNA-376c, miRNA-1268a and demographics and clinical data of the studied neonates

Variables	miRNA-376c		miRNA-1268a	
	Spearman's r	P value	Spearman's r	P value
Gestational age (days)	0.211	0.039*	-0.319	0.001*
Maternal age (years)	0.226	0.025*	-0.298	0.003*
Apgar score at 1 min	0.544	<0.001*	-0.720	<0.001*
Apgar score at 5 min	-0.054	0.711	0.712*	<0.001*
Weight (Kg)	0.208	0.04*	-0.249	0.013 *
Duration of incubation (days)	0.188	0.178	0.198	0.183
Haemoglobin (g/L)	-0.147	0.149	-0.08	0.432
White cell count ($\times 10^9/L$)	-0.258	0.01*	0.309	0.002*
Platelets ($\times 10^9/L$)	0.415	<0.001*	-0.423	<0.001*
pH	0.409	<0.001*	-0.661	<0.001*
PCO ₂	0.552	<0.001*	-0.702	<0.001*
PO ₂	0.587	<0.001*	-0.791	<0.001*
HCO ₃ ⁻	0.490	<0.001*	-0.716	<0.001*
Base deficit	0.201	0.176	-0.156	0.151
Urea (mg/dL)	-0.249	<0.001*	0.573	<0.001*
Creatinine (mg/dL)	-0.229	0.023*	0.526	<0.001*
CRP (mg/dL)	-0.09	0.39	0.412	0.019*
Sodium (mEq/L)	0.222	0.028*	-0.497	<0.001*
Potassium (mEq/L)	-0.269	0.007*	0.153	0.132
Calcium ((mg/dL)	0.376	0.008*	-0.034	0.740
ALT (U/L)	-0.259	0.01*	0.527	<0.001*
AST (U/L)	-0.436	0.003*	0.644	<0.001*

*P<0.05 is significant.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C reactive protein; HCO₃⁻, bicarbonate; PCO₂, partial pressure of carbon dioxide; PO₂, partial pressure of oxygen.

detected no statistically significant difference between neonates with HIE who survived and those who did not survive in terms of miRNA-376c and miRNA-1268a (p=0.124 and p=0.279). miRNA-376c had high sensitivity

but low specificity, while miRNA-1268a had high specificity but low sensitivity, in detecting HIE.

In an experimental animal study, Chen and colleagues¹⁹ documented the potential role of miRNA-124-3p/Bax

Figure 1 Receiver operating characteristic (ROC) curve of (A) miRNA-376c and Apgar score at 1 min and (B) miRNA-1268a.

in the pathophysiology of HIE. In a study of miRNAs obtained from dried spots of blood, Ponnusamy *et al*²⁰ assessed the potential biomarkers for HIE in 30 newborn babies. They studied some selected miRNAs (Let7b, miRNA-21, miRNA-29b, miRNA-124 and miRNA-155) and reported no significant difference among favourable and unfavourable outcome groups for babies with HIE. Other findings that are closely similar to this one used the umbilical cord and/or maternal blood. A previous study by Looney *et al*²¹ reported downregulation of miRNA-374a in the blood obtained from the umbilical cord of infants with HIE. Moreover, Wang *et al*²² selectively evaluated miRNA-210 and miRNA-374a in the blood of the umbilical cord of neonates with HIE and suggested its potential role as a biomarker for severity as well as prognosis of HIE.

Our diagnostic analysis revealed that miRNA-376c has an acceptable sensitivity (88%) but not specificity (68.4%) in differentiating neonates with HIE. In our study, patients had a significantly higher level of miRNA-1268a than the control group.

The ROC curve showed that miRNA-1268a was a considerable discriminator to differentiate neonates with HIE, with a sensitivity and a specificity of 76% and 100% (cut-off point of >2.7). In the combined test with both Apgar score and pH, both sensitivities increased to 89% and the specificity of miRNA-376c increased to 85%.

PPV can predict how likely it is for a person to be diagnosed with a disease in case of a positive test result, while NPV can predict how likely it is for a person to be healthy in case of a negative test result. In our study, the PPV for miRNA-376c was 76%, while it was 77% for miRNA-1268a. The NPV for miRNA-376c was 81%, while it was 100% for miRNA-1268a.

Temporal expression changes of miRNA-23a-5p, miRNA-27a-5p as well as miRNA-31-5p have been detected in experiments on a piglet HIE model to be considered efficient biomarkers for differentiating hypoxia from infection-sensitised hypoxia. Also, miRNA-374a and miRNA-210 have been shown as crucial regulators in neonatal HIE. Nevertheless, therapeutic hypothermia, the current standard treatment for the management of HIE, has been studied in adult rats experiencing traumatic brain injury, altering miRNA-874 and miRNA-451 expression. These experiments differed in methodology and revealed changes in different miRNAs in neonatal HIE.²³ Recently, in 2019, O'Sullivan *et al*²⁴ studied miRNA to assess both the development and severity of HIE. In their study, the authors documented a decline in miR-374a-5p among infants with HIE compared with the controls.

Data from clinical studies of hypothermia for the prevention of brain injury in neonates with HIE suggest that the consensus criteria are likely limiting.^{25 26} There are some caveats required for the individual diagnosis of neonatal HIE. Reported data are often incomplete, unreliable and contradictory, especially for blood gases, adequate imaging of the brain, Apgar scores, as well as

organ dysfunction testing results. Our study is limited by the low number of included cases, being a single-centre experience. Thus, more extensive clinical trials are warranted to assess this association.

In conclusion, the relative expression of miRNA-376c and miRNA-1268a was altered in the cord blood of neonates with HIE. In addition, they have moderate diagnostic accuracy in detecting HIE. There is no association between the relative expression of miRNA-376c and miRNA-1268a in terms of MRI and survival findings.

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