Molecular and Cellular Pediatrics

RESEARCH

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miR223, miR30e, and miR30a show associations with perinatal metabolic acidosis in neonates with hypoxic–ischemic encephalopathy

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Abstract

Objective Hypoxic-Ischemic Encephalopathy (HIE) is a leading cause of neonatal death and long-term disabilities, leading researchers to explore novel candidate biomarkers to improve early diagnosis and guide treatment strategies. miR223, miR30a and miR30e have attracted attention as potential biomarkers for HIE due to its involvement in key cellular processes like inflammation, cell death, and neuroprotection. The rationale of this study was to examine longitudinal maternal and infant circulating miRNA blood levels from T0–T3 to 48 h of life in relation to perinatal metabolic acidosis and neonatal hypoxic–ischemic encephalopathy status.

Study design A prospective observational cohort study involving newborns born from September 2023 to September 2024 was carried out. Healthy newborns were assigned as control group (CG), those with pH < 7.12 without inclusion criteria for hypothermic treatment were assigned to no hypothermic treatment group (NHTG) those that met inclusion criteria for hypothermic treatment were included in the hypothermic treatment group (HTG). Blood samples of the newborns were collected by umbilical cord artery along with maternal venous blood at birth (T0), at 3 (T3) and 48 (T48) hours after birth, the latter were collected from capillary blood.

Results Our results demonstrated consistent upregulation of miR223, miR30e and miR30a at T0 and T3 in NHTG and HTG. Interestingly, miR223 and miR30a were upregulated also in samples obtained by NHTG and HTG mother blood.

Conclusion In this exploratory analysis, miR223, miR30e, and miR30a were found to be associated with perinatal metabolic acidosis and acute hypoxic stress, as defined by cord blood pH and clinical HIE status at presentation, supporting their role as candidate markers of early postnatal response.

Clinical trial registration NCT05986994, Identification of a Pool of MiRNA to Improve Early Management of Perinatal Asphyxia and Hypoxic Ischemic Encephalopathy Prospectively Registered on 14 August 2023.

Keywords MicroRNA, Hypoxic-Ischemic encephalopathy, Asphyxia, Exploratory biomarker, Hypothermia

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Article summary

miR223, miR30e and miR30a *show associations with perinatal metabolic acidosis in neonates*, with elevated levels suggesting their involvement in neonatal hypoxia.

What's known on this subject

Recent data demonstrate that newborns with mild to severe NE(neonatal encephalopathy) have different miRNA profiles than healthy controls. However, research on miRNA in relation to NE is scarce.

What this study adds

This study identifies circulating miRNAs that are associated with perinatal metabolic acidosis and neonatal hypoxic–ischemic encephalopathy status and describes their temporal patterns over the first 48 h of life.

Introduction

Hypoxic-Ischemic Encephalopathy (HIE) is a severe neurological disorder in newborns caused by oxygen deprivation (hypoxia) and reduced blood flow (ischemia) to the brain, typically occurring during or just after birth [1]. It is a leading cause of neonatal death and long-term disabilities, such as cerebral palsy and cognitive impairments [2, 3]. While therapeutic hypothermia can reduce brain damage when applied promptly, predicting the severity of injury and long-term outcomes for infants remains a major challenge [4–7]. Current diagnostic tools do not always provide an accurate assessment of brain injury, leading researchers to explore novel biomarkers to improve early diagnosis and guide treatment strategies [4, 8].

HIE is often preceded by perinatal asphyxia, a condition in which the newborn experiences significant oxygen deprivation during labour or delivery [9]. Asphyxia is marked by physiological changes such as unstable pH levels, organ dysfunction, and visible signs like weak reflexes or poor muscle tone [10]. These changes can signal potential brain injury, but they are not definitive indicators of HIE [2, 10]. Arterial blood gas measurements, including pH levels, often fluctuate, making it difficult to accurately predict which infants will go on to develop HIE. This unpredictability highlights the need for reliable biomarkers that can indicate brain injury early and track its progression [11, 12].

MiRNAs, small non-coding RNA molecules that regulate gene expression, have attracted attention as potential biomarkers for HIE due to their involvement in key cellular processes like inflammation, cell death, and neuroprotection [13–16]. MiRNAs are stable in biological fluids such as blood, making them suitable for non-invasive testing. Importantly, they respond dynamically to brain injury and can reflect the severity and progression of HIE over time. Studies have identified specific miRNAs that are upregulated or downregulated following hypoxic-ischemic events, suggesting that miRNA profiles can provide critical insights into the pathophysiology of HIE [13, 16–18].

In this exploratory biomarker study related to perinatal metabolic acidosis in HIE, miRNAs are measured in maternal samples immediately after birth (T0), and in the infant's blood after birth (T0), 3 h after birth (T3), and 48 h after-birth (T48). This multi-timepoint approach aims to identify miRNAs that change in response to perinatal asphyxia and the onset of HIE, offering a potential timeline of molecular events associated with brain injury.

The rationale behind this study is that miRNA levels could reflect the early response to oxygen deprivation in both the mother and the newborn. By comparing maternal and infant miRNA profiles, we can better understand the infant's risk of developing HIE. Tracking miRNA changes in the newborn at critical stages (from birth to 48 h) provides further insights into how brain injury progresses in HIE, potentially identifying which infants are most likely to benefit from interventions like therapeutic hypothermia.

Materials and methods

Study design

We conducted a prospective observational cohort study involving newborns born at Department of Woman and Child, Buonconsiglio Fatebenefratelli Hospital in Naples from September 2023 to September 2024. The study was approved by Ethics Committee Lazio 1. This study was recorded and publicly accessible at https://clinicaltrials.gov/study/NCT05986994 accessed on 21 November 2024. The study adhered to the international Good Clinical Practice guidelines and informed consent was obtained from the parents of the newborns involved in the study. Investigators were responsible for ensuring ethical conduct, protocol adherence, and the integrity and validity of the collected data, which were gathered from the department's healthcare staff.

This study was carried out at the Neonatal Intensive Care and Neonatology Unit, with a sample size of 12 newborns (over a period of year) in each group.

Participants

Maternal clinical records were reviewed to confirm a negative pregnancy history. Mothers with clinical infection at delivery, intrapartum fever (≥ 38 °C), positive vaginal–rectal swabs, or premature rupture of membranes were excluded. Records were additionally screened for metabolic, vascular, and inflammatory comorbidities. Mothers with pre-gestational diabetes, autoimmune disorders, chronic inflammatory diseases, or severe hypertensive disorders, including severe preeclampsia, were excluded. Mild, clinically managed gestational diabetes or

gestational hypertension, when present, were permitted. Maternal comorbidities across study groups are summarized in Supplementary Table 1.

They were included newborns with gestational age > 35 weeks and body weight > 1,800 g. Exclusion criteria were: withdrawal of informed consent and alteration of the blood sample as loss of RNA, coagulation, missing sample, and high impurity (Fig. 1). Blood samples were collected at birth (T0) from newborns by arterial cord and mothers by peripheral vein. Health newborns with pH between 7.26 and 7.35 obtained by arterial cord blood were assigned to control group (CG). Newborns at risk with metabolic acidosis at birth and with pH < 7.12 obtained by arterial cord blood and were assigned to no hypothermic treatment group (NHTG) due to lack of inclusion criteria of hypothermic treatment. The threshold pH value of 7.12 was chosen based on previous study that demonstrated its associations with respiratory distress syndrome [19].

Newborns with gestational age > 35 weeks, birth weight > 1800 g and hours of life < 6 and were assigned to hypothermic treatment group (HTG) if they met the following criteria:

Criteria A (asphyxia peripartum): pH<7 Base Deficit in extracellular fluid> 12 mEq/L and Apgar score<10 at 10 minutes and/or need for respiratory assistance at 10 minutes
Criteria B (neurological examination abnormalities): presence of at least two neurological signs
Criteria C (abnormalities at aEEG and/or EEG): aEEG or EEG with moderate or severe abnormalities.

Additionally, capillary blood samples (200–300 µL) were collected from newborns at 3 h (T3) and 48 h (T48) of life and transferred into EDTA-containing tubes. The analytical procedures performed did not affect the health status of the newborns participating in the study. Arterial blood gas measurements at T3 and T48 were not obtained for the control group, as these newborns were delivered without complications. We fully acknowledge that the absence of a control group at T3 imposes certain limitations on the study. This omission impacts both the statistical analysis and the clinical interpretation of the results. Without a CG for comparison, we must be cautious in making conclusions about the effects observed at T3.

3 h is the time point of the change in the ABG level and the acute phase after the injury or insult whereas 48 h is the time of discharge from the hospital so well before the

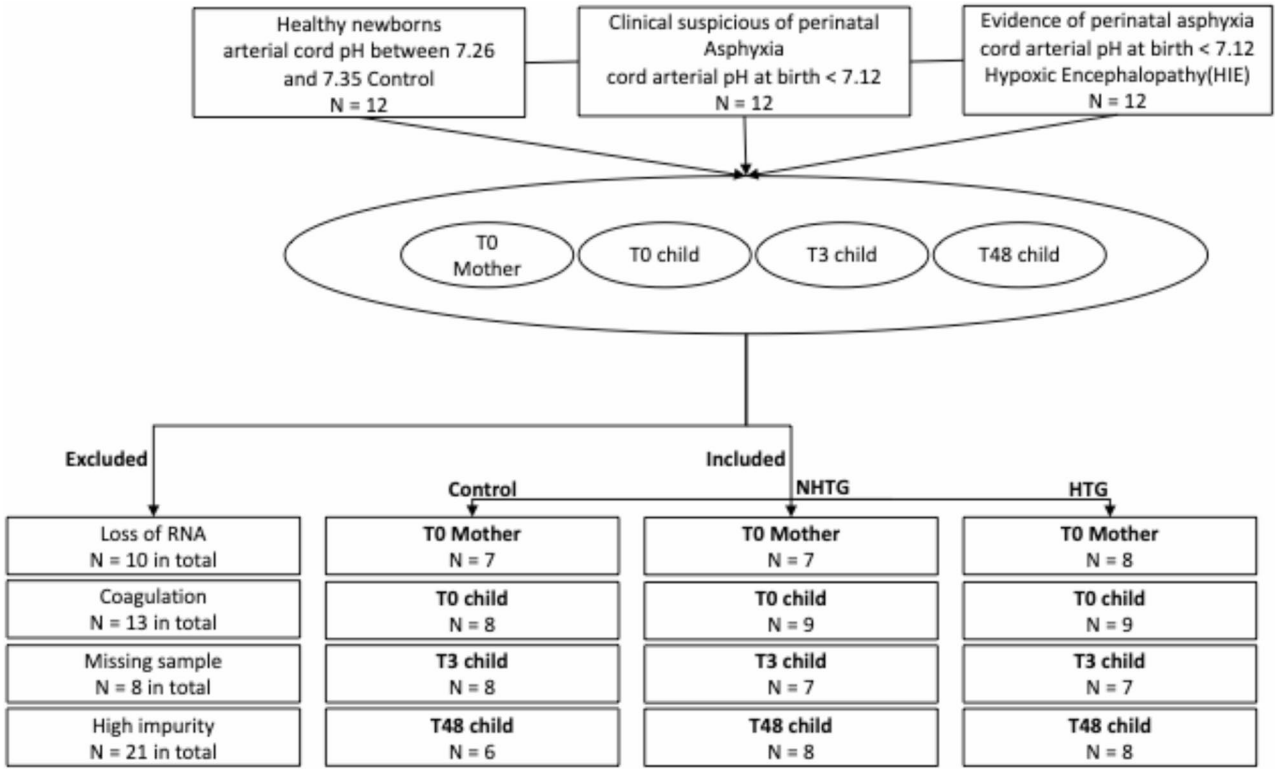

Fig. 1 Study design and sample distribution. Neonates were classified into three groups based on arterial cord pH: healthy controls (CONTROL, pH 7.26–7.35, $n = 12$), suspected perinatal asphyxia without severe acidosis (NHTG, pH < 7.12, $n = 12$), and perinatal asphyxia with evidence of hypoxic-ischemic encephalopathy (HTG, pH < 7.12 with HIE, $n = 12$). Samples were collected at four timepoints (T0 mother, T0 child, T3 child, T48 child). Following quality control for RNA loss, coagulation, sample omission, or high impurity, the final included samples for each group and timepoint are indicated

time of discharge the level of the miRNA come to basal level or stay elevated.

Data were recorded using an Excel database (Version 2410 Build 16.0.18129.20158) by an author who was aware of the study aims. Parameters collected were reported in Fig. 2.

Analysis of GEO dataset GSE181127 using GEO2R on NCBI

To analyse miRNA expression in mild hypoxic–ischaemic encephalopathy (HIE) without therapeutic hypothermia (NHTG; $n = 3$) and in moderate–severe HIE undergoing therapeutic hypothermia (HTG; $n = 24$), relative to control samples ($n = 3$), the GSE181127 dataset was selected from the Gene Expression Omnibus (GEO) database using targeted search terms and its accession number. This dataset comprises RNA sequencing data generated from dried blood spot samples collected at T0. Raw sequencing reads and corresponding annotation files were downloaded from GEO. All downstream analyses were conducted using custom R scripts, including data processing and differential expression analysis.

MiRNA identification

Using the GSE181127 dataset, differential miRNA expression analysis was conducted to compare mild hypoxic–ischaemic encephalopathy (HIE) without therapeutic hypothermia and moderate–severe HIE undergoing therapeutic hypothermia with control samples. The top 10 differentially expressed miRNAs were plotted to visualise expression changes across groups. Statistical significance was assessed using both nominal and adjusted p-values. miR30a was identified as upregulated in the therapeutic hypothermia group compared with controls when evaluated using adjusted p-values (Supplementary Fig. 1). miR30e demonstrated significance in the therapeutic hypothermia group when assessed using nominal p-values only (Table 3). miR223 was included in the analysis based on prior literature supporting its role as a marker of inflammation.

MiRNA validation

Significant miRNAs identified in the GEO2R analysis were cross-referenced with existing literature to validate their known or potential involvement in hypoxic brain injury and asphyxia. Further experimental validation through qPCR or other methods was suggested for miRNAs with novel associations [12, 20].

Whole blood RNA extraction

RNA was extracted from whole blood samples using the GeneJET Whole Blood RNA Purification Kit (K0761; Thermo Scientific™) according to the manufacturer's instructions. Maternal blood samples (500 µL) were obtained from the maternal vein during delivery, while

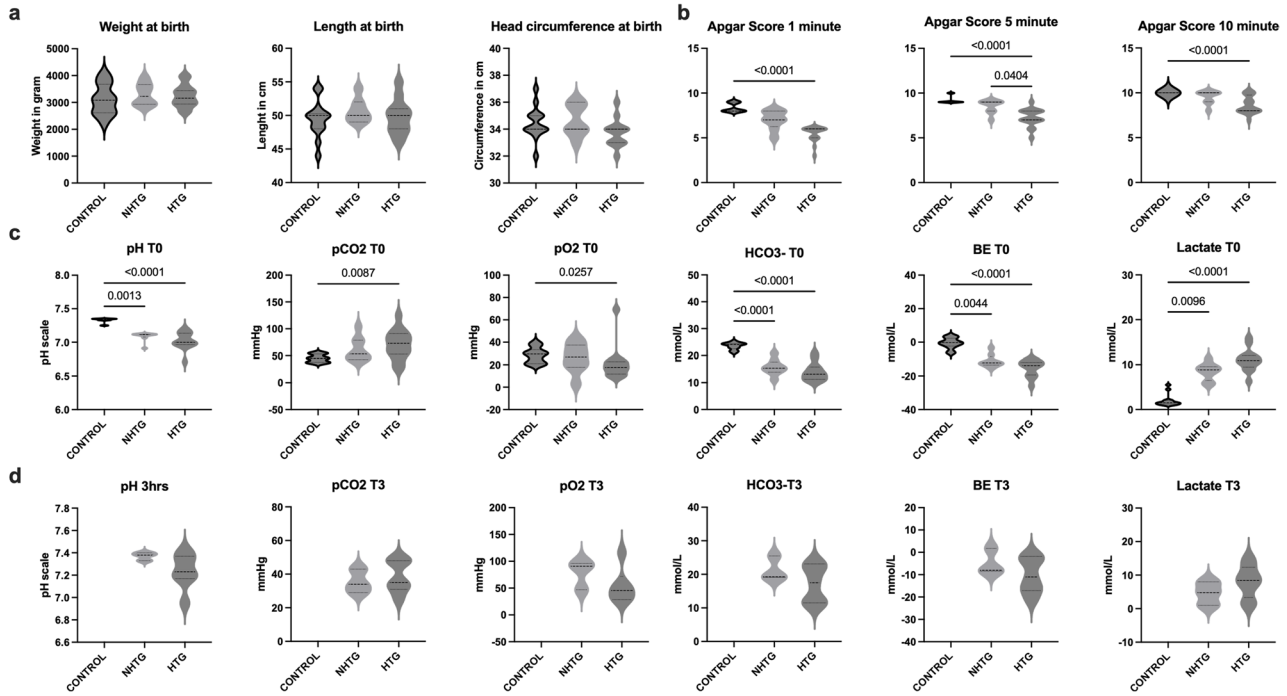

Fig. 2 Perinatal and biochemical characteristics across study groups. **a**. Neonatal anthropometric parameters at birth (weight, length, head circumference). **b**. Apgar scores at 1, 5, and 10 minutes are compared across CONTROL, NHTG, and HTG groups. **c** & **d**. Arterial blood gas parameters at birth (T0 & T3): pH, pCO_2, pO_2, HCO_3^-, base excess (BE), and lactate levels. Each plot represents the distribution with median and interquartile range; individual data points are overlaid

neonatal blood samples (approximately 200–300 µL) were collected from the child via arterial sampling using glass capillaries. After that, the blood was centrifuged at 2000 rpm for 5 min at room temperature centrifuge. Removing the supernatant the whole blood was mixed with 600 µL of Lysis Buffer supplemented with β-mercaptoethanol to enhance RNA stability. The lysate was vortexed vigorously and incubated for 5 min at room temperature. After incubation, 600 µL of 70% ethanol was added to the lysate, and the solution was mixed thoroughly. The mixture was then transferred to a purification column and centrifuged at 12,000 x g for 1 min to bind the RNA. The column was washed with Wash Buffer I and Wash Buffer II followed by centrifugation at 12,000 x g for 1 min after each wash step. Residual ethanol was removed by an additional spin at full speed. The RNA was eluted with 20 µL of Nuclease-Free Water.

MiRNA reverse transcription

For miRNA quantification, reverse transcription was performed using the TaqMan™ MicroRNA Reverse Transcription Kit (Invitrogen). In brief, 10 ng of total RNA was reverse transcribed in a 10 µL reaction volume consisting of 3 µL of TaqMan™ MicroRNA-specific stem-loop primer, 1.5 µL of 10X RT buffer, 1 µL of 100 mM dNTPs, 0.2 µL of RNase inhibitor, and 1 µL of Multiscribe Reverse Transcriptase. The reaction was carried out under the following conditions: 16 °C for 30 min, followed by 42 °C for 30 min, and 85 °C for 5 min to inactivate the reverse transcriptase. The cDNA was then stored at -20 °C until further use.

Real-Time PCR for MiRNA quantification

Real-time PCR for miRNA quantification was performed using the TaqMan™ miRNA Assay (Applied Biosystems) on a StepOne Plus (Thermo Fisher). Each reaction was carried out in a 10 µL volume containing 5 µL of TaqMan™ Fast Advanced Master Mix, 1 µL of 20X TaqMan™ miRNA Assay, 1 µL of cDNA template (from the reverse transcription reaction), and 3 µL of nuclease-free water. The reactions were run in triplicate for each miRNA of interest. Thermal cycling conditions were as follows: Initial denaturation at 95 °C for 20 s followed by 40 cycles of 95 °C for 1 s (denaturation) and 60 °C for 20 s (annealing/extension).

The relative expression of hsa-miR-223*(assay id 002098), hsa-miR-30a-5p (assay id 000417), hsa-miR-30e (assay id 002223) & hsa-miR-23a (assay id 000399 excluded) miRNA was normalized to the endogenous control, U6 snRNA (assay id 001973) & snoRNA202(assay id 001232 excluded due unstable Supplementary Fig. 2), using the 2^-ddCt method.

Statistical analysis

The sample size will be calculated using G*Power 3.1.9.7 for Windows. Due to missing data, the sample size is calculated using Cohen's rules of thumb, estimating that there is a medium effect size in the miRNA expression level among the 3 groups. Setting: effect size = 0.25, alpha = 0.05, power = 0.80, number of groups = 3, number of measurements = 3, correlation between repeated measures = 0.5, sphericity = 1; the total sample size is 36 [21, 22]. Statistical analysis was performed using GraphPad Prism 8. Due to unequal sample sizes across groups and the absence of a normal (Gaussian) data distribution, non-parametric statistical analyses were performed. Differences among groups were assessed using the Kruskal–Wallis test with no matching or pairing assumed. Following a significant Kruskal–Wallis result, Dunn's multiple comparisons test was applied to compare the mean rank of each treatment group with the control group (CG). P values were adjusted for multiple comparisons using a family-wise significance level of $\alpha = 0.05$. Receiver operating characteristic (ROC) analyses were performed to evaluate the discriminatory performance of individual miRNAs between control newborns and the NHTG and HTG groups. Area under the curve (AUC) values, 95% confidence intervals, and associated p-values are reported in Supplementary Tables 2 and 3. Data are presented as mean ± standard error of the mean (SEM). A p-value < 0.05 was considered statistically significant. All figures and analyses were generated using GraphPad Prism.

Computational gene enrichment analysis of target MiRNAs

To gain a better understanding of the biological role of miRNA using a systematic method. The genes for the target miRNA were acquired from three validated or predicated miRNA databases: miRDB, miRwalk, and targetscan [23–26]. Gene Ontology (GO) biological process enrichment analyses was performed using R and the clusterProfiler package, with multiple testing correction applied using the Benjamini–Hochberg method. Comparative enrichment analysis was conducted to identify shared and miRNA-specific biological processes. All enrichment analyses and figure generation were performed using R-based workflows.

Results

Comprehensive blood examination and biochemical profile analysis

In the present study we analysed samples obtained from maternal blood before delivery and samples from newborns before carrying out therapeutic hypothermia according to hospital protocol (Tables 1 and 2).

Table 1 Complete blood count (CBC) parameters at birth. Complete blood count (CBC) parameters measured at birth (T0) in maternal blood from control pregnancies, pregnancies complicated by perinatal asphyxia without hypothermic treatment (NHTG), pregnancies with neonatal hypoxic–ischemic encephalopathy undergoing therapeutic hypothermia (HTG), and neonatal blood from the HTG group. Data are presented as mean ± standard error of the mean (SEM). Adult maternal and neonatal reference ranges are provided for contextual comparison

Parameter	Control – Mother	Asphyxia – Mother	HIE – Mother	HIE Child	Adult (Mother) Reference Range	Neonatal Reference Range
RBC ($\times 10^6$/µL)	4.39 ± 0.19	4.44 ± 0.20	4.09 ± 0.19	4.53 ± 0.14	3.50 – 5.50	4.0 – 6.0
Hemoglobin (g/dL)	12.18 ± 0.35	12.64 ± 0.72	11.88 ± 0.47	16.20 ± 0.43	11.5 – 15.5	14.0 – 22.0
Hematocrit (%)	38.54 ± 1.03	37.81 ± 1.82	35.50 ± 1.45	48.06 ± 1.41	36 – 48	42 – 65
MCV (fL)	88.08 ± 1.88	85.21 ± 2.22	87.10 ± 2.22	106.11 ± 1.55	78 – 98	95 – 115
MCH (pg)	27.88 ± 0.88	28.17 ± 1.15	29.16 ± 0.86	35.82 ± 0.66	27 – 32	30 – 37
MCHC (g/dL)	31.60 ± 0.41	32.99 ± 0.69	31.46 ± 1.87	33.72 ± 0.23	32 – 36	30 – 36
RDW-CV (%)	14.34 ± 0.72	13.30 ± 0.27	13.12 ± 0.29	16.68 ± 0.48	11 – 16	15 – 20
RDW-SD (fL)	45.92 ± 2.84	42.20 ± 1.54	41.28 ± 1.01	65.02 ± 2.35	38 – 50	50 – 70
WBC ($\times 10^3$/µL)	11.68 ± 1.30	11.00 ± 1.11	12.84 ± 1.81	15.38 ± 1.67	4.0 – 11.0	9.0 – 30.0
Neutrophils (%)	80.26 ± 3.96	76.74 ± 1.45	73.06 ± 3.65	70.17 ± 2.17	40 – 75	50 – 75
Lymphocytes (%)	13.30 ± 2.94	15.37 ± 1.02	18.66 ± 3.00	20.28 ± 2.21	20 – 45	20 – 40
Monocytes (%)	5.66 ± 1.07	6.23 ± 0.45	7.52 ± 0.52	7.84 ± 0.55	2 – 12	2 – 12
Eosinophils (%)	0.62 ± 0.15	1.36 ± 0.32	0.56 ± 0.19	2.07 ± 0.64	0 – 7	0 – 5
Basophils (%)	0.16 ± 0.04	0.30 ± 0.06	0.20 ± 0.04	0.28 ± 0.05	0 – 1.5	0 – 1
NRBC / Erythroblasts (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.14 ± 1.17	0-0	0 – 10
Neutrophils ($\times 10^3$/µL)	9.46 ± 1.31	8.49 ± 0.95	9.61 ± 1.87	10.70 ± 1.10	2.0 – 7.5	6.0 – 26.0
Lymphocytes ($\times 10^3$/µL)	1.47 ± 0.34	1.67 ± 0.18	2.21 ± 0.17	3.19 ± 0.59	1.5 – 4.0	2.0 – 11.0
Platelets ($\times 10^3$/µL)	220.2 ± 21.5	206.6 ± 23.5	213.8 ± 9.9	231.3 ± 9.4	150 – 450	150 – 400
MPV (fL)	10.22 ± 0.50	11.27 ± 0.30	11.70 ± 0.58	10.13 ± 0.27	9.2 – 12.1	9.0 – 12.0

Assessment of vital physiological parameters in neonates

The Apgar score is based on five factors: appearance (skin colour), pulse (heart rate), grimace (reflex irritation), activity (muscle tone), and respiration (Fig. 2). Figure 2b illustrates the severe changes in the HTG, which was followed by asphyxia since those were the suspected based on the values of the Apgar score. The time course is at birth, 5 min, and 10 min, as shown in Fig. 2b. The score stays low for the infants in HTG, whereas in NHTG infants returned to normal levels as a CG.

Dynamic changes in arterial blood gas (ABG) values at birth and three hours Post-Birth across all cases

Cases in NHTG and HTG are all submitted to an ABG after the first three hours to assess pH levels, bicarbonate, base excess, and lactate, as these values reduce after hypoxia and can be maintained by hypothermia. Figure 2c and d show that lactates had higher ABG values but lower pH, HCO3, and BE levels compared to the CG. At 3 h, we do not have values to compare to the CG, although they have stabled in both the groups (NHTG and HTG).

The blood gas values obtained from newborns, using arterial cord blood, confirm the mild to severe metabolic acidosis of newborns in NHTG(mild) and HTG(severe). As the blood gas affected generally at 3 h of life we observed a gradual improvement in the parameters following the resuscitation manoeuvres carried out in cases of HTG (Fig. 2).

MiRNA identification through geo dataset analysis

The analysis of publicly available Geo Dataset GSE181127 data identified several differentially expressed miRNAs associated with HIE in neonates Fig. 3. This dataset particularly used as this dataset had the RNA expression tested using dried blood spot at T0. Among these, miR30a was significantly upregulated in response to HIE conditions whereas miR30e(upregulated) and miR223 (downregulated) but not significant in HTG (Table 3).

In this study, miR30e and miR223 were evaluated to assess the effects of inflammation and changes in miRNA expression. miR30a was analysed alongside miR30e because both belong to the same miRNA family, while miR223 was included as a well-established marker of inflammation.

Differential MiRNA expression in the clinical cohort at different time points

As observed in the Geo dataset, we selected three miRNAs for further examination, as shown in Figs. 4 and 5a-c: miR223, miR30a, and miR30e.

Table 2 Biochemical and coagulation parameters at birth. Biochemical and coagulation parameters measured at birth (T0) in maternal blood from control, NHTG, and HTG groups, and in neonatal blood from the HTG group. Data are presented as mean ± standard error of the mean (SEM). Adult maternal and neonatal reference ranges are shown where applicable. Dashes indicate parameters not routinely assessed in maternal samples

Parameter	Control – Mother	Asphyxia – Mother	HIE – Mother	HIE Child	Adult (Mother) Reference Range	Neonatal Reference Range
PT (seconds)	9.94 ± 0.63	9.89 ± 0.57	10.12 ± 1.13	17.03 ± 3.80	9.4 – 12.5	14 – 20
PT-INR	0.82 ± 0.05	0.85 ± 0.08	0.85 ± 0.11	1.43 ± 0.32	0.80 – 1.20	1.2 – 1.8
APTT (seconds)	27.86 ± 2.75	27.13 ± 2.33	26.85 ± 3.13	32.79 ± 4.50	25 – 37	30 – 50
APTT Ratio	0.95 ± 0.09	0.94 ± 0.06	0.92 ± 0.11	1.10 ± 0.15	0.80 – 1.20	0.9 – 1.5
Fibrinogen (Clauss, mg/dL)	497.00 ± 72.09	484.43 ± 107.49	477.50 ± 105.55	166.88 ± 37.33	200 – 400*	125 – 300
Creatinine (mg/dL)	0.60 ± 0.13	0.62 ± 0.08	0.71 ± 0.12	1.00 ± 0.19	0.44 – 1.10	0.6 – 1.2
AST / GOT (U/L)	21.60 ± 4.51	19.71 ± 5.91	21.38 ± 5.32	134.00 ± 142.34	5 – 38	20 – 80
ALT / GPT (U/L)	17.00 ± 6.52	14.67 ± 4.32	21.75 ± 13.29	60.71 ± 87.69	5 – 41	5 – 60
D-Dimer (ng/mL)	-	-	-	4031.11 ± 4845.25	-	< 2000*
Functional Antithrombin III (%)	-	-	-	43.57 ± 8.32	-	30 – 60
Urea (mg/dL)	-	-	-	27.90 ± 5.67	-	10 – 40
Creatinine (mg/dL)	-	-	-	1.00 ± 0.19	-	0.6 – 1.2
Calcium (mg/dL)	-	-	-	9.12 ± 0.60	-	8.0 – 10.8
AST / GOT (U/L)	-	-	-	134.00 ± 142.34	-	20 – 80
ALT / GPT (U/L)	-	-	-	60.71 ± 87.69	-	5 – 60
LDH (U/L)	-	-	-	720.24 ± 141.40	-	400 – 900
Creatine Kinase (U/L)	-	-	-	1486.22 ± 677.53	-	100 – 1500
Troponin-T (ng/mL)	-	-	-	0.16 ± 0.08	-	< 0.20
CK-MB (U/L)	-	-	-	42.18 ± 16.83	-	< 25
Procalcitonin (ng/mL)	-	-	-	3.21 ± 4.06	-	< 2.0
CRP (mg/L)	-	-	-	2.18 ± 3.39	-	< 10

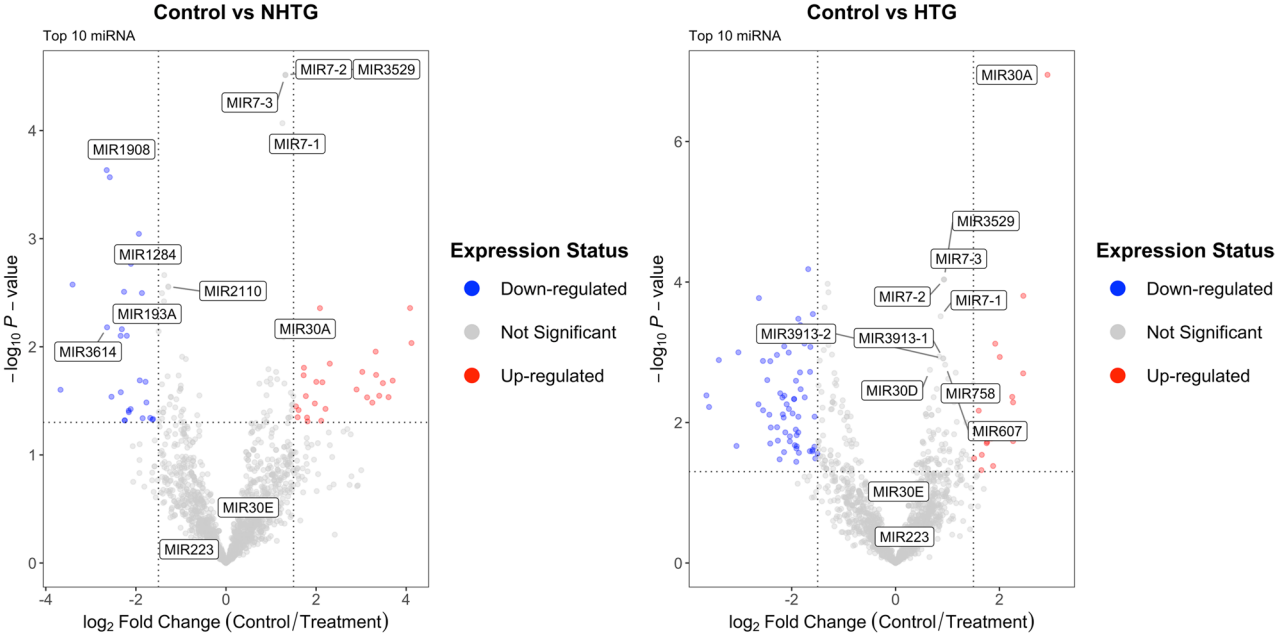

Fig. 3 Differential microRNA expression in neonatal whole blood from the GEO dataset GSE181127. Volcano plots comparing Control vs. NHTG (left panel) and Control vs. HTG (right panel). Each point represents an individual miRNA, plotted as $\log_2$fold change (Control/Treatment) on the x-axis and $-\log_{10}$(unadjusted *p*-value) on the y-axis. Dotted vertical lines indicate fold-change thresholds ($\log_2$ fold change ≥ 1.5), and the horizontal dotted line indicates the nominal significance threshold ($p < 0.05$). MiRNAs highlighted in red are upregulated and those in blue are downregulated relative to controls; grey points indicate miRNAs not meeting significance thresholds. The top 10 miRNAs are annotated based on nominal *p*-value ranking

Table 3 Differentially expressed genes and miRNAs from GEO dataset analysis. Top differentially expressed genes and miRNAs identified from analysis of the GEO dataset GSE181127, comparing Control vs NHTG and Control vs HTG neonatal samples. Results are reported as base mean expression, $\log_2$ fold change, standard error of the $\log_2$ fold change (lfcSE), test statistic, nominal p-value, and adjusted p-value (padj). miRNAs of interest (miR223, miR30A, and miR30E) are highlighted based on prior biological relevance and exploratory findings in the clinical cohort

CTL vs NHTG							
GeneID	**baseMean**	**log2FoldChange**	**lfcSE**	**stat**	**pvalue**	**padj**	**Symbol**
9086	3.349023	4.118138	1.581834	2.603396	0.009231	0.611609	EIF1AY
114882	2.350741	4.086192	1.434157	2.849193	0.004383	0.436539	OSBPL8
54583	2.785275	3.700879	1.597933	2.316041	0.020556	0.740127	EGLN1
132320	2.334094	3.608371	1.654783	2.18057	0.029215	0.740127	SCLT1
5500	2.478809	3.48363	1.51728	2.295971	0.021678	0.740127	PPP1CB
10746	3.193943	3.402208	1.55208	2.192031	0.028377	0.740127	MAP3K2
692199	4.045198	3.332398	1.411104	2.361554	0.018199	0.740127	SNORD84
407029	**868.5523**	**2.085926**	**0.732283**	**2.848526**	**0.004392**	**0.436539**	**MIR30A**
407008	**3465.035**	**-0.488284**	**0.69331**	**-0.704279**	**0.481259**	**0.919848**	**MIR223**
407034	**10783.14**	**0.165741**	**0.232508**	**0.712837**	**0.475946**	**0.91917**	**MIR30E**
CTL vs HTG							
GeneID	**baseMean**	**log2FoldChange**	**lfcSE**	**stat**	**pvalue**	**padj**	**Symbol**
407029	**868.5523**	**2.925553**	**0.551389**	**5.305792**	**1.12E-07**	**0.000147**	**MIR30A**
6653	10.89649	2.460612	0.651059	3.779402	0.000157	0.024561	SORL1
79627	6.827175	2.262701	0.809032	2.796801	0.005161	0.102454	OGFRL1
6648	12.88154	1.918778	0.569527	3.369074	0.000754	0.047652	SOD2
407001	90.55838	-3.642407	1.269407	-2.869376	0.004113	0.099311	MIR218-2
407000	56.64385	-3.591002	1.306996	-2.747523	0.006005	0.110453	MIR218-1
100189213	6.305345	-3.402971	1.057382	-3.2183	0.00129	0.049931	TRI-AAT3-1
100189090	79.2577	-3.026792	0.920037	-3.289859	0.001002	0.049931	TRC-GCA4-1
407008	**3465.035**	**-0.11228**	**0.522562**	**-0.214864**	**0.829873**	**0.959128**	**MIR223**
407034	**10783.14**	**0.345253**	**0.175164**	**1.971032**	**0.04872**	**0.301558**	**MIR30E**

Our results demonstrated consistent upregulation of all considered miRNAs at birth (T0) and after 3 h (T3) (Fig. 5a-c). Whereas miR30e & miR30a were significantly upregulated at T0 in NHTG and at T3 in HTG to the CG. While all the three-miRNA showed distinctive unsignificant variation at T48 suggesting their potential relevance during the early postnatal phase.

In maternal blood only miR223 was substantially elevated in NHTG, however in the other instances there was no significant difference between groups compared to CG (Fig. 4).

Discussion

In the present study, through an exploratory analysis that miR223, miR30e, and miR30a are associated with perinatal metabolic acidosis and acute hypoxic stress, as defined by cord blood pH and clinical HIE status at presentation, with elevated levels suggesting its involvement in NHTG and HTG at T0 and T3. In particular, the present data supports the importance of evaluating miR30e and miR30a at both T0 and T3, as the progressive impact of hypoxia may play a critical role. In fact, although previous papers investigated blood miRNA levels in HIE patients [11–14], one of the novelties of the present study lies in the evaluation of timepoints, which is something missing in all the previous studies. This represents a major factor in understanding the role of miRNA in the transitional aspect at 0, 3, and 48 h. In NHTG, the selected miRNAs exhibit significant upregulation at T0, whereas in HTG, a notable increase in their expression is observed at T3.

Maternal circulating miRNA alterations, particularly miR223, should be interpreted cautiously, as they likely reflect maternal–placental inflammatory or stress-related responses rather than serving as direct indicators of fetal brain injury or neonatal encephalopathy.

A limitation of this study is the use of a single endogenous control (U6) for miRNA normalization; although stability was supported in this cohort (Supplementary Fig. 2), future studies should validate additional reference miRNAs to strengthen normalization robustness.

miR30e and miR30a emerge as the most promising candidate markers in this exploratory analysis. At T3, NHTG's miRNA levels are like those of the CG, which is consistent with ABG findings indicating normalization of the body's stress response. However, in situations of

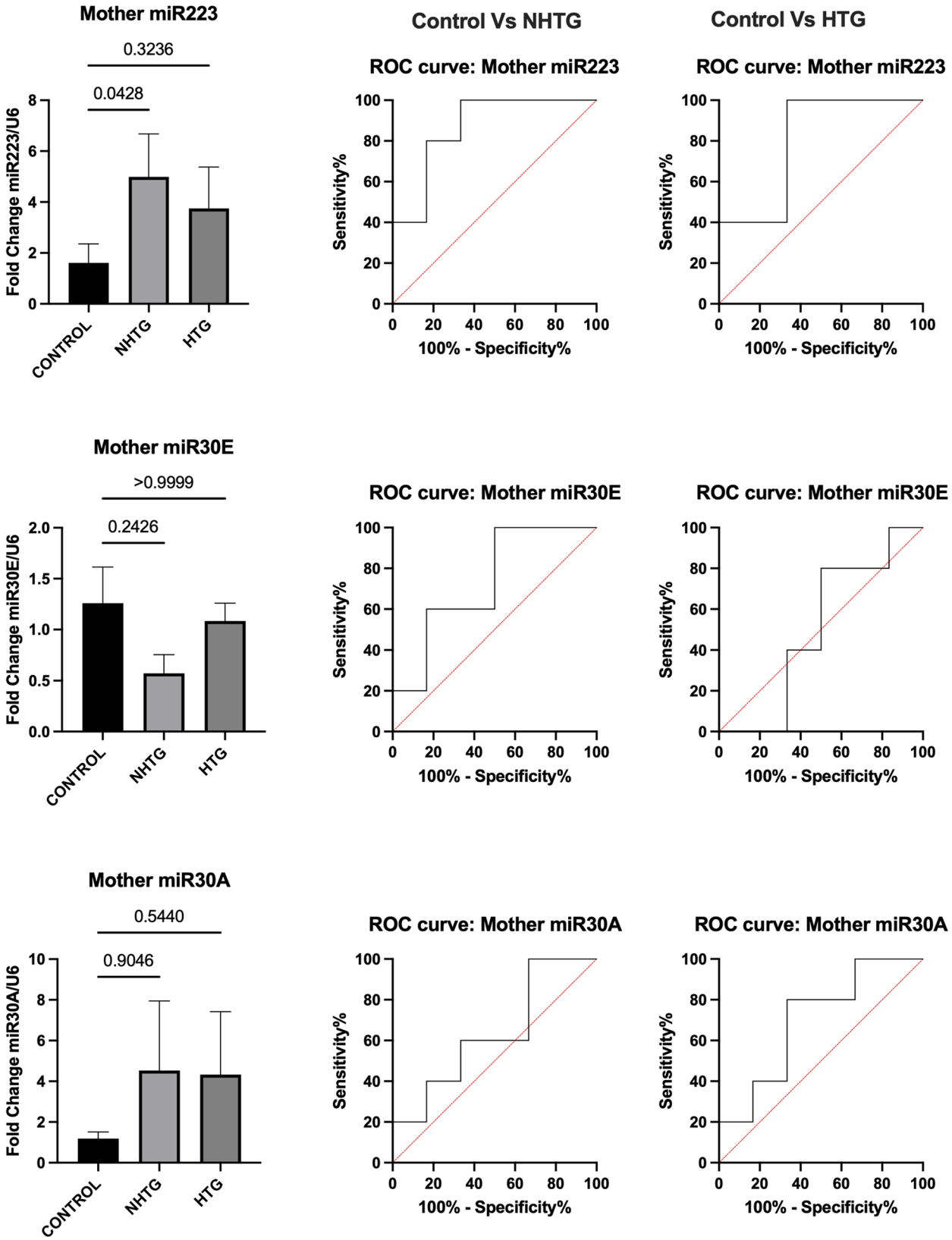

Fig. 4 Maternal circulating miRNA expression at delivery (T0). Relative expression levels of miR223, miR30E, and miR30A in maternal blood across Control, NHTG, and HTG groups at birth (T0). Receiver operating characteristic (ROC) curves illustrate the discriminatory performance of maternal miRNA expression levels in distinguishing Control vs. NHTG and Control vs. HTG groups

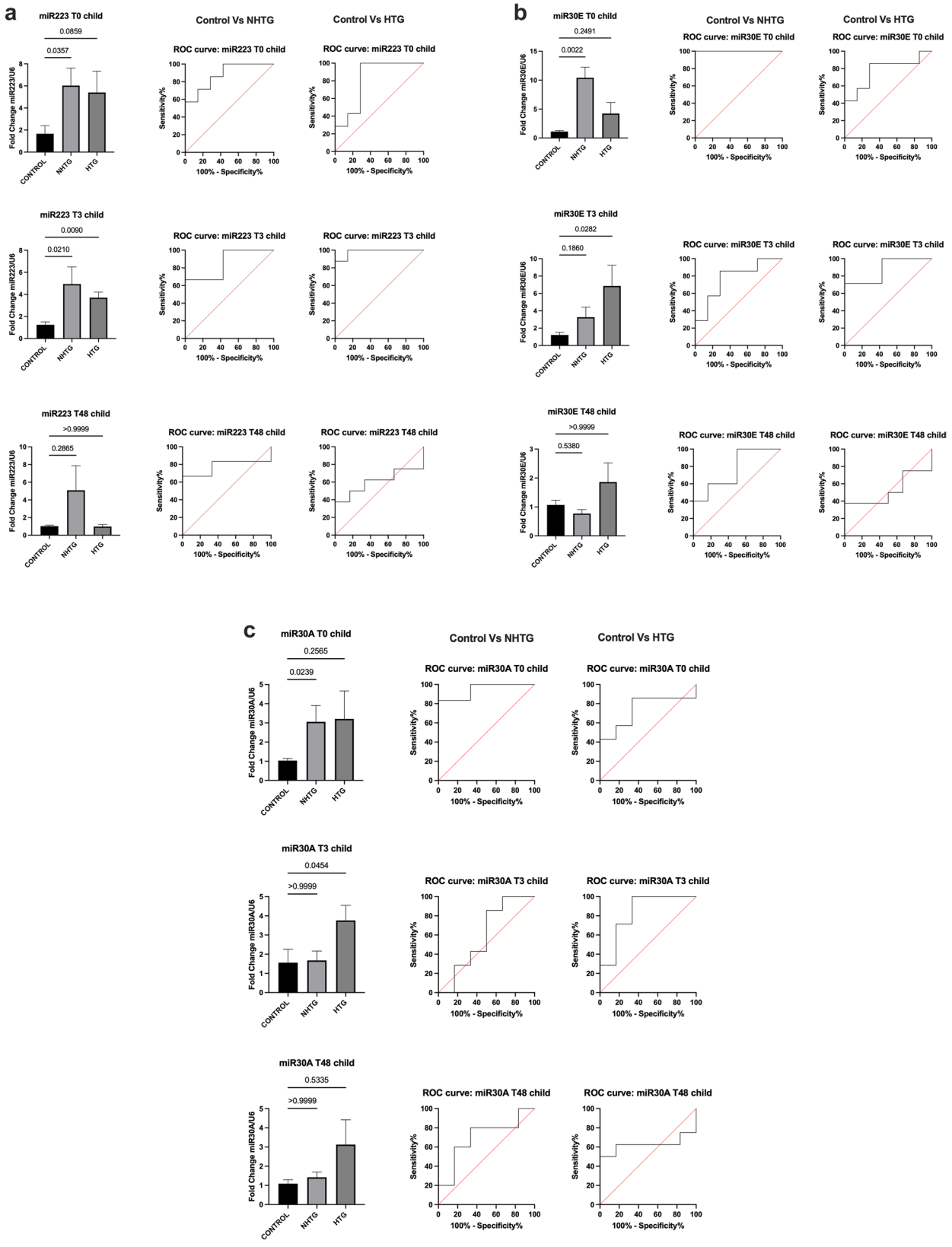

Fig. 5 (See legend on next page.)

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Fig. 5 Temporal changes in neonatal miR223, miR30E, and miR30A expression at birth (T0), 3 h (T3), and 48 h (T48) across study groups. **a**. Neonatal miR223 expression across time points. Left panels show relative expression levels of miR223 (normalized to U6) in neonatal blood samples from Control, NHTG, and HTG groups at birth (T0), 3 hours (T3), and 48 hours (T48). Right panels show ROC curves illustrating the ability of neonatal miR223 expression to distinguish Control from NHTG and HTG groups at each time point, with greater group separation observed at T0 and T3 compared with T48. **b**. Neonatal miR30E expression across time points. Left panels show relative expression levels of miR30E (normalized to U6) in neonatal blood samples from Control, NHTG, and HTG groups at birth (T0), 3 hours (T3), and 48 hours (T48). Right panels show ROC curves depicting the discriminatory performance of miR30E expression in distinguishing NHTG and HTG groups from controls across time points. **c**. Neonatal miR30A expression across time points. Left panels show relative expression levels of miR30A (normalized to U6) in neonatal blood samples from Control, NHTG, and HTG groups at birth (T0), 3 hours (T3), and 48 hours (T48). Right panels show ROC curves demonstrating moderate group discrimination at early time points, with reduced separation between groups by 48 hours post-birth

hypothermia treated patients', systemic inflammation and stress continue, as demonstrated by a significant elevated levels of these miRNAs at T3 in HTG.

In neonatal hypoxic–ischemic encephalopathy (HIE), one of the most significant clinical challenges is the strict therapeutic window for initiating hypothermia, which must begin within the first 6 h of life to confer neuroprotection. Delayed initiation beyond this window has been associated with reduced efficacy and may fail to attenuate the cascade of secondary energy failure, inflammation, and apoptotic cell death that evolves after the initial hypoxic–ischemic insult. However, early identification of neonates who will ultimately develop moderate to severe HIE remains challenging, as neurological signs can be subtle, evolving, or confounded by factors such as sedation, prematurity, or perinatal interventions. This diagnostic uncertainty complicates time-sensitive clinical decision-making and may lead to either missed opportunities for neuroprotection or unnecessary exposure to hypothermia-related risks. In this context, early circulating miRNAs represent a promising class of candidate biomarkers that could support rapid risk stratification during the immediate postnatal period. By providing objective molecular indicators of injury severity within the constrained therapeutic window, miRNA-based diagnostics have the potential to facilitate timely initiation of hypothermia in infants most likely to benefit, thereby addressing a critical barrier to optimizing outcomes in neonatal HIE [1–7].

Indeed, oxygen deprivation results in widespread brain injury, with microRNAs playing a central role in regulating neurodevelopmental and injury-response pathways. As shown in Fig. 6, Gene Ontology enrichment analysis of miR223, miR30a, and miR30e target genes reveals significant enrichment in biological processes related to axonogenesis, synapse assembly, synaptic vesicle cycling, vesicle-mediated transport in synapses, and regulation of synaptic structure and activity. These processes are essential for neuronal connectivity and are particularly vulnerable to hypoxic injury.

Comparative ontology analysis further demonstrates that all three miRNAs are involved in central nervous system neuron development, neuron projection guidance, and regulation of nervous system development, suggesting a coordinated role in the evolution of asphyxia-induced brain damage (Fig. 6). Enrichment of pathways such as actin filament–based movement, regulation of small GTPase–mediated signal transduction, and developmental growth involved in morphogenesis indicates potential effects on cytoskeletal dynamics and neuronal remodeling following hypoxic insult.

In addition, miR223 shows prominent enrichment in inflammatory and stress-related pathways, consistent with its known role as a mediator of neuroinflammation, whereas miR-30a and miR-30e are strongly associated with forebrain development, synaptic transmission, and regulation of regulated secretory pathways (Fig. 6). The miR30 family (miR-30a-5p and miR-30e-5p) is also implicated in apoptosis, differentiation, and inflammatory signaling, processes that are critically involved in hypoxic–ischemic brain injury and neurodevelopmental disorders [12, 13].

Together with previous evidence demonstrating the involvement of these miRNAs in oxidative stress, inflammation, and cell death pathways in hypoxic–ischemic and other brain injury models [27–29].

On the other hand, miR223 is crucial in mediating neuroinflammation and neuronal damage, underscoring its importance in studies of HIE. This miRNA is well-known for regulating inflammation and immune responses, particularly by targeting inflammatory pathways like the NLRP3 inflammasome, a major contributor to neuroinflammation [30]. By inhibiting NLRP3 activity, miR223 effectively reduces the release of pro-inflammatory cytokines, such as IL-1β, which can worsen neuronal injury in HIE [31]. Notably, our previous research has established a direct link between miR223 and the functioning of NCKX2 (Na+/Ca2 + exchanger 2), highlighting its relevance to the progression of HIE, particularly concerning the development of seizures and epilepsy [31, 32].

Conclusion

Neonatal HIE currently represents an important risk factor for the development of short- and long-term neuro psychomotor developmental disorders and treatment with hypothermia still represents the therapeutic gold standard. Despite the technological improvement of standard perinatal monitoring procedures which have

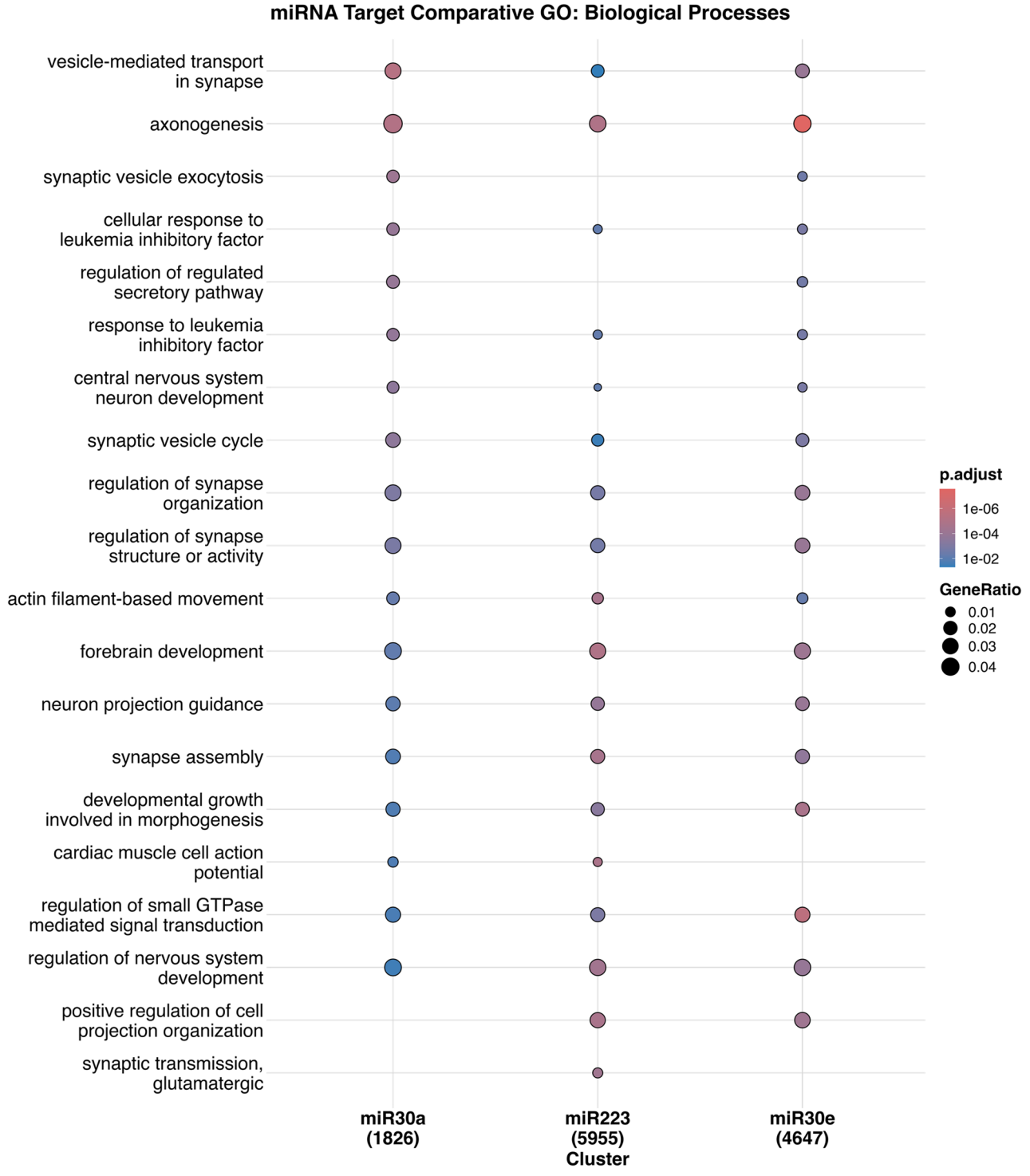

Fig. 6 Comparative Gene Ontology (GO) enrichment analysis of biological processes associated with selected miRNAs. Dot plot showing enriched GO biological process terms for target genes of miR30a ($n = 1826$), miR223 ($n = 5955$), and miR30e ($n = 4647$). Each dot represents a significantly enriched GO term. Dot size corresponds to the GeneRatio, indicating the proportion of genes associated with a given GO term, while dot color represents the adjusted p-value (p.adjust), with red indicating higher significance and blue indicating lower significance. The analysis highlights shared and distinct biological processes among the three miRNAs, with prominent enrichment in synaptic function, neuronal development, axonogenesis, and vesicle-mediated transport pathways

led to a decrease in the incidence of perinatal mortality, the trend in morbidity has not improved as the literature shows an increase in brain lesions even in those children who present a mild degree of birth asphyxia, with a high incidence of brain abnormalities on MRI and several possible neurological disabilities. To date, therefore, there is still an open problem in the selection of newborns deserving of hypothermic treatment. The need to search for new biomarkers of neonatal brain damage is therefore fundamental to identify those newborns who deserve to be subjected to therapeutic hypothermia. From the following work, although it is necessary to increase the sample size to increase statistical power, miR223, miR30e and miR30a appears to be candidate markers showing early postnatal changes associated with perinatal metabolic acidosis and clinical HIE status at presentation. As a future perspective, and in order to evaluate their potential utility as biomarkers in HIE following validation in larger, outcome-linked cohorts, we are currently collecting additional data to further deepen our analysis by examining correlations between miRNA expression levels and short- and long-term outcomes, including mortality, disease severity scores, occurrence of epilepsy, cerebral palsy or motor disabilities, and neurodevelopmental screening assessments at 18 or 24 months of age.

Abbreviations

ABG	Arterial Blood Gas
CG	Control Group
EEG	Electroencephalograph
HIE	Hypoxic-Ischemic Encephalopathy
HTG	Hypothermic Treatment Group
MRI	Magnetic Resonance Imaging
NHTG	No Hypothermic Treatment Group

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40348-026-00220-9.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Acknowledgments

This work was supported by grant CN00000041 "National Center for Gene Therapy and Drugs based on RNA Technology" (concession number 1035 of 17 June 2022-PNRR MUR – M4C2 – Investment 1.4 Call "National Centers", financed by EU- NextGenerationEU), code project (CUP) E63C22000940007 to G.P.

Authors' contributions

Prof Giuseppe Pignataro, Dr. Rohan M Patil, Dr. Matteo Miele and Dr. Giuseppe De Bernardo conceptualized and designed the study, drafted the initial manuscript, and critically reviewed and revised the manuscript.Dr. Maurizio Giordano, Dr. Valentina Fattorusso and Dr. Rohan M Patil designed the data collection instruments, collected data and samples, carried out the initial analyses, and critically reviewed and revised the manuscript.Prof Giuseppe Pignataro conceptualized and designed the study, coordinated and supervised data collection, and critically reviewed and revised the manuscript for important intellectual content.All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Funding

Support: All phases of this study were supported by the following grants: the European Union – Next Generation EU, Mission 4, Component 2, PE0000006, CUP E63C2 2002170007 (Project "A multiscale integrated approach to the study of the nervous system in health and disease" MNESYS), Spoke 3 (to Giuseppe Pignataro); PRIN 2022, Project 2022NRT82A (to Giuseppe Pignataro). Role of Funder/Sponsor: The Italian Ministry of Health funded the project but had no role in the design and conduct of the study.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

Received: 19 September 2025 / Accepted: 6 February 2026
Published online: 18 February 2026

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